

Association of Canine Osteosarcoma and Monocyte Phenotype and Chemotactic Function

J.L. Tuohy, B.D.X. Lascelles, E.H. Griffith, and J.E. Fogle

Background: Monocytes/macrophages are likely key cells in immune modulation in dogs with osteosarcoma (OSA). Increased peripheral monocyte counts are negatively correlated with shorter disease-free intervals in dogs with OSA. Understanding the monocyte/macrophage's modulatory role in dogs with OSA can direct further studies in immunotherapy development for OSA.

Hypothesis/Objectives: That OSA evades the immune response by down-regulating monocyte chemokine receptor expression and migratory function, and suppresses host immune responses.

Animals: Eighteen dogs with OSA that have not received definitive treatment and 14 healthy age-matched controls

Methods: Clinical study—expression of peripheral blood monocyte cell surface receptors, monocyte mRNA expression and cytokine secretion, monocyte chemotaxis, and survival were compared between clinical dogs with OSA and healthy control dogs.

Results: Cell surface expression of multiple chemokine receptors is significantly down-regulated in peripheral blood monocytes of dogs with OSA. The percentage expression of CCR2 (median 58%, range 2–94%) and CXCR2 expression (median 54%, range 2–92%) was higher in control dogs compared to dogs with OSA (CCR2 median 29%, range 3–45%, $P = 0.0006$; CXCR2 median 23%, range 0.2–52%, $P = 0.0007$). Prostaglandin E₂ (PGE₂) (OSA, median 347.36 pg/mL, range 103.4–1268.5; control, 136.23 pg/mL, range 69.93–542.6, $P = .04$) and tumor necrosis factor-alpha (TNF- α) ($P = .02$) levels are increased in OSA monocyte culture supernatants compared to controls. Peripheral blood monocytes of dogs with OSA exhibit decreased chemotactic function when compared to control dogs (OSA, median 1.2 directed to random migration, range 0.8–1.25; control, 1.6, range of 0.9–1.8, $P = .018$).

Conclusions and Clinical Importance: Dogs with OSA have decreased monocyte chemokine receptor expression and monocyte chemotaxis, potential mechanisms by which OSA might evade the immune response. Reversal of monocyte dysfunction using immunotherapy could improve survival in dogs with OSA.

Key words: Bone tumor; Chemoreceptors; Chemotaxis; Immunoregulation.

Appendicular osteosarcoma (OSA), the most common primary bone malignancy in dogs, has a high rate of metastasis and a poor prognosis.^{1,2} The overall median survival time from time of diagnosis for dogs that receive standard-of-care therapy (primary tumor removal and adjuvant chemotherapy) is approximately 10–12 months, with a 20% 2-year survival rate.¹ Metastasis is the primary cause of death in dogs with OSA, with approximately 90% of dogs already harboring occult metastatic disease at diagnosis.¹ Controlling metastatic disease is crucial to improving outcomes in OSA.

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Abbreviations:

APC	allophycocyanin
COX-2	cyclooxygenase-2
DMEM	Dulbecco's Modified Eagle Medium
FACS	flow cytometry
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
fMLP	N-Formyl-L-methionyl-L-leucyl-L-phenylalanine
FSC	forward scatter
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IACUC	Institutional Animal Care and Use Committee
IL-10	interleukin-10
IL-12	interleukin-12
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
NCSU	North Carolina State University
OSA	osteosarcoma
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	phycoerythrin
PGE2	prostaglandin E ₂
qPCR	quantitative RT-PCR
SSC	side scatter
TGF- β	transforming growth factor-beta
TNF- α	tumor necrosis factor-alpha

Tumor-induced immune suppression is one of the mechanisms by which malignancies ensure survival. For example, tumors recruit monocytes and macrophages and induce pro-tumorigenic changes in their phenotypes and function leading to a microenvironment that favors tumor growth.³ OSA cells from dogs secrete Transforming Growth Factor-beta (TGF- β), which likely

contributes to immune suppression as TGF- β is considered an inhibitory immunoregulatory cytokine.⁴⁻⁶ Interestingly, dogs with OSA who develop infections after limb-spare surgery have been observed to almost double their median survival time, potentially via up-regulation of antitumor immunity.⁷ Dogs with OSA and infections were half as likely to die, half as likely to develop metastasis, and these survival effects were because of a delay in metastasis rather than control of local tumor recurrence.⁷ Studies in mice have confirmed these observations and suggest that the antitumor effects of infection could be modulated via monocytes and macrophages. Mice with chronic osteomyelitis that developed OSA experienced improved survival, had increased numbers of inflammatory monocytes, and depletion of monocytes/macrophages in these mice reversed the survival benefit afforded by osteomyelitis.⁸ Understanding the role of immunomodulatory cells such as monocytes in creating a protumorigenic environment in dogs with OSA will inform future investigations to identify therapeutic targets to reverse tumor-induced immune dysfunction and inhibit metastatic disease. To this end, we sought to identify phenotypic and functional differences in monocytes between dogs with OSA and healthy dogs. The objectives of our study were (1) to compare cell surface receptor expression between monocytes from OSA and healthy dogs, (2) to compare mRNA expression and cytokine secretion between monocytes from OSA and healthy dogs, (3) to compare chemotactic differences between monocytes from OSA and healthy dogs, and (4) to analyze associations between OSA survival and peripheral blood monocyte counts/receptor expression. Our hypothesis was that OSA evades the immune response by down-regulating monocyte chemokine receptor expression and migratory function, and suppresses host immune responses.

Materials and Methods

Patient Enrollment and Sample Collection

Client-owned dogs diagnosed with OSA that had not received any tumor-directed therapy or immunomodulatory drugs were enrolled into the study. Because of the enrollment of dogs at a tertiary referral center, some dogs with OSA had been prescribed anti-inflammatory medications by their primary referring veterinarian. A similar population of age-matched medium and large-breed control dogs without known disease that were also not receiving any anti-inflammatory or immunomodulatory medications were recruited for the study by the North Carolina State University (NCSSU) Clinical Studies Core. Verbal and written owner consent were obtained for all dogs participating in the study, and peripheral blood samples were collected under an approved Institutional Animal Care and Use Committee (IACUC) protocol.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and of a Purified Monocyte Population

Approximately 25 mL of peripheral blood was collected into EDTA blood collection tubes from 14 control dogs and 18 dogs with OSA via venipuncture. Five mL blood was submitted for complete blood counts (CBC) in 10 healthy controls and 18 dogs

with OSA. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque®-1077.^a A portion of the PBMCs from 13 healthy controls and 18 dogs with OSA was preserved for cell surface staining, and a portion underwent cell sorting to isolate a purified monocyte population using a MoFlo® high-speed cell sorter.^b Monocyte cell isolation was performed based on forward (FSC) versus side scatter (SSC), and CD14⁺ expression, and the phenotype of the sorted monocytes was confirmed via light microscopy.⁹ Approximately 1×10^6 sorted monocytes from 7 healthy controls and 7 dogs with OSA were centrifuged and resuspended in RNeasy Protect^c cell reagent for PCR analysis. Sorted monocytes from 8 healthy controls and 7 dogs with OSA were used for stimulation cultures, and sorted monocytes from 14 healthy controls and 5 dogs with OSA were used for chemotaxis assays, as outlined below.

Flow Cytometry (FACS) Analysis

A portion of the PBMCs from 13 healthy controls and 18 dogs with OSA were centrifuged, resuspended in 2 mL Phosphate-Buffered Saline (PBS), then stained with the following antibodies against cell surface receptors: anti-CD14,^d anti-CD16,^e anti-CD62L,^f anti-CD32,^g anti-CD11c,^g anti-CCR2,^h anti-CCR7,^h anti-CD43,^h anti-CX₃CR1,^h and anti-CXCR2^h (Table 1). A tube containing unstained cells served as a negative control, and the appropriate isotype controls (IgG2a PE^f, IgG1 FITC^f, rabbit polyclonal^e conjugated antibodies) were applied. Single color compensation controls were established at the start of these experiments, and the data shown here represent multicolor analysis. All antibodies were directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC). The stained cells were incubated for 20 minutes on a shaker at low-speed, in the dark, at room temperature, after which the cells were washed twice with PBS. A FACS Caliberⁱ flow cytometer was used to perform FACS analysis, and the data were analyzed using the accompanying CellQuest™ software. Monocytes were gated based on FSC and SSC (Fig 1a), and gates for determining the percentage of monocytes that stained positive for antibody were set using isotype controls as guidelines.

Total RNA Extraction, Reverse Transcription, and Quantitative RT-PCR

Sorted monocytes from 7 healthy controls and 7 dogs with OSA were used for PCR analysis. The RNeasy minikit^c was used to extract total RNA for quantitative RT-PCR (qPCR) from the sorted monocytes according to the manufacturer's protocol. We verified the quality and quantity of the purified RNA using a

Table 1. List of antibodies used for flow cytometry.

Antibodies	Clone	Isotype	Fluorescence labeling
CD14	TÜK4	mIgG2a	APC, FITC
CD16	LNK16	mIgG1	PE
CD62L	SK11	mIgG2a	PE
CD32	AT10	mIgG1	FITC
CD11c	BU15	mIgG1	FITC
CCR2	Polyclonal	Rabbit	FITC
CCR7	Polyclonal	Rabbit	FITC
CD43	Polyclonal	Rabbit	FITC
CX ₃ CR1	Polyclonal	Rabbit	FITC
CXCR2	Polyclonal	Rabbit	FITC

CD, cluster of differentiation; m, mouse; IgG, immunoglobulin G; APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

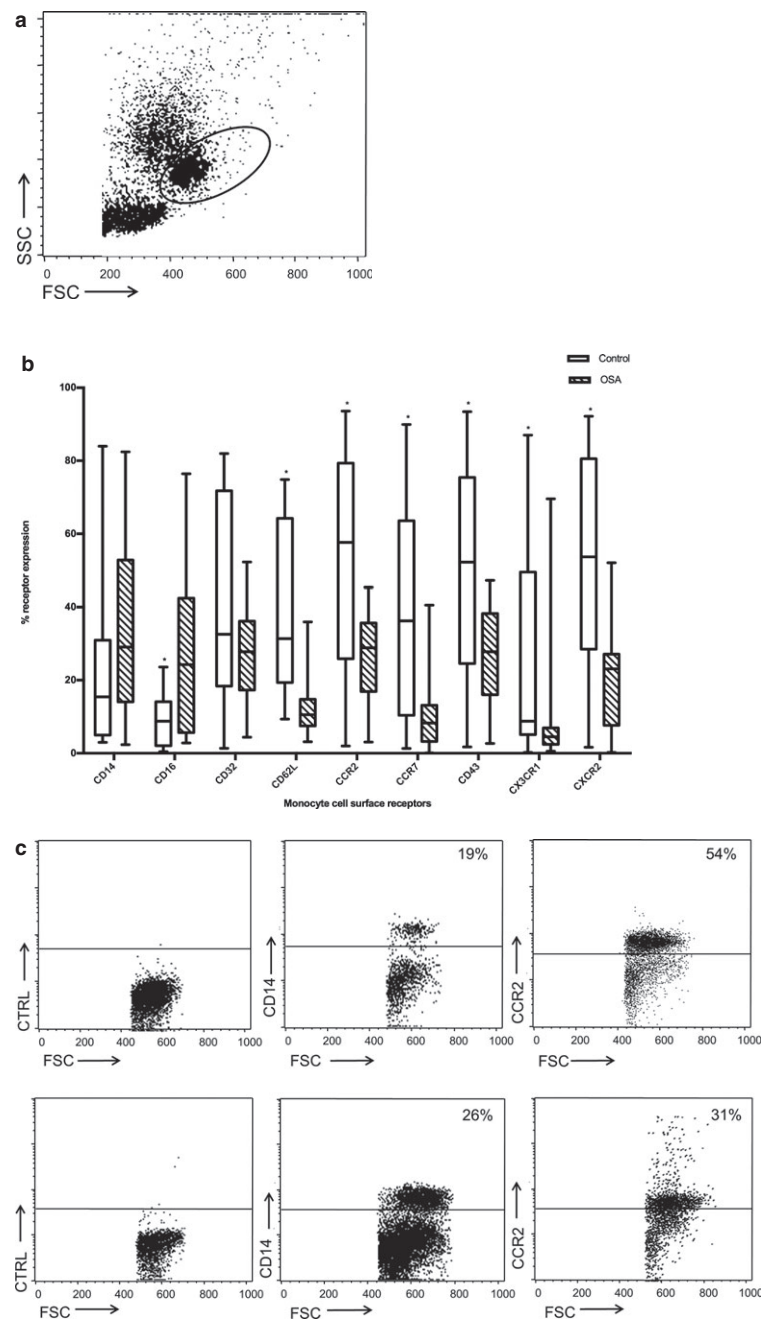


Fig 1. Monocyte surface marker expression is altered in dogs with OSA: Flow cytometry dot plots illustrating cell surface receptor expression in peripheral blood monocytes from dogs with OSA ($n = 18$) and healthy controls ($n = 13$). **(a)** Representative dot plot showing monocyte gating using forward and side scatter characteristics. CD14 staining was used to ensure that the majority of the gated monocytes exhibited positive CD14 cell surface expression. **(b)** Box-and-whisker plot depicting the % positive monocyte cell surface receptor expression of the different receptors (CD14, CD16, CD32, CD62L, CCR2, CCR7, CD43, CX₃CR1, CXCR2), comparing expression between healthy controls and dogs with OSA. The median percentages and ranges of positively staining cells for each antibody in healthy controls are as follows: CD14 (15%, 3–84%), CD16 (9%, 0.5–24%), CD32 (33%, 1–82%), CD62L (31%, 10–75%), CCR2 (58%, 2–94%), CCR7 (36%, 1–90%), CD43 (52%, 2–93%), CX₃CR1 (9%, 0.2–87%), and CXCR2 (54%, 2–92%). The median percentages and ranges of positively staining cells for each antibody in dogs with OSA are as follows: CD14 (29%, 2–82%), CD16 (24%, 3–76%), CD32 (28%, 4–52%), CD62L (11%, 3–36%), CCR2 (29%, 3–45%), CCR7 (8%, 0.1–41%), CD43 (28%, 3–47%), CX₃CR1 (5%, 0.6–70%), and CXCR2 (23%, 0.2–52%). Significant differences are marked with an asterisk ($P < .05$). Chemokine receptors have significantly decreased expression, and CD16 expression is significantly increased in monocytes of dogs with OSA. **(c)** Representative dot plots showing the percentage cell surface expression of CD14 and CCR2 in monocytes of a healthy control (upper panel) versus an OSA dog (lower panel). There was no significant difference between CD14 expression in OSA compared to control dogs. A portion of the CD14 positive monocytes in dogs with OSA were noted to have increased forward scatter, denoting larger cell sizes. CCR2 expression is significantly decreased in dogs with OSA ($P = .0006$). Representative dot plots for isotype controls are displayed for each dog.

NanoDrop 2000c spectrophotometer^g, and also used gel electrophoresis to confirm RNA quality (Figure S1a). cDNA was subsequently synthesized in a Techne TC4000^j thermal cycler using the Promega[©] RT kit^k according to manufacturer's directions. Primers for qPCR to detect mRNA expression levels of Interleukin-10 (IL-10), Interleukin-12 (IL-12), Tumor Necrosis Factor-alpha (TNF- α), and Cyclooxygenase-2 (COX-2) were selected based on previous reports (Table 2),^{10,11} and purchased from Integrated DNA Technologies.¹ The PerfeCTa[®] SYBR[®] Green FastMix[®] for iQ[™] PCR kit^m was used for mRNA expression detection, and the reactions performed with the iCycler iQ[™] real-time PCR detection system.ⁿ All samples were run in triplicate. The thermal cycling conditions were as follows: cycle 1 (95.0°C for 8 minutes) 1X, cycle 2 (95.0°C for 30 seconds, 56.0°C for 20 seconds, 72.0°C for 40 seconds) 40X, cycle 3 (melting curve starting at 60.0°C for 10 seconds, with stepwise increases of 0.5°C for 70X to achieve 95°C), and cycle 4 (hold at 4.0°C). The housekeeping gene, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), was used for normalization of results. The CT value of GAPDH was consistent across experiments and between animals for all PCR results reported here (CT = ~21). The PCR product was separated using agarose gel electrophoresis, the DNA was sequenced by GENE-WIZ[®] DNA sequencing services, and the sequences were subjected to alignment searches in the NCBI database to confirm matches with the gene of interest (Figure S1b). The $2^{-\Delta\Delta CT}$ method was used to derive the normalized relative mRNA expression level of the target genes.

Prostaglandin ELISA

Sorted monocytes from 8 healthy controls and 7 dogs with OSA were used for stimulation experiments—half of these monocytes served as unstimulated controls and incubated for 6 hours at 37°C and 5% CO₂. The other half were stimulated with 100 ng/10⁶ monocytes of lipopolysaccharide (LPS^o) and incubated similarly for 6 hours at 37°C and 5% CO₂. The resultant supernatants were collected, stored at -20°C until they were assessed for PGE₂ levels using a commercially available ELISA kit^o according to manufacturer's directions.

IL-10, IFN- γ , TNF- α Magnetic Bead Panel Assay

Sorted monocytes from 5 healthy controls and 5 dogs with OSA were used for stimulation experiments as described for the prostaglandin ELISA. The resultant supernatants were collected,

Table 2. Quantitative RT-PCR primers to detect mRNA expression levels of proinflammatory and anti-inflammatory cytokines, and COX-2.

Target	(5'-3')	Position (bp)
IL-10 (F)	GCGACGCTGTCACCGATT	379-396
IL-10 (R)	CTGGAGCTTACTAAATGCGCTCTT	460-437
IL-12 (F)	TGGCTGCTATTCACAAGCTCAAGT	638-661
IL-12(R)	TGGTTTGATGATGTCTCTGATGAAG	708-684
TNF- α (F)	AGCCAGTAGCTCATGTTGTAGCAA	759-781
TNF- α (R)	GCGACTATCAGCTGGTTGTCTGT	358-380
COX-2 (F)	CTGTTCCACCCATGTCAA	-
COX-2 (R)	GCAGTTTTCGCCGTAGAATC	-

F, forward primer; R, reverse primer.

stored at -20°C until they were assessed for IL-10, IFN- γ , and TNF- α levels using a commercially available canine cytokine magnetic bead panel kit (MILLIPLEX[®] MAP)^p according to manufacturer's directions. IL-12 was not available as an analyte in the kit, thus IFN- γ levels were analyzed as a surrogate, as IL-12 is known to induce IFN- γ secretion.

Chemotaxis Assay

Sorted monocytes from 14 healthy controls and 5 dogs with OSA were used for migration assays. Monocyte migration was assessed using a NeuroProbe ChemoTx^q 96-well disposable cell migration system with 8 μ m polycarbonate framed filters. Sorted monocytes were resuspended in 10⁶/mL of cell culture media, labeled with 1 μ g calcein-AM^r /10⁶ monocytes, and the cells incubated for 30 minutes in the dark at room temperature. Following incubation, the cells were resuspended at 10⁶/mL in Dulbecco's Modified Eagle Medium (DMEM^s). Twenty-five μ L of the cell suspension were deposited on each filter. The lower chamber, ie, the 96-well plate, contained the following: 100% heat-inactivated fetal bovine serum (FBS^s), 100 ng/mL monocyte chemoattractant protein-1 (MCP-1^t), 100 nM N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP^a), 100 ng/mL CCL19^t as positive chemoattractants, and PBS as a negative control. The positive chemoattractants were either placed individually in separate wells, or combined together in a single well. For total cell counts, 25 μ L of cells was placed directly into the lower well to quantify maximal fluorescence. The plate was incubated for 4 hours at 37°C, after which the nonmigrated cells on top of the filters were gently wiped off using a cell scraper. Cellular fluorescence in the bottom wells was quantified using a fMax fluorescence microplate reader^u at 485 and 538 nm wavelength, and the corresponding SoftMax Pro software. Filter fluorescence was also quantified to confirm that cells were not adherent to the underside of the filter. Degree of migration was calculated as a percentage of migrated cells out of the known cell numbers placed in bottom wells. Cell migration was expressed as a migration index, calculated by dividing directed cell chemotaxis (cells migrating in response to a chemokine, quantified by degree of fluorescence) by random cell chemotaxis (cells migrating in response to media alone, quantified by degree of fluorescence). Each sample was assayed in triplicate.

Statistical Analyses

Statistical analyses were performed to compare monocyte cell surface receptor expression levels between groups using the non-parametric Mann-Whitney *U*-test because of the non-normal distribution of the data. Comparison of relative mRNA expression of each gene and of PGE₂ levels between groups were analyzed with the Mann-Whitney *U*-test. All statistical tests were carried out as 2-sided tests, and a *P*-value of <.05 was considered significant. Comparison of peripheral blood monocyte counts between dogs with OSA and healthy controls was performed using a 2-tailed T-Test. Pearson's correlation coefficient was used to determine the strength and direction of a possible linear relationship between monocyte surface receptors and peripheral blood monocyte counts. Two-way ANOVAs and least-squares means were used to test for differences in monocyte surface receptors because of status and monocyte counts (high versus low). To analyze the chemotaxis data, one-way ANOVAs were run and means were compared using Welch's adjustment for heterogeneous variation. Kaplan-Meier survival analysis was used to compare survival between groups, with proportional hazards regression analysis to test the effect of chemokine receptor expression on survival. Survival was calculated as the number of days from diagnosis of disease to death attributed to disease, or last follow-up if the patient was still

alive. A dog was determined to have died of disease if metastasis or local tumor recurrence was the cause, and dogs with an unknown cause of death were presumed to be dead because of disease. Dogs were censored from the analysis if they were still alive at last follow-up. All analyses were performed using SAS software (Version 9.4, Cary, NC). All statistical analyses were performed in consultation with the NCSU Biostatistics consulting group (Dr. E. Griffith).

Results

Study Population

The mean (\pm SD) age at time of OSA diagnosis was 8.7 (\pm 2.1) years. All dogs with OSA were purebred, with the most common pure breeds being Labrador Retriever (5 [28%]), Rottweiler (3 [17%]), Great Dane (3 [17%]), and Golden Retriever (2 [11%]). There were 8 (44%) spayed females, and 10 (56%) castrated males.

Monocyte Surface Receptor Expression

The primary flow cytometry canine monocyte gates were based on typical forward and side scatter characteristics. Once that forward versus side light scatter gate was established, we ensured that greater than 98% of the CD14+ cells were found within this gate. As CD14 expression on peripheral blood monocytes varies greatly, this ensured that we included the CD14^{lo} monocytes for surface receptor analysis (Fig 1a). Monocytes exhibited positive surface expression of CD14, CD16, CD32, CD62L, CCR2, CCR7, CD43, CX₃CR1, CXCR2, with low levels of CD11c. Irrelevant isotype controls were used for each experiment—the isotype control binding of monocytes was <5%. The median percentages and ranges of positively staining cells for each antibody are listed in the legend accompanying Fig 1b. Comparison of monocyte surface receptors between untreated dogs with OSA and healthy controls revealed a significant decrease in surface receptor expression of CD62L ($P < .00001$), CCR2 ($P = .0006$), CCR7 ($P = .0004$), CD43 ($P = .008$), CX₃CR1 ($P = .002$), CXCR2 ($P = .0007$) in dogs with OSA, and a significant increase in CD16 expression in dogs with OSA ($P = .005$) (Fig 1b). Fig 1c depicts representative dot plots with the percentage of cell surface receptor expression in monocytes of a healthy control versus an OSA dog.

A peripheral blood monocyte count of >400 cells/ μ L has been significantly associated with decreased disease-free interval in dogs with OSA.¹² Others have reported associations between peripheral blood monocyte counts and outcomes in humans with OSA.¹³ Hence, we evaluated the flow cytometry data in conjunction with peripheral blood monocyte counts, using 400 cells/ μ L as a differentiation point between high and low monocyte counts according to previously reported findings.¹² There was no significant difference in the peripheral blood monocyte counts of dogs with OSA (mean = 514.7 cells/ μ L, SD = 377.9 cells/ μ L) compared to healthy controls (mean = 331.9 cells/ μ L, SD =

192.6 cells/ μ L; $P = .16$). Comparison of receptor expression between dogs with high (>400 cells/ μ L) versus low (<400 cells/ μ L) monocyte counts revealed a dichotomy of CD14, CD16 expression in dogs with OSA— CD14 expression is high ($P = .01$) and CD16 expression is low ($P = .02$) when monocyte counts are high (Table 3). Least-squares means from the 2-way ANOVA comparing relationships between monocyte counts and receptor expression are shown in Table S1.

In healthy controls, there were strong significant positive correlations between chemokine receptors, whereas in dogs with OSA, there were fewer and less robust significant positive correlations between chemokine receptors (Fig 2). The Pearson's correlation values with corresponding P -values are represented in Table S2.

Relative mRNA Expression of Monocyte IL-10, IL-12, TNF- α , and COX-2

TNF α , IL-10, and IL-12 are inflammatory mediators secreted by monocytes and macrophages, and differential levels of TNF α , IL-10, and IL-12 secretion have been described for the various human monocyte subsets,^{14,15} reflecting subset-specific functional differences. COX-2 expression in monocytes reflects a proinflammatory state.^{16–18} We compared relative mRNA expression of IL-10, IL-12, TNF α , and COX-2 in dogs with OSA versus healthy controls to discern potential variation in cytokine secretion that might reflect functional differences. mRNA expression analysis of monocytes from untreated dogs with OSA and healthy controls revealed no significant differences in relative mRNA expression levels of IL-10 ($P = .44$), IL-12 ($P = .41$), and TNF- α ($P = .14$) between the 2 groups. The relative mRNA expression of COX-2 was decreased in dogs with OSA compared to healthy controls, but the difference was not significant ($P = .08$).

Evaluation of IL-10, IFN- γ , TNF- α , and prostaglandin E₂ secretion from monocytes

As mRNA levels might not consistently correspond with protein secretion, we analyzed monocyte secretion of the cytokines IL-10, IFN- γ , TNF- α , and PGE₂. Similar to our mRNA evaluation, we compared cytokine secretion from monocytes of dogs with OSA versus healthy controls to assess for functional differences. Secretion of cytokines such as TNF- α , IL-10, IFN- γ , and PGE₂ has been noted to differ between human monocyte subsets, and can be affected by disease.^{15,17} PGE₂ ELISA and the MILLIPLEX[®] assay revealed increased PGE₂ levels ($P = .04$) and increased TNF- α levels ($P = .02$), respectively in supernatants of cultured monocytes stimulated with LPS of untreated dogs with OSA compared to normal controls (Fig 3a, b). The median concentrations and ranges of PGE₂ concentrations were 347.36 pg/mL, 103.4–1268.5 pg/mL for dogs with OSA, and 136.23 pg/mL, 69.93–542.6 pg/mL for healthy controls. The median concentrations and ranges of TNF- α levels were 934.7 pg/mL, 644.3–2709 pg/mL for dogs with OSA, and 267.2 pg/mL, 70.4–670.4 pg/mL for healthy

Table 3. Comparison of receptor expression between dogs with high (>400 cells/ μ L, n = 11) versus low (<400 cells/ μ L, n = 17) monocyte counts. In dogs with OSA, when monocyte counts are high, there is a dichotomy of CD14, CD16 expression—CD14 is high ($P = .01$) and CD16 is low ($P = .02$).

Chemokine receptor	Monocyte counts	Control Dogs		Dogs with OSA	
		Median (% expression)	<i>P</i> -value	Median (% expression)	<i>P</i> -value
CD14	High	11.9	.90	37.5	.01
	Low	7.7		13.9	
CD16	High	2.8	.15	5.7	.02
	Low	11.3		37.3	

controls. Levels of IL-10 and IFN- γ were low to undetectable in both groups.

Monocyte Chemotaxis

The results of our phenotypic analysis of monocytes between dogs with OSA and healthy controls revealed marked decreases in chemokine receptor expression in dogs with OSA (Fig 1b,c). Therefore, we asked whether monocyte chemotaxis might be altered by OSA. As described in our methods, monocytes from OSA and healthy dogs were assessed by the NeuroProbe ChemoTx[®] cell migration system. Comparison of monocyte chemotaxis between dogs with OSA and healthy controls revealed significantly decreased chemotaxis in dogs with OSA when CCL19 ($P = .018$), and when a mixture of all 4 chemoattractants ($P = .018$) was used (Fig 4). With CCL2 or fMLP as chemoattractants, there was also decreased monocyte chemotaxis in dogs with OSA compared to healthy controls, but the difference did not reach significance (CCL2 $P = .067$; fMLP $P = .067$).

Survival Analysis

As a peripheral monocyte count of >400 cells/ μ L has been significantly associated with decreased disease-free interval in dogs with OSA,¹² we performed a Kaplan-Meier survival analysis comparing dogs with OSA with high or low monocyte counts, using 400 cells/ μ L as a cut-off point. One dog died of unknown cause, and was assumed to have died from OSA. We did not find a statistically significant difference in median survival between the 2 groups ($P = .15$, Fig 5). Our regression analysis revealed that increasing percentages of CCR2 expression on peripheral monocytes was significantly associated with increasing survival in dogs with OSA ($P < .0001$).

Discussion

The goal of this study was to determine phenotypic and functional differences between peripheral blood monocytes from untreated dogs with OSA versus healthy age-matched controls. Our hypothesis was that evasion of the immune response by OSA is due in part to down-regulation of monocyte chemokine receptor expression and migratory function, and suppression of host immune responses. We demonstrated a significant

decrease in monocyte chemokine receptors, increased PGE₂ and TNF- α secretion by monocytes, and decreased monocyte chemotaxis in dogs with OSA.

Monocytes display a variety of cell surface receptors, including leukocyte chemokine receptors. CD62L, also known as L-selectin, is a cell adhesion molecule present on immune cells such as monocytes, lymphocytes, and NK cells. CD62L mediates the trafficking of these cells to sites of inflammation and peripheral lymphoid tissue by binding to glycoproteins.^{19,20} CCR2, a chemokine receptor for MCP-1, is especially prominent in mediating monocyte migration.²¹ Similarly, CCR7 regulates migration of leukocytes to secondary lymphoid organs via binding of its ligands CCL19 and CCL21.²⁰ CD43 can be found on a variety of immune cells including lymphocytes, granulocytes, monocytes, macrophages, and NK cells, and it plays a role in cell adhesion and migration.^{22,23} CX₃CR1, and CXCR2, both G protein-coupled chemokine receptors, mediate lymphocyte, monocyte and granulocyte migration.²⁰ There are few reports describing the effect of OSA on monocyte surface receptor expression in dogs. For example, down-regulation of MHC class II and CD80 in canine myeloid cells when exposed to tumor cell lines including OSA *in vitro* has been observed.²⁴ In humans, the prevalence of an immunosuppressive phenotype in peripheral blood monocytes of OSA patients has been observed.¹³ However, to the authors' knowledge, there are no reports evaluating phenotypic changes in monocytes of clinical dogs with OSA.

We assessed the expression of monocyte surface receptors using flow cytometry, and demonstrated that peripheral blood monocytes from dogs with OSA exhibited significantly decreased chemokine receptor expression, namely of CD62L, CCR2, CCR7, CD43, CX₃CR1, and CXCR2. This pronounced down-regulation of monocyte chemokine receptors of dogs with OSA suggests a peripheral sequestration of monocytes, inhibited from migrating to sites of need such as the primary tumor or metastatic lesions. Two of the chemokine receptors, CCR2 and CXCR2, had significantly higher expression on monocytes of healthy controls with low monocyte counts compared to monocytes of dogs with OSA that had high or low monocyte counts. This supports our theory that the decreased expression of chemokine receptors in dogs with OSA reflects a peripheral sequestration. There was no difference in the mean peripheral monocyte counts between dogs with

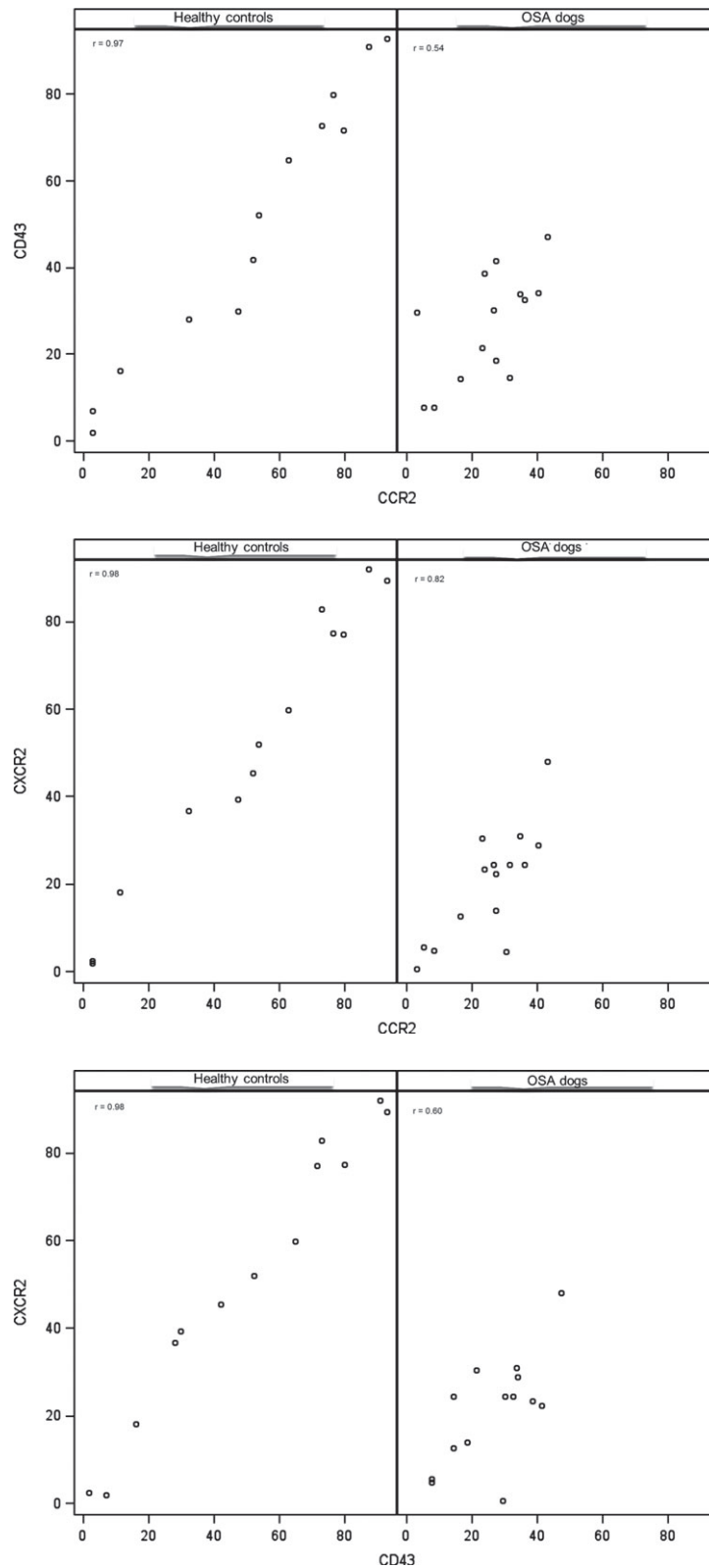


Fig 2. Chemokine receptor correlation is disrupted in OSA: Correlations between chemokine receptors in healthy controls (n = 13) versus dogs with OSA (n = 18). Representative dot plots displaying correlations between pairs of monocyte chemokine receptors CD43 and CCR2 (n = 13 for healthy controls; n = 14 for dogs with OSA), CXCR2 and CCR2 (n = 13 for control dogs; n = 15 for dogs with OSA), CXCR2 and CD43 (n = 13 for healthy controls; n = 14 for dogs with OSA). There are strong positive correlations between chemokine receptors in healthy controls, compared to the less robust correlations between receptors in dogs with OSA.

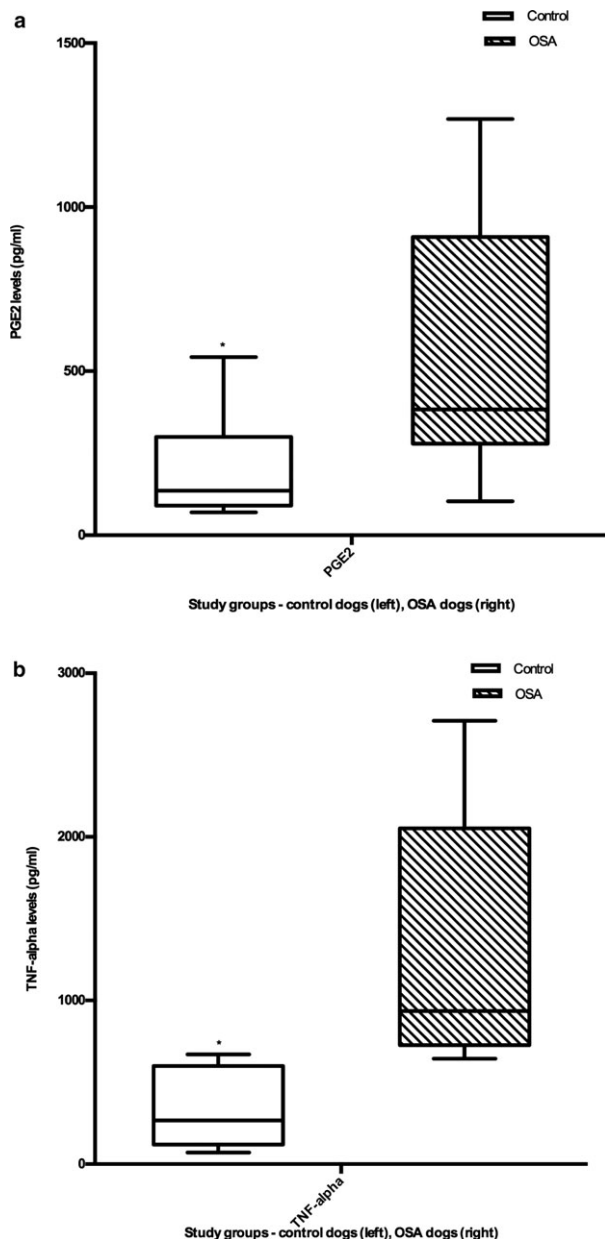


Fig 3. Monocyte PGE₂ and TNF- α secretion are increased in OSA: Box-and-whisker plots displaying the levels of (a) PGE₂ (pg/mL) in monocyte culture supernatants of healthy controls (n = 8) versus dogs with OSA (n = 7), and (b) TNF- α (pg/mL) in monocyte culture supernatants of healthy controls (n = 5) versus dogs with OSA (n = 5). Monocytes from dogs with OSA secreted significantly higher levels of PGE₂ ($P = .04$) and TNF- α ($P = .02$) compared to healthy control monocytes.

OSA and healthy controls, which could indicate that this parameter is not as sensitive for detecting a peripheral sequestration compared to analyzing the relationship between chemokine receptors and monocyte counts in both groups of dogs. Alternatively, the lack of difference in mean monocyte counts could also refute our theory of peripheral monocyte sequestration in OSA. We found a strong positive correlation between expression of chemokine receptors in healthy controls,

whereas the correlation was less robust in dogs with OSA, suggesting a disturbance in the regulation of these chemokine receptors.

A retrospective study of dogs with OSA found that dogs with peripheral monocyte counts above 400 cells/ μ L had a significantly shorter disease-free interval.¹² Similarly, in human OSA patients, a low peripheral lymphocyte-to-monocyte ratio (≤ 3.43) was associated with poorer prognosis for survival and disease-free interval.²⁵ One possible explanation is that higher monocyte counts reflect a peripheral sequestration of monocytes in dogs that had more rapid tumor progression, and our findings of suppressed expression of multiple monocyte chemokine receptors in dogs with OSA suggest such an association. We did not detect a significant overall difference in peripheral monocyte counts of dogs with OSA versus healthy controls in our study population. Our Kaplan-Meier survival analysis comparing dogs with OSA with high (>400 cells/ μ L) versus low (<400 cells/ μ L) monocyte counts did not reveal significant differences in survival between groups. However, we did find that increasing percentages of CCR2 expression on monocytes of dogs with OSA was significantly associated with increasing survival, which, in conjunction with our chemotaxis data, suggests that suppression of chemokine receptors and chemotaxis is a form of tumor-mediated monocyte dysfunction.

Additionally, we found that dogs with OSA with a peripheral monocyte count of >400 cells/ μ L exhibited a dichotomy of CD14, CD16 expression on monocytes—CD14 expression was high and CD16 expression was low, when compared to dogs with monocyte counts <400 cells/ μ L. Human monocytes have been classified into subsets, with different phenotypes and functions. Earlier classification schemes split human monocytes into CD14^{high}CD16⁻ “inflammatory, classical” and CD14^{low}CD16⁺ “resident, nonclassical” monocytes.^{26,27} Recent studies have shown that the CD16⁺ monocytes can be further characterized into intermediate and nonclassical subsets, with the intermediate subset expressing higher CD14 and lower CD16 levels, and the nonclassical subset conversely expressing lower CD14 and higher CD16 levels.¹⁵ The function of these monocyte subsets has not been fully defined, but the intermediate subset appears to produce proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, express high levels of MHC II antigen processing and presentation genes, and are potent stimulators of T cells.¹⁵ There are no corresponding studies differentiating canine monocytes into subsets. Based upon human classification, our data suggest that there is an increase in intermediate monocytes in dogs with OSA with monocyte counts >400 cells/ μ L. Potentially, these intermediate monocytes, with their proinflammatory and immunostimulatory capacity, are effective antitumor monocytes that are sequestered in the periphery in OSA.

Monocyte migration can reportedly be affected by neoplasia. Peripheral monocyte migration is inhibited by lactate concentrations common in tumor microenvironments, and this loss of migratory ability is not because of a reduction in cell viability.²⁸ Chemokine

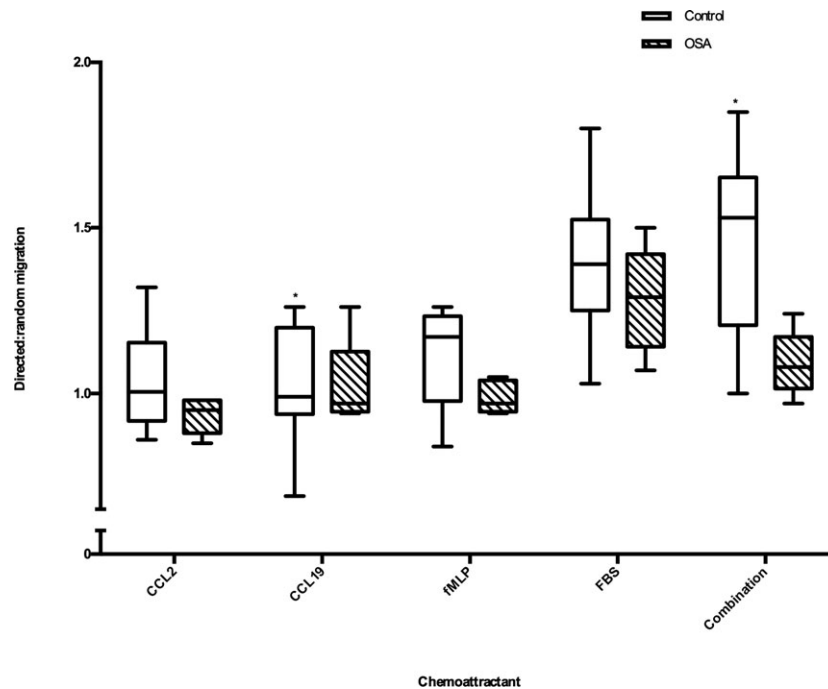


Fig 4. Monocyte chemotaxis is decreased in OSA: Box-and-whisker plot comparing the mean levels of random: directed monocyte migration for the various chemokines used, either alone or in combination (CCL2, CCL19, FBS, fMLP) between healthy controls ($n = 14$) and dogs with OSA ($n = 5$). Monocyte migration is significantly reduced in dogs with OSA when CCL19 ($P = .018$) or a combination of chemokines ($P = .018$) was used as chemoattractants. Decreased migration in dogs with OSA was also observed with CCL2 or fMLP as chemoattractants, but the difference did not reach significance (CCL2 $P = .067$; fMLP $P = .067$).

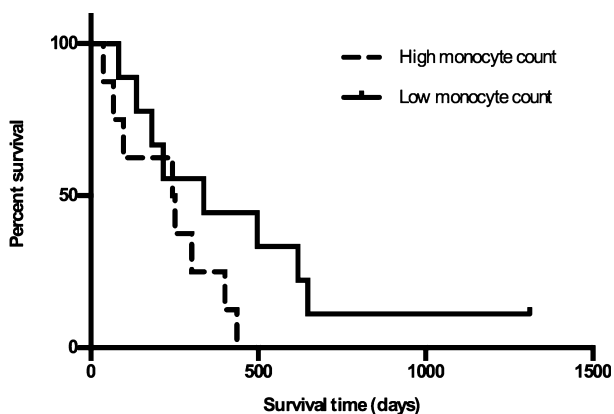


Fig 5. Peripheral blood monocyte counts do not affect survival in OSA: Kaplan-Meier analysis of survival times comparing dogs with OSA with high (>400 cells/ μL ; $n = 8$) versus low (<400 cells/ μL ; $n = 9$) monocyte counts did not reveal any significant difference between the 2 groups ($P = .15$). Median survival of dogs with high monocyte counts was 247.5 days, versus 337 days in dogs with low monocyte counts.

receptors such as CCR5 are down-regulated in monocytes and macrophages in human patients with head and neck squamous cell carcinoma, leading to dysregulation of chemotactic receptor-ligand signaling and decreased monocyte/macrophage migratory ability.²⁹ Based on findings by other investigators and our demonstration of profound down-regulation of

monocyte chemokine receptors in dogs with OSA, we hypothesized that OSA inhibits monocyte chemotaxis as an immunosuppressive strategy. Using an in vitro migration assay, we demonstrated decreased monocyte chemotaxis in dogs with OSA compared to healthy controls. This difference was significant when CCL19 was used as a chemoattractant, and when supraphysiologic combinations of chemoattractants were used—a combination of FBS, CCL2, CCL19, and fMLP. CCL19 is a ligand for the chemokine receptor CCR7, and is a known monocyte chemoattractant.³⁰ There was also decreased monocyte migration in dogs with OSA when other known chemokines such as CCL2 or fMLP were used, and even though the differences were not significant, the proximity of the P -value (.067) to significance (.05) suggests that use of these chemokines deserves further evaluation. CCL2 is also known as monocyte chemoattractant protein, binds CCR2, and together with fMLP, which binds to G protein-coupled receptors, is used as chemoattractants for monocytes. The significant down-regulation of CCR2 and CCR7 on monocytes of dogs with OSA demonstrated by our flow cytometry data supports the observation that monocyte migration is decreased in dogs with OSA. The primary disadvantage of our method for assessing migration was the high level of random migration observed, potentially because of excessive stimulation of monocytes by the cell sorting process. As a result, we included supraphysiologic combinations of chemoattractants to counter this potential issue.

There could be a multi-faceted explanation for the down-regulation of monocyte chemokine receptors in dogs with OSA. Similar to our findings, the loss of CX₃CR1 expression in human monocytes increased glioma growth.³¹ However, in contrast to our observations, this tumor-promoting effect is not because of loss of migratory capability. In fact, the absence of CX₃CR1 was associated with an increase in monocyte infiltration into the tumor microenvironment, where the monocytes were theorized to differentiate into tumor-associated macrophages for promotion of tumor growth. Evaluation of the relationship between monocyte chemokine receptor expression and the infiltration of monocytes/macrophages in the tumor microenvironment in OSA is needed to explore whether this phenomenon occurs in dogs.

The higher PGE₂ levels that we observed in monocyte culture supernatants of dogs with OSA are consistent with our hypothesis that OSA suppresses monocyte chemotaxis and the host immune response. PGE₂ is a main metabolite resulting from the conversion of arachidonic acid by cyclooxygenases (COX-1 and COX-2), and increased levels of COX-2 and PGE₂ have been observed in various tumors.^{32–35} PGE₂ secretion from human carcinoma cell lines inhibits monocyte chemotaxis by down-regulating cell surface expression of chemokine and adhesion receptors CCR5 and Mac-1, respectively.³⁶ Our finding of increased PGE₂ secretion from monocytes of dogs with OSA correlates with our observation that monocyte chemotaxis is inhibited in OSA. Increased PGE₂ levels have been shown to effect global immunosuppression, contributing to immunopathology in many cancers.³⁷ For example, PGE₂ disables the innate immune response by inhibiting neutrophils, monocytes and macrophages, and disrupts the cross-talk between dendritic cells and T cells that is essential for activation of the adaptive immune response. PGE₂ also skews T helper cells toward a type 2 pro-tumorigenic instead of a type 1 antitumor response, and promotes the accumulation of T regulatory and myeloid-derived suppressor cells, which can be utilized by the tumor to suppress an effective antitumor response.³⁷ A recent study in human OSA showed inhibition of human OSA cells by decreasing PGE₂ levels via microRNA modulation, demonstrating the role of PGE₂ in tumor promotion.³⁸ Specifically in OSA of dogs, PGE₂ is increased in both canine OSA cell lines and in naturally occurring tumors,³⁹ COX-2, microsomal PGE₂ synthase-1, and PGE₂ receptor are demonstrated to be increased in OSA lesions using immunohistochemistry staining,⁴⁰ and OSA cells from dogs secrete PGE₂.⁴¹ Our findings of increased PGE₂ in dogs with OSA are in accordance with these previous reports, and emphasize the ability of PGE₂ to induce immunopathology in OSA.

We did not find significant differences in relative mRNA expression of IL-10, IL-12, TNF- α , and COX-2 in monocytes from OSA compared to healthy dogs. There was a decreasing trend in COX-2 expression in dogs with OSA, but the difference from healthy controls was not significant. This is contrary to what we expected, given the higher PGE₂ secretion from

monocytes of dogs with OSA, and the overexpression of COX-2 that has been reported in OSA of dogs.^{40,42} Potentially, there might be other enzymes such as microsomal PGE₂ synthase-1 that could be concurrently influencing the level of PGE₂ secretion. However, as the difference in COX-2 expression between OSA and healthy dogs was not statistically significant, whether COX-2 expression in monocytes of dogs with OSA is truly decreased needs to be further evaluated, as other influences such as miRNA activity can affect mRNA transcription. Because of potential disparities between mRNA and protein expression, we evaluated monocyte secretion of TNF- α , IFN- γ , and IL-10, using IFN- γ as representative of IL-12 activity. There were low to undetectable levels of IFN- γ and IL-10 from both groups, and higher levels of TNF- α secreted from monocytes of dogs with OSA. This was an unexpected finding, as TNF- α is a proinflammatory cytokine. However, TNF- α induces a number of other changes, particularly in the context of the tumor microenvironment. TNF- α induces the up-regulation of IL-34 expression in OSA cell lines, and IL-34 has been associated with progression of disease and promotion of angiogenesis in OSA.⁴³ TNF- α has also been shown to increase the production of PGE₂ as a mechanism for effecting bone resorption.⁴⁴ Potentially, the increase in TNF- α in monocytes of dogs with OSA reflects tumor-driven mechanisms for OSA promotion rather than induction of a proinflammatory monocyte response. Other immunomodulatory cytokines such as TGF- β are utilized by OSA for tumor promotion. TGF- β , generally known to dampen immune responses and promote tumor growth, is secreted by OSA cells from dogs,⁶ and has also been implicated in the pathogenesis of human OSA.⁴⁵ Collectively, these findings indicate a complex microenvironment that promotes immune dysfunction and tumor growth.

One potential limitation of this study is the use of clinical dogs and random source healthy controls, instead of purpose-bred dogs which provide a genetically uniform study population. However, one strong advantage of using nonpurpose-bred dogs is the genetic diversity that accurately reflects that of the general pet population. This diversity, together with the exposure to shared environmental variables with humans, contributes to the unique canine model of spontaneously occurring OSA that bears striking similarities to the human disease. Such a model is extremely valuable for studying the disease to improve outcomes for both humans and dogs.

In conclusion, this study demonstrated that monocyte chemokine receptors are markedly down-regulated in dogs with OSA compared to healthy controls, and monocytes from dogs with OSA exhibit decreased chemotactic capacity and secrete higher levels of PGE₂ and TNF- α . Our findings strongly suggest that OSA in dogs evades the immune system by suppressing chemokine receptor expression and increasing PGE₂ secretion in monocytes, thus inhibiting their migration, leading to sequestration in the periphery. Increased PGE₂ levels could also promote immunosuppression, as PGE₂ can

exert a multitude of immunopathologic effects such as interfering with lymphocyte proliferation and the antitumor cytotoxicity of T cells, inhibit NK cell, dendritic cell, neutrophil, monocyte and macrophage effector functions. Increased TNF- α secretion could be a tumor-promoting mechanism through manipulation of the tumor microenvironment and elevating PGE₂ production. Understanding the mechanisms by which OSA causes dysregulation of the immune response will provide insight into developing novel immunotherapeutics to reverse tumor-induced immunopathology and reduce metastatic disease in OSA.

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

Footnotes

- ^a Sigma-Aldrich, St. Louis, MO (Histopaque, fMLP, LPS)
^b Beckman-Coulter, Atlanta, GA (Cell sorter)
^c Qiagen, Germantown, MD (RNAProtect, RNEasy minikit)
^d AbDSerotec, Raleigh, NC (CD14)
^e AbCam, Cambridge, MA (CD16, CD32, rabbit polyclonal)
^f BD Biosciences Pharmingen, San Diego, CA (CD62L, IgG2a PE, IgG1 FITC)
^g Thermo Scientific, Waltham, MA (CD11c, FBS)
^h Bioss Inc, Woburn, MA
ⁱ BD Biosciences, San Jose, CA (FACS Calibur)
^j Bibby Scientific, Burlington, NJ (TC4000 thermal cycler)
^k Promega, Madison, WI (Promega RT kit)
^l Integrated DNA Technologies, IO (Primers for PCR)
^m Quanta Biosciences, Gaithersburg, MD (PCR SYBR Green kit)
ⁿ Bio-Rad, Hercules, CA (iCycler)
^o Cayman Chemical, Ann Arbor, MI (PGE2 ELISA kit#514010)
^p EMD Millipore, Billerica, MA (CCYTOMAG-90K MILLI-PLEX MAP kit)
^q NeuroProbe, Gaithersburg, MD (Chemotaxis assay)
^r AnaSpec Inc., Fremont, CA (Calcein)
^s Corning CellGro® (Mediatech Inc), Manassas, VA (DMEM)
^t R&D Systems, Minneapolis, MN (MCP-1, CCL19)
^u Molecular Devices, Sunnyvale, CA (Plate-reader)

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Agarose gel electrophoresis for RNA and DNA assessment.

Table S1. Least-squares means from the 2-way ANOVA comparing relationships between monocyte counts and receptor expression.

Table S2. Pearson's correlations between chemokine receptors in OSA dogs (n = 18) compared to healthy controls (n = 13).