Original article

Immune function and serum vitamin D in shelter dogs: A case-control study

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ABSTRACT

This study sought to establish a baseline understanding of immune function and its association with serum vitamin D in shelter dogs. Ten apparently healthy shelter dogs housed in the Arizona Humane Society for ≥ 7 days and 10 apparently healthy, age, breed, and sex-matched control dogs were included. Serum 25-hydroxyvitamin D (25[OH]D), the major circulating vitamin D metabolite, was measured using high performance liquid chromatography. Whole blood samples were stimulated with lipopolysaccharide (LPS), lipoteichoic acid, or phosphate buffer solution, and tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 were measured using a canine-specific multiplex bead-based assay. Phagocytosis of opsonized-*Escherichia coli* and *E. coli*-induced oxidative burst were evaluated with flow cytometry.

Shelter dogs had decreased percentages of granulocytes and monocytes (GM) that had phagocytized opsonized-*E coli* ($P = 0.019$) and performed *E. coli*-induced oxidative burst ($P = 0.011$). There were no significant differences in TNF-α, IL-6, IL-10, or 25(OH)D concentrations between shelter and control dogs. Serum 25(OH)D concentrations had a weak positive association with the intensity of GM *E. coli*-induced oxidative burst ($r^2 = 0.23$, $P = 0.03$). There was a moderate inverse association between serum 25(OH)D concentration and LPS-stimulated TNF-α production in shelter dogs ($r^2 = 0.40$, $P = 0.04$). These results demonstrate immune dysregulation in vitro in shelter dogs housed for ≥ 7 days when compared to age, breed, and sex-matched control dogs. While serum 25(OH)D concentrations did not differ between shelter and control dogs, significant associations between 25(OH)D concentration and immune function parameters in vitro were identified.

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Introduction

Vitamin D is a hormone that is best known for its role in bone metabolism and regulation of calcium and phosphorus homeostasis; however, vitamin D also has an important immunologic role identified in many different species (Willis et al., 2012; Djukic et al., 2014; Rodriguez-Lecompte et al., 2016; Garcia-Barragan et al., 2018; Jaffey et al., 2018a, 2018b; 2018c). Several in vitro studies in humans have demonstrated that calcitriol, the active metabolite of vitamin D, increases production of antimicrobial peptides (Martineau et al., 2007, 2017), modulates inflammation via decreased production of tumor necrosis factor (TNF)-α (Martineau et al., 2007), as well as enhances induction of both leukocyte reactive nitrogen/oxygen species (Rockett et al., 1998; Sly et al., 2001) and phagocytosis (Bachhetta et al., 2014). These immunomodulatory functions highlight the importance of vitamin D in mucosal immunity. Numerous studies have shown that serum concentrations of 25-hydroxyvitamin-D (25[OH]D), the major circulating analog of vitamin D, are consistently lower in humans with community acquired pneumonia and upper respiratory tract infections than their healthy control counterparts (Cannell et al., 2006; Urashima et al., 2010; Pletz et al., 2014; Sainsbury et al., 2014; Hayward et al., 2018). Moreover, a recent meta-analysis concluded that oral vitamin D supplementation to school children was safe and protected against acute respiratory tract infections (Martineau et al., 2017).

Hypovitaminosis-D has been identified in association with multiple canine infectious diseases including blastomycosis (O'Brien et al., 2018), leishmaniasis (Rodriguez-Cortes et al., 2017), babesiosis (Dvir et al., 2019), and spirocercosis (Rosa et al., 2013), which suggests low vitamin D could contribute to the development of these infections through vitamin D deficiency-related immune dysregulation. This theory on pathogenesis is especially interesting if applied to dogs in animal shelters. Community acquired infections including canine infectious

respiratory disease complex have a high prevalence in animal shelters and represent a substantial drain on resources, decreases adoption rates, and can result in euthanasia. The specific reason for the high frequency of infections in shelter dogs is unknown and likely multifactorial; however, there are several variables that have the potential to impact immune function in shelter dogs, including unknown prior health care or food availability. Shelter dogs could have hypovitaminosis-D because dogs are dependent on food to fulfill their vitamin D requirements (How et al., 1994). There is little currently known about the immune function of shelter dogs, their vitamin D status, or whether an association could exist between them.

The identification of dysregulation of specific components of the immune response in shelter dogs will allow for a better understanding of the pathogenesis of community-acquired infections, as well as pave the way for future studies to investigate targeted therapies to reconcile immune function in hopes of decreasing the incidence, morbidity, or both, of community-acquired infections in shelters. Moreover, the identification of hypovitaminosis-D or associations between serum vitamin D and immune function would provide the rationale needed for future studies to determine whether oral vitamin D supplementation in shelter dogs has the potential to augment immune function. As such, there are three main objectives to this study: (1) to compare granulocyte/monocyte (GM) phagocytic and oxidative burst capacities, as well as leukocyte production of TNF-α, interleukin (IL)-6, and IL-10 in shelter and control dogs; (2) to compare serum 25(OH)D concentrations in shelter and control dogs; and (3) to determine if serum 25(OH)D concentrations are associated with GM phagocytic and oxidative burst functions or leukocyte production of TNF-α, IL-6, or IL-10. We hypothesized that shelter dogs would have decreased serum 25(OH)D concentrations, GM phagocytic and oxidative burst functions, and leukocyte production of TNF-α, IL-6, and IL-10 compared to non-shelter control dogs. Further, we hypothesized that serum 25(OH)D concentrations would be associated with GM phagocytic and oxidative burst functions, as well as leukocyte production of TNF-α, IL-6, and IL-10.

Material and methods

Animals

The study protocol was approved by the Midwestern University Animal Care and Use Committee (Protocol number 2932 CRC 72; Approval date, 24 May 2019). A convenience sample of 10 apparently healthy dogs from the Arizona Humane Society was enrolled in this study. Dogs housed in the shelter for $\geq$7 days were selected from a list that included medical record number and time spent in the animal shelter only. A single veterinarian (NBS) made selections and had no previous knowledge of signalment or medical history of eligible dogs. Inclusion criteria consisted of being housed in the shelter for $\geq$7 days, unremarkable physical examination (performed by a veterinarian), testing negative for *Dirofilaria immitis* antigen and antibodies to *Anaplasma phagocytophilum, Anaplasma platys, Ehrlichia canis, Ehrlichia ewingii,* and *Borrelia burgdorferi* C6 peptide using a commercial ELISA-based kit (SNAP 4Dx Plus Test kit, IDEXX Laboratories). Exclusion criteria included dogs pregnant or lactating, if they had a recent surgical procedure, if any illness was recorded since the time of shelter admission (e.g. clinical signs associated with respiratory or gastrointestinal disease, or both), or if they had been administered medications other than routine parasitic prevention or fenbendazole (administered prophylactically to all dogs). A second population of 10 apparently healthy, non-shelter dogs were enrolled as a control population, which were matched by age ($\pm$2 years), breed, and sex (including castration status). The control dogs were determined to be apparently healthy based on history and physical examination. Control dogs could not have had any illnesses, received any medications, with the exception of routine parasitic prevention within 60 days of enrollment. Owner consent was obtained prior to sample collection from all dogs.

Blood sample collection and processing

A 10 mL blood sample was collected from each dog via jugular venipuncture into two tubes (serum separator tube and a tube with lithium heparin as an anticoagulant) and processed within 1 h of sample collection.

Leukocyte cytokine production

Whole blood from the tubes containing lithium heparin were diluted 1:2 with RPMI 1640 culture medium containing 200 U of penicillin/mL and 200 mg of streptomycin/mL, transferred to 24-well plates, and stimulated with lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 (final concentration, 100 ng/mL, Sigma Aldrich, St Louis, MO), lipoteichoic acid (LTA) from *Streptococcus faecalis* (final concentration, 1 μg/mL, Sigma Aldrich), or phosphate-buffered saline (PBS)-negative control substance. Plates were incubated for 24 h at 37 °C in 5% CO_2 in the dark. Following incubation, the plates were centrifuged (400g for 7 min) at 21 °C. The supernatant was collected and stored at −80 °C for batch analysis. For analysis, samples were thawed, and then TNF-α, IL-6, and IL-10 were measured in supernatant with a canine cytokine-specific multiplex bead-based assay (Milliplex MAP, EMD Millipore Corp) as described elsewhere (Jaffey et al., 2018a). The median fluorescence intensity and cytokine concentration in each sample was measured in duplicate with appropriate controls and associated data analysis software (Milliplex Analyst version 5.1, EMD Millipore Corp). The lower limit of detection for each cytokine in our laboratory was 48.8 pg/mL. Furthermore, the intra-assay coefficient was <5%, and the inter-assay coefficient of variation was <15%.

Phagocytosis of E. coli

Phagocytic function of GM was determined using a commercial test kit (PhagoTest; Orpegen Pharma), validated for use in canines. Briefly, 100 μL of heparinized whole blood was combined with 20 μL of either FITC-labeled, opsonized-*E. coli* strain LE392 or washing solution (negative control) and the samples were incubated in a 37 °C water bath for 10 min. The samples were placed on ice to arrest phagocytosis and 100 μL of quenching solution was added to quench surface bound FITC-labeled-*E. coli*. The cells were washed, erythrocytes lysed, and all cells were washed again before 200 μL of DNA staining solution (containing R-phycoerythrin, which binds to eukaryotic diploid DNA) was added to facilitate exclusion of aggregated artifacts of bacteria or cells that do not have intact DNA to bind.

Oxidative burst induced by E. coli

Oxidative burst function in GM was determined using a commercial test kit (PhagoBurst; Orpegen Pharma), validated for use in canines. Briefly, 100 μL of heparinized whole blood from each dog was incubated with 20 μL of opsonized-*E. coli* strain LE392 or control solution for 10 min in a 37 °C water bath. Next, samples were incubated with 20 μL of dihydrorhodamine-123 as a substrate for oxygen intermediates for 10 min at 37 °C in a water bath. After cessation of this reaction, erythrocytes were lysed, the cells were washed, and 200 μL of DNA staining solution (containing R-phycoerythrin, which binds to eukaryotic diploid DNA) was added to facilitate exclusion of aggregated artifacts of bacteria or cells.

Flow cytometry

Flow cytometry was performed at the Midwestern University College of Veterinary Medicine Immunology Laboratory using a flow cytometer (Guava easyCyte HT, Luminex Corporation) and associated data analysis software (GuavaSoft 3.2, Luminex). A minimum of 20,000 events/sample were recorded (Fig. 1). For assessment of phagocytosis, data was recorded as the percentage of GM cells having internalized FITC-labeled *E. coli,* as well as their mean fluorescent intensity (MFI), a method of quantifying the phagocytosed bacteria per cell. Data for assessment of oxidative burst was recorded as the percentage of GM cells having produced reactive oxygen metabolites and the MFI, the relative robustness of oxidative burst reaction produced per cell.

Serum 25(OH)D measurement

Whole blood samples in serum separator tubes were centrifuged (1500 g, 7 min). Serum was harvested within 1 h of sample collection, placed in airtight, freezer-resistant plastic tubes, and stored at −80 °C for batch analysis. Serum concentrations of 25 (OH)D were determined from thawed serum using a modification of a high-performance liquid chromatography method previously reported (Jaffey et al., 2018d).

Statistical analysis

Statistical analysis was performed by commercial software (SigmaStat, Systat Software). Normality was determined using the Shapiro–Wilk test. Descriptive data was normally distributed and thus presented as mean $\pm$ standard deviation (SD). When the measured leukocyte cytokine production fell below the lower limit

of detection for the assay (i.e. 48.8 pg/mL), data were recorded at the lower limit of detection for statistical purposes. Two-tailed, unpaired *t*-tests were used to compare serum 25(OH)D concentrations and immune function parameters between shelter and control dogs. A two-way repeated measured analysis of variance (ANOVA) was used for between-group comparisons (factor 1) of leukocyte production of each TNF-α, IL-6, and IL-10 after stimulation with LPS, LTA, or PBS (factor 2), and post hoc Bonferroni *t*-tests were used for pairwise multiple comparisons. Data from both the shelter dog and its corresponding control dog counterpart were required to be available in order to be included in two-way repeated measures ANOVA tests. Simple linear regression analyses were used to investigate if serum 25(OH)D concentration (independent variable) could predict immunologic outcomes (dependent variables). Linear regression analyses were first performed on data from the entire study population (i.e. shelter and control dogs combined), and then just within shelter dogs. A *P*-value of <0.05 was considered statistically significant.

Results

A total of 20 dogs were included in this prospective case-control study. Demographic data are summarized in Table 1. All shelter dogs >1 year ($n = 9$) were offered a complete dry diet formulated for adult dogs (Hill's Science Diet Adult Dry, Hill's Pet Nutrition) once daily and those aged 0.5–1 year ($n = 1$) were offered a complete dry diet formulated for juvenile dogs (Hill's Science Diet Puppy Dry; Hill's Pet Nutrition) twice daily. The amount of offered food was based on an institution-devised feeding regimen. Diet information was available for nine of the 10 control dogs and comprised one each of: Purina HA Chicken Dry (Nestle Purina); Natural Balance Potato and Duck Dry (The J.M Smucker Company) with $\frac{1}{2}$ cup of cooked
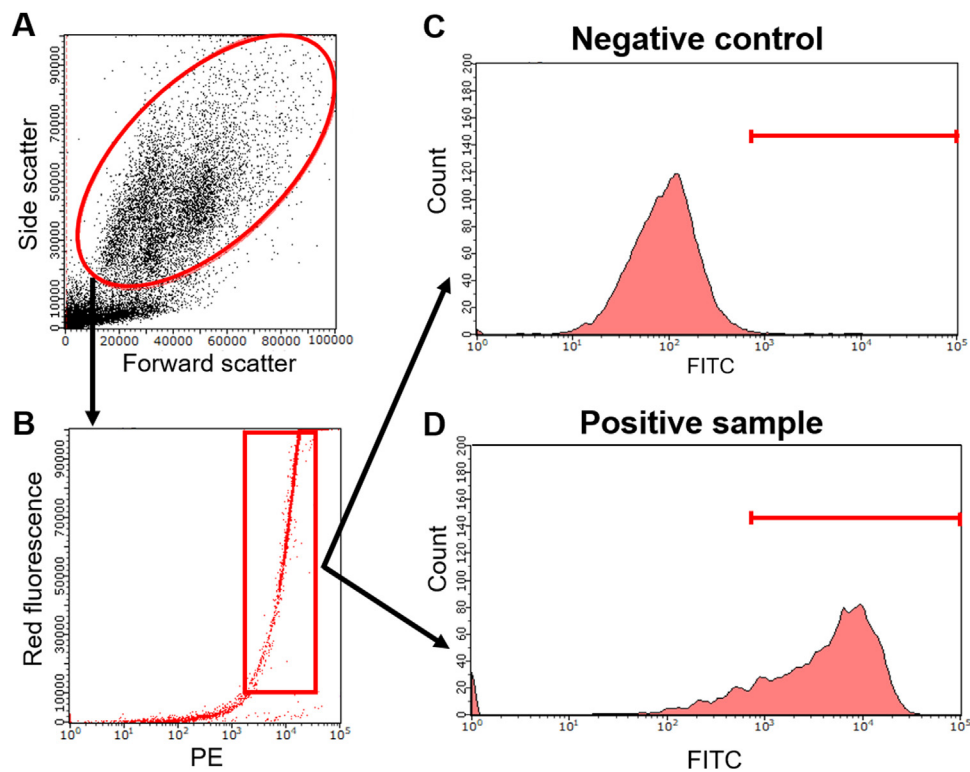

Fig. 1. Gating scheme for flow cytometry of phagocytosis and oxidative burst following exposure to FITC-labeled *Escherichia coli*. Granulocytes and monocytes were gated on a forward versus side scatter plot (A). Subsequently, R-phycoerythrin (PE)-labeled DNA stain was used to exclude aggregates of bacteria or dead cells, with positive-staining cells identified and gated (B). Finally, FITC-staining of these gated cells was recorded in a histogram to determine percentage of FITC-positive cells and their mean fluorescent intensity. Representative histograms of a negative control (C) and a sample containing a majority of FITC-positive cells (D) are shown. The red lines represent gating limits for FITC-positivity.

Table 1
Demographic data for shelter and control dogs.

Variable	Shelter	Control
Breed (*n*)	Chihuahua (4), GSD (2), Pug-mix (1), Pit bull terrier-mix (1), Labrador retriever (1)	Chihuahua (4), GSD (2), Pug-mix (1), Pit bull terrier-mix (1), Labrador retriever (1)
Sex	Male-intact (4), male-neutered (3), female-spayed (2), female-intact (1)	Male-intact (4), male-neutered (3), female-spayed (2), female-intact (1)
Age (years)[a]	4.5 (3.4)	4.7 (3.3)
Bodyweight (kg)[a]	14.4 (9.9)	17.7 (15.5)

GSD, German shepherd dog.
[a] Data presented as mean (standard deviation).
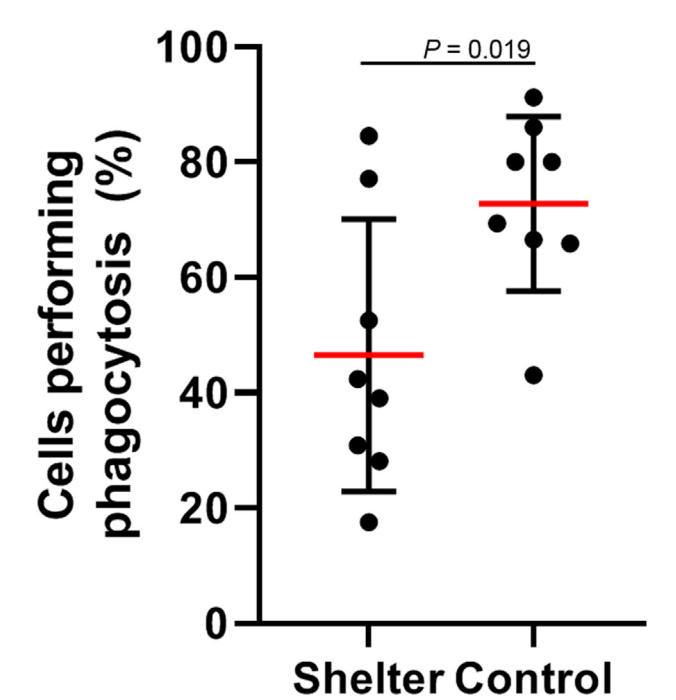
Fig. 2. Mean ± standard deviation (SD) of the percentage of granulocytes and monocytes that phagocytized opsonized-*Escherichia coli* in shelter and control dogs. The red line represents the mean, the black lines correspond to the SD above and below the mean. The black circles represent data for individual dogs.

green beans; Purina Pro Plan Sensitive Skin/Stomach Salmon and Rice Dry (Nestle Purina); Hill's Large Breed Puppy Chicken and Barley Dry (Hill's Pet Nutrition) with occasional Dentastix Beef Flavor (Mars); Pedigree Small Dog Roasted Chicken, Rice &Vegetable Dry (Mars); Purina Beneful Originals with Real Beef Dry (Nestle Purina); Members Mark Salmon & Pea Dry (Sam's Club); Nutro Small

Breed Chicken, Lentil, and Sweet Potato Dry (Mars) with four Milk-Bones (The J.M Smucker Company) per day; and Kirkland Signature Chicken and Rice Can (Diamond Pet Foods). The majority of control dogs (*n* = 7) were offered food two daily and the remaining two dogs were allowed free access to their food.

Serum 25(OH)D measurement

There was not a significant difference between serum 25(OH)D concentrations of shelter dogs (mean, 33.1 ng/mL; SD, 9.9; *n* = 10), compared to control dogs (mean, 32.1 ng/mL; SD, 10.7; *n* = 10; *P* = 0.83).

Phagocytic function and association with serum 25(OH)D concentration

Shelter dogs were found to have a significantly decreased percentage of GM that phagocytized opsonized-*E. coli* (mean, 46.5%; SD, 23.6; *n* = 8) compared to control dogs (mean, 72.8%; SD, 15.1; *n* = 8; *P* = 0.019; Fig. 2). In contrast, there was not a significant difference in the number of phagocytized opsonized-*E. coli* per cell between shelter dogs (mean, 9065.2 bacteria/cell; SD, 4510.8; *n* = 8) and control dogs (mean, 8544.7 bacteria/cell; SD, 2370.1; *n* = 8; *P* = 0.78). There were no significant associations between serum 25(OH)D concentration and the percentage of GM that phagocytized opsonized-*E. coli* or in the number of phagocytized opsonized-*E. coli* per cell (Tables 2 and 3).

Oxidative burst and association with serum 25(OH)D concentration

Shelter dogs had a significantly decreased percentage of GM that performed *E. coli*-induced oxidative burst (mean 48.3%; SD 24.2; *n* = 10) compared to control dogs (mean, 74.1%; SD, 14.8; *n* = 10; *P* = 0.011; Fig. 3). There was not a significant difference in the *E. coli*-induced oxidative burst intensity per cell between shelter dogs (mean 20940.3 per cell; SD 16862.0; *n* = 10) and control dogs (mean,

Table 2
Results of simple linear regression analyses for association between serum 25-hydroxyvitamin D (independent variable) and various immunologic dependent variables in the entire population (i.e. shelter dogs and control dogs).

Dependent variable	*n*	r^2	Coefficient	SE	*P*
Phagocytosis percentage	18	0.00	0.05	0.55	0.93
Phagocytosis per cell	18	0.03	−59.89	79.90	0.46
Oxidative burst percentage	20	0.03	0.41	0.54	0.46
Oxidative burst intensity per cell	20	0.23	688.32	293.30	0.03
LPS – TNF-α	20	0.07	−113.41	100.37	0.27
LPS – IL-6	20	0.08	−19.11	15.24	0.23
LPS – IL-10	20	0.01	−12.14	27.16	0.66
LTA – TNF-α	20	0.06	−105.73	95.57	0.28
LTA – IL-6	20	0.10	−12.68	9.62	0.20
LTA – IL-10	20	0.12	−74.46	47.63	0.14
PBS – TNF-α	20	0.01	3.27	7.75	0.68
PBS – IL-6	20	0.01	−2.10	6.72	0.76
PBS IL-10	20	0.00	0.47	2.37	0.84

SE, standard error; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PBS, phosphate buffered saline; TNF, tumor necrosis factor; IL, interleukin.
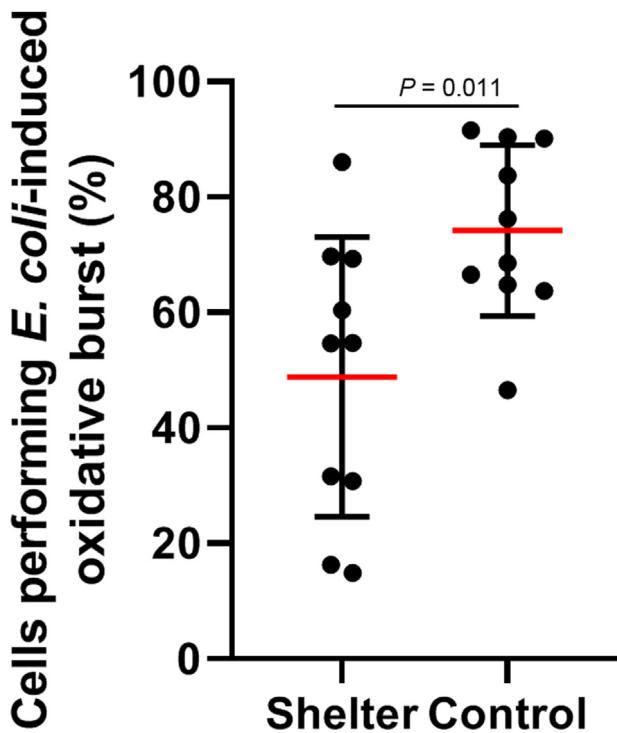
Table 3
Results of simple linear regression analyses for association between serum 25-hydroxyvitamin D (independent variable) and various immunologic dependent variables in the shelter dogs only.

Dependent variable	n	r^2	Coefficient	SE	P
Phagocytosis percentage	8	0.03	−0.35	0.83	0.69
Phagocytosis per cell	8	0.02	−52.32	159.27	0.75
Oxidative burst percentage	10	0.00	−0.06	0.80	0.94
Oxidative burst intensity per cell	10	0.25	791.72	481.67	0.14
LPS − TNF-α	10	0.40	−326.27	141.08	0.04
LPS − IL-6	10	0.02	−4.84	12.74	0.71
LPS − IL-10	10	0.07	−30.49	39.70	0.47
LTA − TNF-α	10	0.27	−279.39	163.09	0.13
LTA − IL-6	10	0.08	−10.32	12.06	0.42
LTA − IL-10	10	0.28	−141.71	80.20	0.12
PBS − TNF-α	10	0.02	4.42	12.51	0.73
PBS − IL-6	10	0.07	8.17	10.79	0.47
PBS IL-10	10	0.14	2.26	1.97	0.29

SE, standard error; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PBS, phosphate buffered saline; TNF, tumor necrosis factor; IL, interleukin.
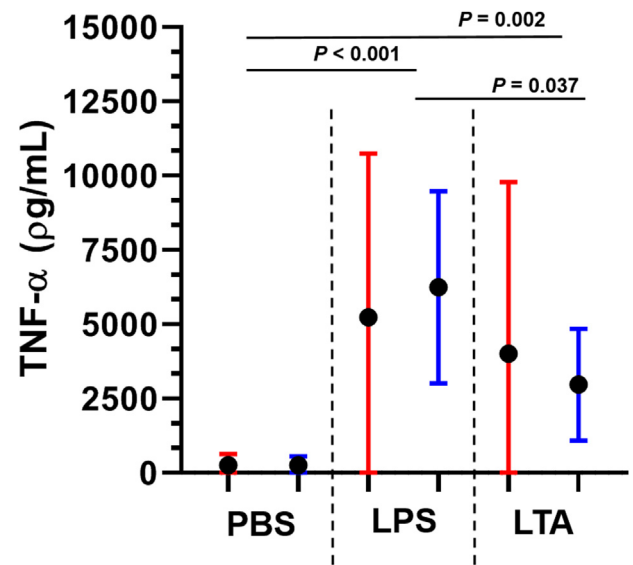

Fig. 4. Two-way repeated measures analysis of variance comparing leukocyte cytokine production of tumor necrosis factor (TNF)-α between shelter dogs (red lines, n = 10) and control dogs (blue lines, n = 10) after incubation of whole blood with phosphate buffered saline (PBS), lipopolysaccharide (LPS), or lipoteichoic acid (LTA). The black circles represent the mean and the bars the standard deviation. There was not a significant between group difference in leukocyte production of TNF-α (P = 0.993) and this difference did not depend on incubation of whole blood with PBS, LPS, or LTA (P = 0.491). Leukocyte production of TNF-α, independent of group was significantly greater for whole blood incubated with LPS compared with PBS (P < 0.001) and LTA (P = 0.037). Moreover, leukocyte production of TNF-α was significantly greater in whole blood incubated with LTA compared with PBS (P = 0.002).

Fig. 3. Mean ± standard deviation (SD) of the percentage of granulocytes and monocytes that performed *E. coli*-induced oxidative burst in shelter dogs and control dogs. The red line represents the mean, the black lines correspond to the SD above and below the mean. The black circles represent data for individual dogs.
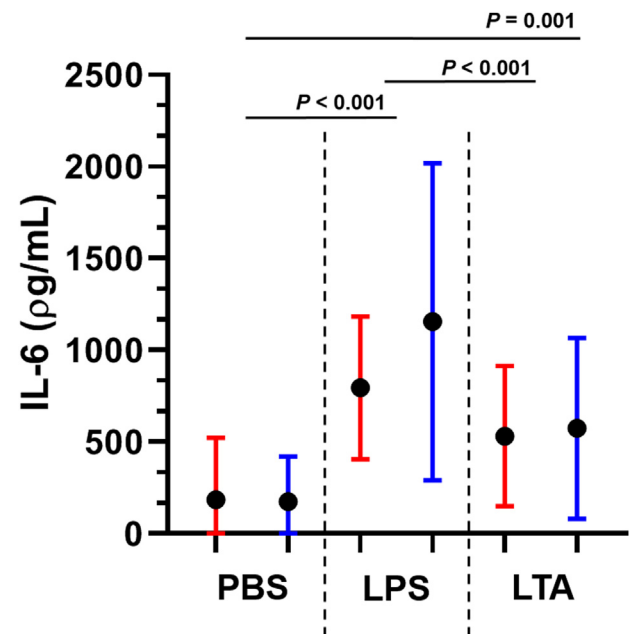
21619.6/cell; SD, 12114.8; n = 10; P = 0.92). Simple linear regression analysis revealed that there was a significant, but weak, positive association between serum 25(OH)D concentration and *E. coli*-induced oxidative burst intensity per cell in the entire population (i.e. shelter dogs and control dogs; Table 2), but not when this analysis was performed in shelter dogs alone (Table 3). There was not an association between serum 25(OH)D and the percentage of GM that performed *E. coli*-induced oxidative burst (Tables 2 and 3).

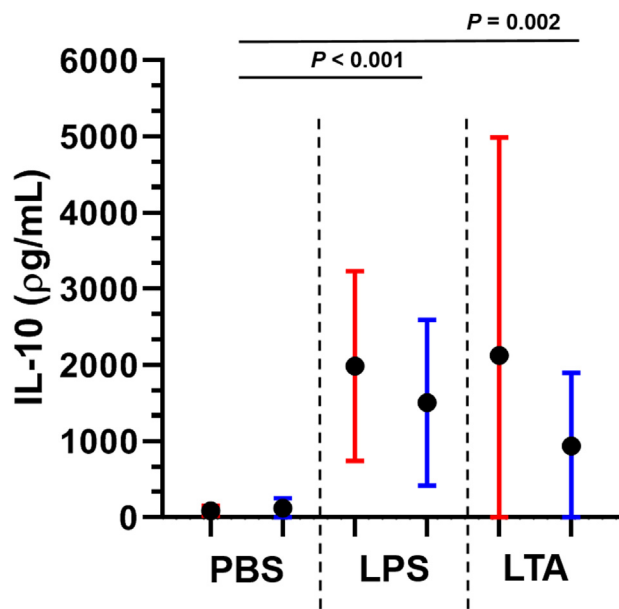*Leukocyte cytokine production and its association with serum 25(OH) D concentration*

There was no significant difference in leukocyte production of TNF-α, IL-6, or IL-10 between shelter dogs and control dogs,

Fig. 5. Two-way repeated measures analysis of variance comparing leukocyte cytokine production of interleukin (IL)-6 between shelter dogs (red lines, n = 10) and control dogs (blue lines, n = 10) after incubation of whole blood with phosphate buffered saline (PBS), lipopolysaccharide (LPS), or lipoteichoic acid (LTA). The black circles represent the mean and the bars the standard deviation. There was not a significant between group difference in leukocyte production of IL-6 (P = 0.501) and this difference did not depend on incubation of whole blood with PBS, LPS, or LTA (P = 0.126). Leukocyte production of IL-6, independent of group was significantly greater for whole blood incubated with LPS compared with PBS (P < 0.001) and LTA (P < 0.001). Moreover, leukocyte production of IL-6 was significantly greater in whole blood incubated with LTA compared with PBS (P = 0.001).

Fig. 6. Two-way repeated measures analysis of variance comparing leukocyte cytokine production of interleukin (IL)-10 between shelter dogs (red lines, n = 10) and control dogs (blue lines, n = 10) after incubation of whole blood with phosphate buffered saline (PBS), lipopolysaccharide (LPS), or lipoteichoic acid (LTA). The black circles represent the mean and the bars the standard deviation. There was not a significant between group difference in leukocyte production of IL-10 (P = 0.244) and this difference did not depend on incubation of whole blood with PBS, LPS, or LTA (P = 0.281). Leukocyte production of IL-10, independent of group was significantly greater for whole blood incubated with LPS (P < 0.001) or LTA (P = 0.002) compared with PBS.

irrespective of whether leukocytes were stimulated (i.e. with LPS or LTA) or unstimulated (Figs. 4–6). When leukocyte production of TNF-α, IL-6, and IL-10 were compared based on the type of stimulant, irrespective of group (i.e. shelter dogs or control dogs), LPS-stimulated leukocytes produced significantly greater TNF-α and IL-6, but not IL-10, than LTA-stimulated leukocytes (Figs. 4–6). In addition, as expected, stimulation of leukocytes with LPS or LTA resulted in significantly greater TNF-α, IL-6, and IL-10 production, compared to production by unstimulated leukocytes (Figs. 4–6).

Associations between serum 25(OH)D concentration and leukocyte cytokine production in the entire cohort (i.e. shelter dogs and control dogs combined) were determined initially. There was no association between serum 25(OH)D concentration and leukocyte production of TNF-α, IL-6, or IL-10 when stimulated with LPS, LTA, or left unstimulated (Table 2). Next, we were interested in whether associations existed between serum 25(OH)D concentration and leukocyte cytokine production within shelter dogs alone. There was a significant moderate inverse association between serum 25(OH)D concentration and LPS-stimulated leukocyte production of TNF-α (Table 3); however, there was no association between serum 25(OH)D and LTA-stimulated or unstimulated leukocyte production of TNF-α. Similarly, there was no association identified between serum 25(OH)D concentration and leukocyte production of IL-6 or IL-10 whether stimulated (i.e. with LPS or LTA) or unstimulated (Table 3).

Discussion

Vitamin D has potent immunomodulatory effects in a diverse spectrum of species that confers protective effects for the host (Willis et al., 2012; Djukic et al., 2014; Rodriguez-Lecompte et al., 2016; Garcia-Barragan et al., 2018; Jaffey et al., 2018a, 2018b, 2018c). Many of these protective effects serve to support mucosal innate immunity and hypovitaminosis-D has been associated with

the development of infections in humans (Cannell et al., 2006; Urashima et al., 2010). Further, oral vitamin D supplementation has a protective effect against acute respiratory infections in humans (Martineau et al., 2017). Community acquired infections in animal shelters are common and have far-reaching negative effects. To the authors' knowledge, little is known regarding immune function in shelter dogs that could account for the high occurrence of infections in shelter populations. In order to better understand if immune dysregulation or vitamin D could play a role, we investigated several important immune function parameters and their association with serum 25(OH)D concentration in shelter dogs and age, breed, and sex-matched control dogs.

Shelter dogs had a significantly decreased percentage of GM that underwent phagocytosis, despite there being no difference in absolute number of phagocytized *E. coli* per cell between groups. To the author's knowledge, there are no studies that have specifically investigated phagocytic function in shelter dogs; however, the results from the current study corroborate findings of reduced phagocytic potential in other species exposed to acute physical and psychological stressors (Garbuliński et al., 1991; Chen et al., 2002; Palermo-Neto et al., 2003; Baccan et al., 2004; Sesti-Costa et al., 2010). With the knowledge that shelter dogs have reduced phagocytic function and the potential contributory role of acute environmental stressors, future studies aimed at the improvement of phagocytic function, reduction of stress, or both could have a substantial effect on these dogs.

Shelter dogs were also found to have a lower percentage of leukocytes that had undergone oxidative burst, though the intensity of the oxidative burst per cell did not differ between groups. Interestingly, there was a significant positive association between serum 25(OH)D concentration and oxidative burst intensity per cell when the entire cohort was examined (shelter dogs and control dogs). This finding aligns with research conducted in other species. A 2010 study demonstrated that superoxide production of neutrophils decreased when participants were tasked with psychologically-stressful endeavors (Khanfer et al., 2010). Additionally, piglets have decreased oxidative burst intensity associated with weaning-related stress (Sauerwein et al., 2005). Further, in vitro and in vivo studies in humans have revealed that oxidative burst is increased following incubation with and oral supplementation of, respectively, vitamin D (Cohen et al., 1986; Abu-Amer and Zvi, 1993; Onwuneme et al., 2015).

There was no significant difference in leukocyte production of TNF-α, IL-6, or IL-10 between shelter dogs and control dogs, regardless of the type of stimulant. Dogs housed in shelters are exposed to a plethora of pathogens and acute stressors, which could lead to an immunologic hyporesponsive state known as endotoxin tolerance. Endotoxin tolerance is a transient genetic reprogramming of innate immune cells following exposure to stimuli including endotoxin, peptidoglycan, surgical stress, ischemia, and TNF-α. This syndrome results in a dampened pro-inflammatory response with exposure to subsequent stimuli and is meant to protect the host against the deleterious effects of an aberrant inflammatory state (Cavaillon, 1995; Ogle et al., 1997; Cook, 1998; Neviere et al., 2000; Kawasaki et al., 2001; Biswas and Lopez-Collazo, 2009; Jaffey et al., 2018c). One potential reason for the lack of difference in leukocyte cytokine production could be that only apparently healthy shelter dogs were included. This approach was purposefully strict to evaluate our objective, but may not be representative of the typical shelter population.

There was a significant inverse association between serum 25(OH)D and LPS-stimulated leukocyte cytokine production of TNF-α in the shelter dogs. This finding supports results from previous in vitro studies in dogs that incubation of blood with calcitriol attenuates leukocyte production of TNF-α in a concentration-dependent manner. TNF-α is associated with numerous

pathologies, including antioxidant depletion (Oguma et al., 2006; Ferro et al., 2014), initiation of cell apoptosis and tissue necrosis (Mukhopadhyay et al., 2006; Powe and Castleman, 2009; Fu-Tao et al., 2014), and trapping of neutrophils within inflammatory foci (Dore and Sirois, 1996; Mukhopadhyay et al., 2006). In humans, TNF-α has also been associated with respiratory disease severity and duration (Matsuda et al., 1995; Noah et al., 1995; Rutigliano and Graham, 2004). The role of TNF-α in shelter dogs is unknown, but it is reasonable to suspect the deleterious effects could also extend to this population. The results from this study supports the theory that oral supplementation of vitamin D to shelter dogs has potential, pending future clinical trials, to attenuate leukocyte production of TNF-α as it does in humans (Schleithoff et al., 2006; Willis et al., 2012; Ghavamzadeh et al., 2014). This could have important clinical implications in shelter dogs in the future.

There was no association between serum 25(OH)D concentration and the percentage of cells that underwent phagocytosis, or the number of phagocytized-*E. coli* bacteria. This is of particular interest because studies in mice have shown a relationship between decreased phagocytosis and hypovitaminosis D (Djukic et al., 2014). In addition, human peripheral blood samples were found to have markedly upregulated phagocytic activity when stimulated with calcitriol (Tokuda and Levy, 1996). It is possible that we did not appreciate this association because the effect of vitamin D and its metabolites on phagocytic function is dependent on the type of leukocyte or pathogen. The Djukic et al. study focused on the phagocytosis of *E. coli* in microglial cells, while the Tokuda et al. study focused on the phagocytosis of fluorescent carboxyl monospheres in monocytes. The current study evaluated phagocytic function of opsonized-*E. coli* in granulocytes and monocytes combined. The evaluation of granulocytes and monocytes combined was used in the current study because our goal was to best reflect in vivo conditions of shelter dogs. Further studies are warranted to determine if the effect of vitamin D on phagocytosis in dogs is dependent on the type of leukocyte, pathogen, or both.

Serum 25(OH)D concentrations were not significantly different between shelter and control dogs. The majority of circulating 25(OH)D is bound to vitamin D-binding protein (80–90%) and albumin (10–20%). These negative acute phase proteins decrease with systemic inflammatory stimuli, which results in increased renal loss of unbound filtered vitamin D (Nykjaer et al., 1999; Reid et al., 2011). We had anticipated that acute stressors and exposure to pathogens in shelter dogs, even in the absence of overt illness, would have resulted in decreased serum 25(OH)D concentrations. Future studies that measure serum 25(OH)D concentrations in a large randomized population of dogs at the time of shelter admission are needed to better understand vitamin D status in these dogs.

One of the major strengths of this study was its use of age, breed, and sex-matched (including neutering status) control dogs, which eliminated confounding from immunologic influences such as senescence or the immunosuppressive effect of sex hormones (Evans et al., 2000; Jansson and Holmdahl, 1998). One possible limitation of this study was the fact that all shelter dogs were vaccinated and administered prophylactic antiparasitic medications at the time of shelter admission (per institution policy). This represented an uncontrolled variable that could have influenced immunologic test results; however, vaccination would have likely augmented immune function in these dogs, as that is the foundation of their effectiveness to induce immunity against pathogens. Shelter dogs were deemed apparently healthy based on an unremarkable physical examination, negative for common vector-borne infections, and were excluded if any illnesses were recorded. It is possible that some of these apparently healthy dogs were subclinical for disease processes (e.g. respiratory infection, enteropathogens, kidney disease) that could have impacted their serum 25(OH)D concentrations; however, if a clinically relevant

occult disease were present we would have expected shelter dogs to have had significantly lower serum 25(OH)D concentrations than their non-shelter control counterpart. Further, we performed immunologic testing at a single time-point. It is possible that immune function test results would be different if measured at different time-points post-shelter admission. There could also be value in understanding if and when a change in immune function occurs after shelter admission. It is important that the results from this study are not extrapolated to justify supplementation of oral vitamin D to shelter dogs. Clinical trials aimed at investigating safety, dosing, and immunomodulatory effects of oral vitamin D are needed before clinical application.

Conclusions

Dogs housed in a shelter for ≥7 days demonstrated immune dysregulation in vitro that could be a contributory factor to the high occurrence of infections in shelter dogs. Although serum 25(OH)D concentrations were not significantly different between shelter and control dogs, they were predictive of GM oxidative burst function and stimulated leukocyte production of TNF-α in vitro. These findings suggest the possibility that oral vitamin D supplementation in shelter dogs has the potential to improve immune function. Future studies investigating the safety and in vivo immunologic effects of oral vitamin D supplementation in shelter dogs are warranted.

Conflict of interest statement

This work was supported by Maddie's Fund. Maddie's fund played no role in the study design, the collection, analysis, or interpretation of data, or the decision to submit the manuscript for publication. None of the authors have any financial or personal relationships that could influence or bias the content of this paper.

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References

Abu-Amer, Y., Zvi, B., 1993. Impaired bone marrow-derived macrophage differentiation in Vitamin D deficiency. Cellular Immunology 151, 356–368.

Baccan, G.C., Oliveira, R.D.R., Mantovani, B., 2004. Stress and Immunological phagocytosis: possible nongenomic action of corticosterone. Life Sciences 75, 1357–1368.

Bachhetta, J., Zaritsky, J.J., Sea, J.L., Chun, R.F., Lisse, T.S., Zavala, K., Nayak, A., Wesseling-Perry, K., Westerman, M., Hollis, B., et al., 2014. Suppression of iron-regulatory hepcidin by vitamin D. Journal of the American Society of Nephrology 25, 564–572.

Biswas, S.K., Lopez-Collazo, E., 2009. Endotoxin tolerance: new mechanisms, molecules, and clinical significance. Trends in Immunology 30, 475–487.

Cannell, J.J., Vieth, R., Umhau, J.C., Holick, M.F., 2006. Epidemic influenza and vitamin D. Epidemiology and Infection 134, 1129–1140.

Cavaillon, J.M., 1995. The nonspecific nature of endotoxin tolerance. Trends in Microbiology 3, 320–324.

Chen, W., Sun, L., Tsai, C., Song, Y., Change, C., 2002. Cold-stress induced the modulation of catecholamines, cortisol, immunoglobulin M, and leukocyte phagocytosis in *Tilapia*. General and Comparative Endocrinology 126, 90–100.

Cohen, M.S., Mesler, D.E., Snipes, R.G., Gray, T.K., 1986. 1,25-dihydroxyvitamin D3 activates secretion of hydrogen peroxide by human monocytes. Journal of Immunology 136, 1049–1053.

Cook, J.A., 1998. Molecular basis of endotoxin. Annals of the New York Academy of Sciences 851, 426–428.

Djukic, M., Onken, M.L., Schutze, S., Redlich, S., Gotz, A., Hanisch, U., Bertsch, T., Ribes, S., Hanenberg, A., Schneider, S., et al., 2014. Vitamin D deficiency reduces the immune response, phagocytosis rate, and intracellular killing rate of microglial cells. Infection and Immunity 82, 2585–2594.

Dore, M., Sirois, J., 1996. Regulation of p-selectin expression by inflammatory mediators in canine jugular endothelial cells. Veterinary Pathology 33, 662–671.

Dvir, E., Rosa, C., Handel, I., Mellanby, R.J., Schoeman, J.P., 2019. Vitamin D status in dogs with babesiosis. The Onderstepoort Journal of Veterinary Research 86, e1–e5.

Evans, M.R., Goldsmith, A.R., Norris, S.R.A., 2000. The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). Behavioral Ecology and Sociobiology 47, 156–163.

Ferro, T.J., Hocking, D.R., Johnson, A., 2014. Tumor necrosis factor-alpha alters pulmonary vasoreactivity via neutrophil-derived oxidants. American Journal Physiology 265, 462–471.

Fu-Tao, Z., Yi, D., Shah, Z., Xing, D., Yuan, G., Dong, M.L., Ding, M.X., 2014. TNF/TNFR1 pathway and endoplasmic reticulum stress are involved in ofloxacin-induced apoptosis of juvenile canine chondrocytes. Toxicology and Applied Pharmacology 276, 121–128.

Garbuliński, T., Obmińska-Domoradzka, B., Switała, M., Debowy, J., 1991. Responses of neutrophils and lymphocytes in the cold stress: effects of nonsteroid anti-inflammatory drugs. Polish Journal of Pharmacology and Pharmacy 43, 353–359.

Garcia-Barragan, A., Gutierrez-Pabello, J.A., Alfonseca-Silva, E., 2018. Calcitriol increases nitric oxide production and modulates microbicidal capacity against *Mycobacterium bovis* in bovine macrophages. Comparative Immunology Microbiology and Infectious Diseases 59, 17–23.

Ghavamzadeh, S., Mobasseri, M., Madhavi, R., 2014. The effect of vitamin D supplementation on adiposity, blood glycated hemoglobin, serum leptin and tumor necrosis factor-α in Type 2 diabetic patients. International Journal of Preventive Medicine 5, 1091–1098.

Hayward, S., Harding, R.M., McShane, H., Tanner, R., 2018. Factors influencing the higher incidence of tuberculosis among migrants and ethnic minorities in the UK. F1000Res 7, 461.

How, K.L., Hazewinkel, H.A., Mol, J.A., 1994. Dietary vitamin D dependence of cat and dog due to inadequate cutaneous synthesis of vitamin D. General and Comparative Endocrinology 96, 12–18.

Jaffey, J.A., Amorim, J., DeClue, A.E., 2018a. Effect of calcitriol on in vitro whole blood cytokine production in critically ill dogs. The Veterinary Journal 236, 31–36.

Jaffey, J.A., Amorim, J., DeClue, A.E., 2018b. Effects of calcitriol on apoptosis, toll-like receptor 4 expression, and cytokine production on endotoxin-primed canine leukocytes. American Journal of Veterinary Research 79, 1071–1078.

Jaffey, J.A., Amorim, J., DeClue, A.E., 2018c. Effects of calcitriol on phagocytic function, toll-like receptor 4 expression, and cytokine production in canine leukocytes. American Journal of Veterinary Research 79, 1064–1070.

Jaffey, J.A., Backus, R.C., McDaniel, K.M., DeClue, A.E., 2018d. Serum vitamin D concentrations in hospitalized critically ill dogs. PLoS One 13, e0194062.

Jansson, L., Holmdahl, R., 1998. Estrogen-mediated immunosuppression in autoimmune diseases. Inflammation Research 47, 290–301.

Kawasaki, T., Ogata, M., Kawasaki, C., Tomihisa, T., Okamoto, K., Shigematsu, A., 2001. Surgical stress induces endotoxin hyporesponsiveness and an early decrease of monocyte mCD14 and HLA-DR expression during surgery. Anesthesia and Analgesia 92, 1322–1326.

Khanfer, R., Phillips, A.C., Carroll, D., Lord, J.M., 2010. Altered human neutrophil function in response to acute psychological stress. Psychosomatic Medicine 72, 636–640.

Martineau, A.R., Wilkinson, K.A., Newton, S.M., Floto, R.A., Norman, A.W., Skolimowska, K., Davidson, R.N., Sorensen, O.E., Kampmann, B., Griffiths, C.J., et al., 2007. IFN-(gamma)- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin ll-37. Journal of Immunology 178, 7190–7197.

Martineau, A.R., Jolliffe, D.A., Hooper, R.L., Greenberg, L., Aloia, J.F., Bergman, P., Dubnov-Raz, G., Esposito, S., Ganmaa, D., Ginde, A.A., et al., 2017. Vitamin D supplementation to prevent acute respiratory tract infections: systematic review and meta-analysis of individual participant data. The BMJ 356, i6583.

Matsuda, K., Tsutsumi, H., Okamoto, Y., Chiba, C., 1995. Development of interleukin 6 and tumor necrosis factor alpha activity in nasopharyngeal secretions of infants and children during infection with respiratory syncytial virus. Clinical and Diagnostic Laboratory Immunology 2, 322–324.

Mukhopadhyay, S., Hoidal, J.R., Mukherjee, T.K., 2006. Role of TNFalpha in pulmonary pathophysiology. Respiratory Research 7, 125.

Neviere, R.R., Li, F.Y., Singh, T., Myers, M.L., Sibbald, W., 2000. Endotoxin induces a dose-dependent myocardial cross-tolerance to ischemia-reperfusion injury. Critical Care Medicine 28, 1439–1444.

Noah, T.L., Henderson, F.W., Wortman, I.A., Devlin, R.B., Handy, J., Koren, H.S., Becker, S., 1995. Nasal cytokine production in vital acute upper respiratory infection of childhood. The Journal of Infectious Diseases 171, 584–592.

Nykjaer, A., Dragun, D., Walther, D., Vorum, H., Jacobsen, C., Herz, J., Melsen, F., Christensen, E.I., Willnow, T.E., 1999. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell 96, 507–515.

O'Brien, M.A., McMichael, M.A., Le Boedec, K., 2018. 25-hydroxyvitamin D concentrations in dogs with naturally acquired blastomycosis. Journal of Veterinary Internal Medicine 32, 1684–1691.

Ogle, C.K., Guo, X., Ogle, J.D., 1997. Induction of endotoxin tolerance in rat bone marrow cells by in vivo infusion of tumor necrosis factor. Critical Care Medicine 25, 827–833.

Oguma, K., Sano, J., Kano, R., Watari, T., Hasegawa, A., 2006. In vitro effect of recombinant human tumor necrosis factor-α on canine neutrophil apoptosis. Research in Veterinary Science 80, 162–166.

Onwuneme, C., Blanco, A., O'Neill, A., Watson, B., Molloy, E.J., 2015. Vitamin D enhances reactive oxygen intermediates production in phagocytic cells in term and preterm infants. Pediatric Research 79, 654–661.

Palermo-Neto, J., Massoco, C.O., Robespierre de Souza, W., 2003. Effects of physical and psychological stressors on behavior, macrophage activity, and ehrlich tumor growth. Brain, Behavior, and Immunity 17, 43–54.

Pletz, M.W., Terkamp, C., Schmacher, U., Rohde, G., Schutte, H., Welte, T., Bals, R., 2014. Vitamin D deficiency in community-acquired pneumonia: Low levels of 1,25(OH)2D are associated with disease severity. Respiratory Research 15, 53.

Powe, J.R., Castleman, W.L., 2009. Canine Influenza virus replicates in alveolar macrophages and induces TNF-α. Veterinary Pathology 46, 1187–1196.

Reid, D., Toole, B.J., Knox, S., Talwar, D., Harten, J., O'Reilly, D., Blackwell, S., Kinsella, J., McMillan, D.C., Wallace, A.M., et al., 2011. The relation between acute changes in the systemic inflammatory response and plasma 25-hydroxyvitamin D concentrations after elective knee arthroplasty. The American Journal of Clinical Nutrition 93, 1006–1011.

Rockett, K.A., Brookes, R., Udalova, I., Vidal, V., Hill, A.V.S., Kwiatkowski, D., 1998. 1,25-dihydroxyvitamin D3 induces nitric oxide synthase and suppresses growth of *Mycobacterium tuberculosis* in a human macrophage-like cell line. Infection and Immunity 66, 5314–5321.

Rodriguez-Cortes, A., Martori, C., Martinez-Florez, A., Clop, A., Amills, M., Kubejko, J., Llull, J., Nadal, J.M., Alberola, J., 2017. Canine leishmaniasis progression is associated with vitamin D deficiency. Scientific Reports 7, 3346.

Rodriguez-Lecompte, J.C., Yitbarek, A., Cuperus, T., Echeverry, H., van Dijk, A., 2016. The immunomodulatory effect of vitamin D in chickens is dose-dependent and influenced by calcium and phosphorus. Poultry Science 95, 2547–2556.

Rosa, C.T., Schoeman, J.P., Berry, J.K., Mellanby, R.J., Dvir, E., 2013. Hypovitaminosis D in dogs with spirocercosis. Journal of Veterinary Internal Medicine 27, 1159–1164.

Rutigliano, J.A., Graham, B.S., 2004. Prolonged production of TNF-α exacerbates illness during respiratory syncytial virus infection. Journal of Immunology 173, 3408–3417.

Sainsbury, J.P., Trajtman, A., Stalker, A.T., Embil, J.M., Keynan, Y., 2014. Vitamin D binding protein polymorphism protects against development of blastomycosis. Journal de Mycologie Medicale 24, 328–331.

Sauerwein, H., Schmitz, S., Hiss, S., 2005. The acute phase protein haptoglobin and its relation to oxidative status in piglets undergoing weaning-induced stress. Redox Report 10, 295–302.

Schleithoff, S.S., Zittermann, A., Tenderich, G., Berthold, H.K., Stehle, P., Koerfer, R., 2006. Vitamin D supplementation improves cytokine profiles in patients with congestive heart failure: a double-blind, randomized, placebo-controlled trial. The American Journal of Clinical Nutrition 83, 754–759.

Sesti-Costa, R., Baccan, G.C., Chedraoui-Silva, S., Mantovani, B., 2010. Effects of acute cold stress on phagocytosis of apoptotic cells: the role of corticosterone. Neuroimmunomodulation 17, 79–87.

Sly, L.M., Lopez, M., Nauseef, W.M., Reiner, N.E., 2001. 1alpha,25-dihydroxyvitamin D3-induced monocyte antimycobacterial activity is regulated by phosphatidylinositol 3-kinase and mediated by the NADPH-dependent phagocyte oxidase. Journal of Biological Chemistry 276, 35482–35493.

Tokuda, N., Levy, R.B., 1996. 1,25-Dihydroxyvitamin D3 stimulates phagocytosis but suppresses HLA-DR and CD13 antigen expression in human mononuclear phagocytes. Experimental Biology and Medicine 211, 244–250.

Urashima, M., Segawa, T., Okazaki, M., Kurihara, M., Wada, Y., Ida, H., 2010. Randomized trial of vitamin D supplementation to prevent seasonal influenza A in schoolchildren. American Journal of Clinical Nutrition 91, 1255–1260.

Willis, K.S., Smith, D.T., Broughton, K.S., Larson-Meyer, D.E., 2012. Vitamin D status and biomarkers of inflammation in runners. Open Access Journal of Sports Medicine 3, 35–42.