

# Age-associated changes to pathogen-associated molecular pattern-induced inflammatory mediator production in dogs

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## Abstract

**Objective** – To determine whether older dogs will have a more pronounced pro-inflammatory response and blunted anti-inflammatory response to pathogen-associated molecular patterns (PAMPs) compared with younger dogs.

**Design** – Prospective.

**Setting** – University teaching hospital.

**Animals** – Thirty-eight privately owned sexually altered dogs of various ages.

**Interventions** – Blood was collected for HCT, WBC count, plasma biochemical analysis, and whole blood culture. Whole blood was stimulated with lipopolysaccharide (LPS) or, lipoteichoic acid or, peptidoglycan or, addition of phosphate-buffered saline. Tumor necrosis factor (TNF), interleukin (IL)-6, and IL-10 production from whole blood were compared among young, middle aged, and geriatric dogs.

**Measurements and Main Results** – LPS, lipoteichoic acid, and peptidoglycan stimulated significant TNF, IL-6, and IL-10 production from canine whole blood compared with phosphate-buffered saline. Whole blood from geriatric dogs had a blunted IL-10 response to LPS stimulation and middle-aged dogs had increased LPS-induced TNF production compared with the other groups.

**Conclusion** – PAMPs from gram-positive and gram-negative bacteria stimulate TNF, IL-6, and IL-10 production from canine whole blood. The inflammatory mediator response to PAMPs from gram-negative bacteria alters with age and may be one factor contributing to mortality in geriatric dogs with sepsis.

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**Keywords:** cell culturing, cytokines, immunology, inflammation

## Introduction

Sepsis is the systemic inflammatory response to infection and is associated with an increased mortality compared with simple infections. Bacteria are the most common cause of infection and sepsis in dogs.<sup>1–7</sup> One of the initiating factors for the inflammatory response to bacterial infection is immune cell activation by pathogen-associated molecular patterns (PAMPs). PAMPs are

highly conserved molecular motifs from microorganisms that are recognized by the innate immune system and include mediators such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), and peptidoglycan (PG). Activation of the immune system by PAMPs results in production of a myriad of inflammatory mediators including tumor necrosis factor (TNF), interleukin (IL)-6, IL-1 $\beta$ , alpha-chemokine ligand (CXCL)-8, and IL-10 resulting in systemic inflammation, hemodynamic derangement, disordered coagulation, and organ dysfunction.<sup>8,9</sup> Morbidity and mortality from sepsis are the result of the systemic inflammatory response rather than the actual infection.<sup>10,11</sup>

A direct relationship between age and mortality during sepsis has been identified in people and experimental murine studies.<sup>12–14</sup> Proposed mechanisms to account for this relationship are centered around altered host defense mechanisms in geriatric individuals.

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Immunoaging, a term describing age-related changes to the immune system, involves downregulation of some aspects of the immune system (ie, immunosenescence) while other aspects of the immune systems appear to be upregulated.

Increasing age has been associated with increased and sustained production of inflammatory mediators in people and mice.<sup>15–18</sup> Healthy, older people with experimentally induced endotoxemia have significantly greater plasma concentrations of TNF and soluble TNF receptors and a longer duration of fever than healthy younger people.<sup>18</sup> It is important to note that this sustained inflammatory response was demonstrated during experimentally induced endotoxemia (ie, administration of LPS, not live bacteria) so the sustained inflammatory response could not be attributed to decreased bacterial clearance leading to persistent immune cell stimulation. A similar pro-inflammatory shift in the elderly has been documented in ex vivo studies as well.<sup>19,20</sup> However, environmental factors may have contributed to immunoaging because people living in adverse economic environments are more likely to have a pro-inflammatory shift compared with people living in affluent environments.<sup>21</sup> In experimental models of sepsis, geriatric mice had a significantly greater pro-inflammatory mediator production and higher mortality rates than young or middle-aged mice and this difference in mortality was associated with failure to downregulate pro-inflammatory mediator production in geriatric mice.<sup>14,17,22</sup>

To the authors' knowledge, there are no studies addressing the role of age on host defense mechanisms during sepsis in dogs. This study is presented as the first step toward understanding immunoaging in dogs. We hypothesized that PAMPs would induce significant inflammatory mediator production from canine whole blood and that whole blood from geriatric dogs would have a more pronounced pro-inflammatory response and blunted anti-inflammatory response to PAMPs compared with whole blood from younger dogs. To investigate this hypothesis, we stimulated whole blood from young, middle aged, and geriatric dogs with LPS or, LTA or, PG or, a control (phosphate-buffered saline [PBS]) and compared production of 2 pro-inflammatory mediators, TNF and IL-6, and an anti-inflammatory mediator, IL-10, among the stimulants and age groups.

## Materials and Methods

### Animals

Dogs were recruited for enrollment into this study by electronic and paper solicitation to the faculty, staff, and students of the University of Missouri, College of Veterinary Medicine. Owners provided written consent for

**Table 1:** Group classification based on age and weight<sup>24–26</sup>

Weight (kg)	Age (y)	Group
<12.4	0.5–2.9	Young
	3–9.9	Middle-aged
	>9.9	Geriatric
12.5–34	0.5–2.5	Young
	2.6–6.9	Middle-aged
	>6.9	Geriatric
	0.5–1.9	Young
>34	2–5.9	Middle-aged
	>5.9	Geriatric

dogs to participate, and this study was approved by the University of Missouri Animal Care and Use Committee. Enrollment criteria included sexually altered dogs >0.5 years of age that were apparently healthy on complete physical examination performed at enrollment. Dogs were excluded from study if they were <0.5 years of age, were sexually intact, had a history of illness within 2 months before enrollment, or were of a breed reported to have an immunodysfunction syndrome related to sepsis (eg, Rottweiler, Doberman Pinscher).<sup>23</sup> Additionally, dogs were excluded if vaccination or any medication (eg, nonsteroidal anti-inflammatory drugs, glucocorticoids, antimicrobials) other than parasite preventatives were administered in the 2 months before sample collection. Dogs were assigned to 1 of 3 age groups: young, middle aged, or geriatric based upon chronological age and body weight (Table 1).<sup>24–26</sup> The desired sample size was 12 dogs per group. This number was based on a power calculation performed on previous data.<sup>a</sup>

### Blood collection

Blood was collected aseptically from the jugular vein into potassium EDTA, lithium heparin, and sodium heparin tubes<sup>b</sup> for hematology, plasma biochemical analysis, and whole blood culture, respectively.

### Whole blood culture

Whole blood culture was performed using a modification of a previously described technique.<sup>27–29</sup> The collected blood was diluted with modified Roswell Park Memorial Institute media<sup>c</sup> (200 U penicillin/mL, 200 µg streptomycin/mL, and 200 mM L-glutamine) in a 1:2 dilution and added to the wells of a 24 well plate. Either LPS (50 and 100 ng/mL) from *Escherichia coli* 0127:B8<sup>d</sup> or, LTA (500 and 1000 ng/mL) from *Streptococcus faecalis*<sup>d</sup> or, PG (500 and 1000 ng/mL) from *Staphylococcus aureus*<sup>d</sup> or, PBS were added to individual wells. These concentrations of LPS, LTA, and PG were selected based on preliminary work in our laboratory.<sup>e</sup> The plates were gently rocked for 5 minutes to

thoroughly mix the blood mixture and incubated at 37°C in room air with 5% CO<sub>2</sub>. After 24 hours, the plates were centrifuged (2500 × g × 7 min) and the supernatant was collected. Samples were stored at –80°C until batch analyses.

### Assays

**HCT, WBC count, and plasma biochemistry:** HCT, WBC count, and plasma biochemical analysis were performed by a commercial veterinary diagnostic laboratory.<sup>f</sup> HCT and WBC count were evaluated by Coulter Counter.<sup>g</sup> Biochemical analysis of plasma was evaluated using an automated wet chemistry analyzer.<sup>h</sup>

**TNF:** Whole blood culture supernatant TNF activity was evaluated using a cell kill bioassay as described previously.<sup>30</sup> An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl tetrazolium bromide) colorimetric assay<sup>d</sup> was used to assess cell viability. Canine recombinant TNF<sup>i</sup> was used to construct a standard curve to quantify the activity of TNF in the test wells. The lower limit of detection for this assay is 0.5 ng/mL.

**IL-6 and IL-10:** Canine-specific ELISAs for IL-6 and IL-10 were used to determine the concentration of IL-6 and IL-10, respectively, in whole blood culture supernatant.<sup>j</sup> The assays were performed in duplicate according to the manufacturer's instructions. The lower limits of detection for these assays are 31.3 and 15.6 pg/mL for IL-6 and IL-10, respectively.

**Statistics:** Data were analyzed using commercially available software.<sup>k</sup> The Kolmogorov-Smirnov statistical test for normality was used to determine if data were normally distributed. Weight and vital, hematologic and biochemical parameters were compared among age groups. Supernatant inflammatory mediator production was compared among treatments in the overall population of dogs and among groups within each individual treatment. Statistical comparisons were made using a 1 way analysis of variance or Kruskal-Wallis 1 way analysis of variance on ranks for parametric and nonparametric data, respectively. A Tukey multiple comparison procedure was used for post hoc analysis, when appropriate. A *P*-value of <0.05 was considered statistically significant. Data are expressed as mean (SD) or median, Q1, Q3 unless otherwise noted.

## Results

### Population characteristics

A total of 38 dogs met the inclusion criteria for this study. Numbers, gender characteristics, age ranges, and weight ranges of the three groups are listed in Table 2. Breeds represented in the young group included: Mixed Breed (*n* = 6), Golden Retriever (*n* = 2), and 1

of each of the following breeds: Australian Shepherd, French Bulldog, Great Dane, and Shetland Sheepdog. Breeds represented in the middle-aged group included: mixed breed (*n* = 9), and 1 of each of the following breeds: Brittany Spaniel and Keeshond. Breeds represented in the geriatric group included: mixed breed (*n* = 8), Labrador Retriever (*n* = 2), Weimaraner (*n* = 2), and 1 of each of the following breeds: English Springer Spaniel, Golden Retriever, and Border Collie.

### Vital parameters, HCT, WBC count, and plasma biochemical analysis

Results of the vital parameters, HCT, WBC count, and plasma biochemical profile are displayed in Table 2. The BUN concentration was significantly higher in the young group compared with the geriatric group (*P* = 0.017). Plasma total calcium concentration was significantly higher in young group compared with the geriatric (*P* < 0.01) and middle-aged (*P* = 0.027) groups. Plasma phosphorus concentration was significantly higher in the young group compared with the geriatric (*P* < 0.001) and middle-aged (*P* = 0.002) groups. There were no significant differences in weight, temperature, heart rate, respiratory rate, HCT, WBC count, blood glucose, sodium, potassium, albumin, total protein, globulin, alanine transaminase, and alkaline phosphatase among groups.

### TNF production from whole blood

Supernatant TNF activity from blood stimulated with the PBS did not significantly differ among age groups (*P* = 0.818). Supernatant TNF activity from whole blood stimulated with LPS, LTA, and PG was significantly greater than PBS for all age groups (*P* < 0.001) (Figure 1 a–c). However, there was no significant difference in supernatant TNF activity among the various concentrations of LPS, LTA, and PG tested (LPS, *P* = 0.093; LTA, *P* = 0.557; PG, *P* = 0.866). Whole blood from middle-aged dogs produced significantly more TNF than geriatric dogs (*P* < 0.001) and young dogs (*P* < 0.002) when stimulated with 50 ng/mL LPS. There was no significant difference in supernatant TNF activity from whole blood stimulated with LTA (*P* ≥ 0.137) or PG (*P* ≥ 0.724) among age groups.

### IL-6

Supernatant IL-6 concentration from whole blood stimulated with the PBS did not significantly differ among age groups (*P* = 0.454) (Figure 2a–c). LPS, LTA, and PG stimulated significant production of IL-6 from canine whole blood compared with PBS (*P* < 0.001) at each of the concentrations tested, regardless of age group. LPS increased supernatant IL-6 concentration approximately 6-fold and LTA and PG increased supernatant

**Table 2:** Comparison of physical examination, physiologic, hematologic, and biochemical parameters among young, middle-aged, and geriatric dogs

Parameter	Reference interval	Young	Middle	Geriatric	P-value
Number, gender	NA	12 dogs; 7 FS, 5 MC	11 dogs; 6 FS, 5 MC	15 dogs; 6 FS, 9 MC	NA
Age (y)	NA	1.4 (0.5)	4.0 (1.0)	9.2 (2.5)	NA
Weight (kg)	NA	23.6 (10.9)	20.6 (5.7)	26.0 (9.2)	0.197
Rectal temperature (C°)	37.5–39.0	38.5 (0.6)	38.38 (0.5)	38.5 (0.9)	0.490
Heart rate	80–120	115.6 (23.2)	106.9 (25.0)	112.3 (9.2)	0.331
Respiratory rate	14–22	36.0 (13.3)	34.9 (13.4)	27.8 (15.0)	0.162
WBCC (10 <sup>3</sup> /μL)	4.53–14.9	9.1 (1.8)	8.0 (2.0)	8.2 (1.5)	0.222
WBCC (10 <sup>9</sup> /L)	4.53–14.9	9.1 (1.8)	8.0 (2.0)	8.2 (1.5)	0.222
HCT (%)	37–57	48.3 (4.4)	51.6 (3.6)	47.3 (5.4)	0.165
Glucose (mmol/L)	4.5–7.4	5.7 (0.6)	5.1 (0.6)	5.5 (0.6)	0.114
Glucose (mg/dL)	81–133	102.4 (11.2)	92.5 (11.6)	99.2 (11.2)	0.114
Urea nitrogen (mmol/L)	2.9–10	8.25 (3.5)	7.9 (3)	5 (1.5)*	0.017
Urea nitrogen (mg/dL)	8–28	23.1 (9.8)	22.1 (8.4)	13.9 (4.2)*	0.017
Sodium (mEq/L)	143–152	148.5 (1.7)	150.0 (1.8)	149.2 (1.5)	0.132
Sodium (mmol/L)	143–152	148.5 (1.7)	150.0 (1.8)	149.2 (1.5)	0.132
Potassium (mEq/L)	3.4–4.9	4.0 (0.3)	4.0 (0.4)	3.9 (0.3)	0.375
Potassium (mmol/L)	3.4–4.9	4.0 (0.3)	4.0 (0.4)	3.9 (0.3)	0.375
Albumin (g/L)	29–40	33 (2)	34 (3)	31 (3)	0.112
Albumin (g/dL)	2.9–4.0	3.3 (0.2)	3.4 (0.3)	3.1 (0.3)	0.112
Total plasma protein (g/L)	52–74	58 (5)	64 (5)	61 (7)	0.068
Total plasma protein (g/dL)	5.2–7.4	5.8 (0.5)	6.4 (0.5)	6.1 (0.7)	0.068
Globulin (g/L)	22–36	26 (4)	30 (5)	31 (6)	0.071
Globulin (g/dL)	2.2–3.6	2.6 (0.4)	3.0 (0.5)	3.1 (0.6)	0.071
Calcium (mmol/L)	2.3–2.8	2.6 ± 0.1	2.5 (0.1)*	2.5 (0.1)*	<0.03
Calcium (mg/dL)	9.2–11.3	10.5 (0.3)	10.1 (0.4)*	9.9 (0.4)*	<0.03
Phosphorus (mmol/L)	0.6–1.6	1.5 (0.3)	1.2 (0.2)*	1.1 (0.2)*	<0.01
Phosphorus (mg/dL)	2.0–5.0	4.8 (1.0)	3.6 (0.7)*	3.3 (0.7)*	<0.01
ALT (U/L)	9–58	35.4 (12.8)	34.3 (14.8)	38.1 (13.6)	0.908
ALP (U/L)	5 129	49.6 (30.4)	70.3 (83.3)	45.4 (27.7)	0.934

\*Significant difference from the young group.

FS, female spayed; MC, male castrated; ALT, alanine transaminase; ALP, alkaline phosphatase; WBCC, WBC count.

IL-6 concentrations approximately 4-fold, compared with PBS in all age groups. Supernatant IL-6 concentration was not significantly different among the various concentrations of LPS, LTA, and PG tested (LPS,  $P = 0.588$ ; LTA,  $P = 0.427$ ; PG,  $P = 0.722$ ). There was no significant difference in LPS-, LTA-, or PG-induced IL-6 production from whole blood among the young, middle-aged or geriatric groups ( $P \geq 0.454$ ).

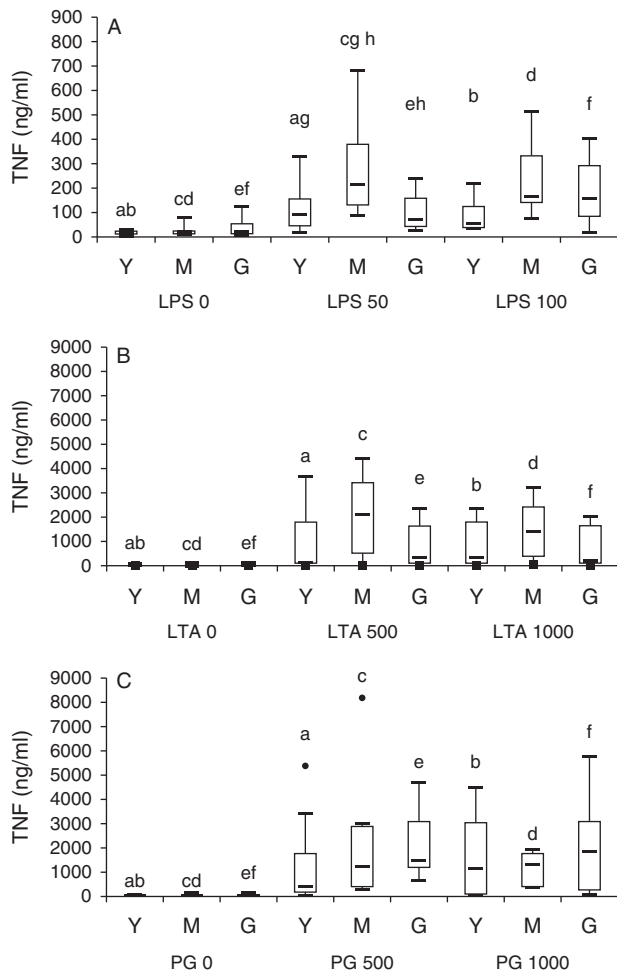
### IL-10

Supernatant IL-10 concentration from whole blood incubated with the PBS did not significantly differ among age groups ( $P = 0.155$ ) (Figure 3a–c). Supernatant IL-10 concentrations were significantly greater from whole blood stimulated with LPS, LTA, and PG compared with PBS ( $P < 0.001$ ) in all groups. There was not a significant difference in IL-10 production among the various concentrations of LPS, LTA, and PG tested (LPS,  $P = 0.999$ ; LTA,  $P = 0.164$ ; PG,  $P = 0.892$ ). LPS stimulation resulted in 15.8, 12.5, and 10.5-fold greater supernatant IL-10 concentration compared with PBS in the young, middle-aged, and geriatric group, respectively.

Whole blood from geriatric dogs produced significantly less IL-10 after LPS stimulation compared with young dogs ( $P < 0.02$ ) but there was no significant difference in IL-10 production from whole blood stimulated with LPS between the middle-aged group and the geriatric or young groups ( $P \geq 0.265$ ). There was no significant difference in LTA or PG-induced IL-10 production from whole blood among the age groups ( $P \geq 0.836$ ,  $P \geq 0.760$ , respectively).

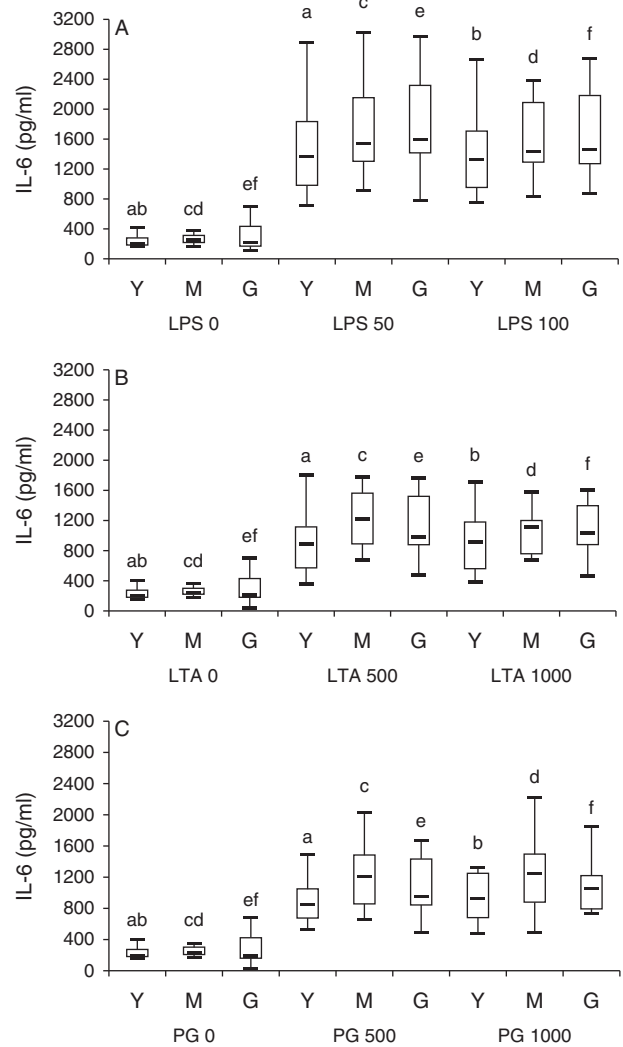
### Discussion

This is the first study to demonstrate differences in PAMP-induced inflammatory mediator production among dogs in different age groups. In this study, LPS, LTA, and PG stimulated significant TNF, IL-6, and IL-10 production from whole blood. Compared with young dogs, whole blood from geriatric dogs had a blunted IL-10 response to LPS and whole blood from middle-aged dogs had significantly more LPS-induced TNF production compared with whole blood from the young or geriatric groups.



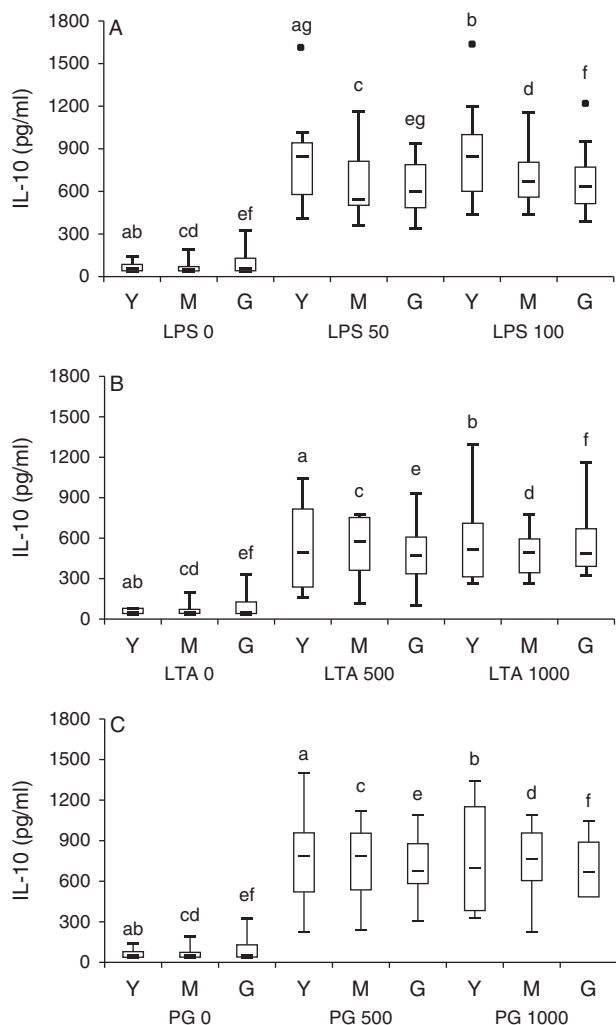
**Figure 1:** Tumor necrosis factor activity from whole blood culture after stimulation with (a) lipopolysaccharide (LPS) 0, 50, and 100 ng/mL, (b) lipoteichoic acid (LTA) 0, 500, 1000 ng/mL, and (c) peptidoglycan (PG) 0, 500, 1000 ng/mL from the young (Y), middle-aged (M), and geriatric (G) groups. Data are presented as box and whisker plots. The upper and lower edges of the box represent the 75th and 25th percentiles, respectively, whereas the line within the box is the median value. Whiskers represent the range. Outliers are indicated by a closed circle. Same letter designates a significant difference in tumor necrosis factor (TNF) activity among treatment or group. *Note:* The *y*-axis is 10 × lower for LPS than for LTA and PG.

To the authors' knowledge, this is the first reported use of whole blood culture to evaluate TNF, IL-6, and IL-10 response to PAMP in dogs. In multiple species, LPS, LTA, and PG induce inflammatory mediator production, including TNF, IL-1, IL-6, and CXCL-8, from whole blood culture.<sup>28,29,31–39</sup> Whole blood culture is an inexpensive and rapid *ex vivo* method for studying leukocyte responsiveness to stimuli and has been used to evaluate the inflammatory response and novel anti-inflammatory therapies in other species.<sup>31,32,35,40,41</sup> Whole blood culture maintains interactions between



**Figure 2:** Interleukin (IL)-6 production from whole blood culture after stimulation with (a) lipopolysaccharide (LPS) 0, 50, and 100 ng/mL, (b) lipoteichoic acid (LTA) 0, 500, 1000 ng/mL, and (c) peptidoglycan (PG) 0, 500, 1000 ng/mL from the young (Y), middle-aged (M), and geriatric (G) groups. Data are presented as box and whisker plots. The upper and lower edges of the box represent the 75th and 25th percentiles, respectively, whereas the line within the box is the median value. Whiskers represent the range. Same letter designates a significant difference in IL-6 activity among treatments.

populations of blood cells and plasma and more appropriately mimics physiological conditions *in vivo* including maintenance of plasma milieu and monocyte to lymphocyte ratio, compared with isolated peripheral blood mononuclear cells.<sup>35,42</sup> Maintaining the *in vivo* plasma milieu provides cytokines, growth factors, and other proteins necessary for LPS and PG activation of leukocytes in some species.<sup>35,43–45</sup> Compared with techniques involving isolated peripheral blood mononuclear cells, whole blood culture is less technically



**Figure 3:** Interleukin (IL)-10 production from whole blood culture after stimulation with (a) lipopolysaccharide (LPS) 0, 50, and 100 ng/mL, (b) lipoteichoic acid (LTA) 0, 500, 1000 ng/mL, and (c) peptidoglycan (PG) 0, 500, 1000 ng/mL from the young (Y), middle-aged (M) and geriatric (G) groups. Data are presented as box and whisker plots. The upper and lower edges of the box represent the 75th and 25th percentiles, respectively, whereas the line within the box is the median value. Whiskers represent the range. Outliers are indicated by a closed circle. Same letter designates a significant difference in IL-10 activity among treatment or group.

demanding and requires smaller volumes of blood. Based on our data, whole blood culture may be used in the future to study pathogen-associated molecular pattern-induced TNF, IL-6, and IL-10 production from canine leukocytes, *ex vivo*.

Immunoaging has become an area of intense research in human medicine.<sup>46</sup> It has been hypothesized that immunoaging contributes to increased morbidity and mortality observed in the elderly.<sup>47</sup> Despite extensive

research and large multicenter studies, sepsis continues to result in high morbidity and mortality and the incidence of sepsis continues to increase by 1.5% annually in people.<sup>47,48</sup> Elderly people are significantly more likely to suffer mortality during sepsis than younger people.<sup>36,49</sup> Increased age has been associated with increased and sustained production of pro-inflammatory mediators in people and mice.<sup>16–18</sup> Using whole blood culture, Gabriel and Rink<sup>20</sup> demonstrated that older people produced significantly more IL-1 $\beta$ , IL-6, and CXCL-8 in response to LPS stimulation compared with young people. Inflammation, not necessarily infection per se, is associated with complications in sepsis and severe sepsis. Sequelae such as multiple organ dysfunction syndrome (MODS), multiple organ failure syndrome, and acute respiratory distress syndrome are known to result from the inflammatory response. The development of MODS, multiple organ failure syndrome, and acute respiratory distress syndrome drastically affects morbidity and mortality in septic patients.<sup>50,51</sup>

TNF is the prototypical pro-inflammatory cytokine that is involved in the innate immune response to a myriad of stimuli, including the innate immune response to pathogens. Our study demonstrated that middle-aged dogs produced significantly more TNF than young or geriatric dogs. We expected that whole blood from young dogs would produce less TNF than older dogs because infants and young pigs have reduced TNF production capacity.<sup>52,53</sup> However, because an increased TNF response to pathogens has been documented in geriatric people, we were surprised that whole blood from geriatric dogs produced less LPS-induced TNF than whole blood from middle-aged dogs.<sup>16,18,21</sup> It is hypothesized that people in affluent environments have less pathogen exposure resulting in downregulation of pro-inflammatory responses during old age. Indeed, geriatric people living in affluent environments have decreased TNF production capacity compared with younger adult people.<sup>21</sup> Similar environmental factors may have contributed to our findings as all of the dogs in this study were from relatively affluent homes. Nevertheless, it appears that dogs have maximal TNF production capacity during middle age, which may be clinically important in the treatment of critically ill dogs. Additionally, the similarities in immune response between aging people and dogs may indicate the usefulness of dogs in modeling human sepsis.

Sepsis is characterized by disparity among the inflammatory and anti-inflammatory response. In this study, whole blood from geriatric dogs produced significantly less anti-inflammatory mediator IL-10 in response to LPS compared with young dogs. Our findings are similar to those in other species. Activation of peripheral blood

monocytes results in more robust production of IL-10 in neonatal foals compared with adult horses.<sup>54</sup> IL-10 deficiency has resulted in a more pronounced bacteremia, increased TNF production, and early mortality in mice with experimentally induced bacteremia.<sup>55</sup> People also have a decline in IL-10 production associated with advancing age.<sup>21,56</sup> Downregulation of IL-10 production is associated with a greater overall mortality risk in people.<sup>57</sup> Because sepsis and MODS are the end result of an overzealous inflammatory response, blunting of the anti-inflammatory response to pathogens may contribute to higher mortality rates in older dogs.

Age did not alter LPS, LTA, or PG-induced IL-6 production or LTA or PG-induced TNF or IL-10 production in this study. Our results differed from those in people, mice, and nonhuman primates where LPS-induced IL-6 production is amplified in the geriatric population.<sup>14,15,17,20,58,59</sup> Both LTA and PG activate the immune system via toll-like receptor (TLR)-2 while LPS signals through TLR-4. TLRs are membrane spanning proteins that are vital to the innate immune system's response to conserved molecular motifs. There is evidence that poor immune responses in the elderly animals are a result of diminished expression and function of TLRs.<sup>60</sup> Although both TLR-2 and TLR-4 are downregulated with increasing age, TLR-4 appears to be more dramatically affected in mice.<sup>60,61</sup> This alteration in TLR expression may be species specific because in people there is no evidence of TLR-2 or TLR-4 downregulation with age.<sup>62</sup> It is possible that dogs have differential downregulation of TLRs which would explain the difference in response to TLR-2 and TLR-4 ligands.

There are several limitations to this investigation that should be noted. First, this was an *ex vivo* investigation and thus it is unknown if these findings directly correlate to what happens *in vivo*. The number of dogs enrolled met the desired sample size, which was based on power calculations performed on previous data.<sup>a</sup> Given that biologic variation exists, it is possible that our results would have been altered by an increased sample size. Our population was heterogeneous with respect to breeds involved; however, breeds included have not previously been identified to have immunodeficiency syndromes.<sup>23</sup> As is shown in Table 2, there were some differences in biochemistry analysis among our 3 groups. Younger dogs had significantly greater plasma total calcium and phosphorus concentration than middle-aged or geriatric dogs and geriatric dogs had significantly lower BUN concentrations than the young dogs. While we are uncertain if these differences in plasma biochemical parameters altered our results, they have reported previously as expected age-related changes.<sup>63–65</sup>

It was challenging to find owners to volunteer geriatric dogs that were not receiving medications. Many of the older geriatric dogs volunteered for this study were receiving medications for osteoarthritis (eg, nonsteroidal anti-inflammatory drugs, glucocorticoids). All dogs that had received medication in the previous 2 months were excluded from our study based on our exclusion criteria. Thus, our geriatric population was younger than expected. Although the geriatric dogs enrolled fit our definition of 'geriatric,' it is possible that dogs older than those in our group could have a different response with regards to the products of inflammation. Conversely, our 'young' population was also diverse including dogs from 6 months to 2 years of age. Perhaps including a fourth population of immature dogs would have produced a different result.

In conclusion, people and dogs share some similarities with regards to immunaging, particularly decreased production capacity for TNF and IL-10 in the elderly. Based on our findings, age alters the inflammatory mediator response to PAMPs from gram-negative bacteria and may be 1 factor contributing to mortality in geriatric dogs with sepsis. Ultimately, this study provides insight into the complex immune system changes associated with aging in dogs. These changes may benefit the clinician in prognosticating and treating septic canine patients.

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### Footnotes

- <sup>a</sup> Dr. Amy DeClue, unpublished data.
- <sup>b</sup> Tyco Healthcare Group LP, Mansfield, MA.
- <sup>c</sup> Corning Inc., Corning, NY.
- <sup>d</sup> Sigma-Aldrich, St Louis, MO.
- <sup>e</sup> Nickell J, Chang C, DeClue A. The effects of glucose on pathogen associated molecular pattern-induced tumor necrosis factor production from canine whole blood. International Veterinary Emergency and Critical Care Society Meeting, Chicago, IL, 2009.
- <sup>f</sup> University of Missouri, Veterinary Medical Diagnostic Laboratory, Columbia, MO.
- <sup>g</sup> Beckman Coulter, Miami, FL.
- <sup>h</sup> Olympus AU400e, Olympus America, Center Valley, PA.
- <sup>i</sup> Endogen, Rockford, IL.
- <sup>j</sup> Quantikine Canine IL-6 Immunoassay; Quantikine Canine IL-10 immunoassay, R and D Systems, Minneapolis, MN.
- <sup>k</sup> SigmaStat, Systat Software Inc., Chicago, IL.

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