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SYMPOSIUM

Upregulation of Proinflammatory Cytokine Production in Response to Bacterial Pathogen-Associated Molecular Patterns in Dogs with Diabetes Mellitus Undergoing Insulin Therapy

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Abstract

Background:

Metabolic alterations associated with diabetes mellitus alter innate immunity. Dogs often develop infectious or inflammatory complications related to diabetes mellitus, yet little is known about the effects of diabetes mellitus on the immune system in this species.

Methods:

Prospective evaluation in dogs with poorly regulated spontaneous type 1 diabetes mellitus (T1DM). *In vitro* leukocyte cytokine response to lipopolysaccharide (LPS), lipoteichoic acid (LTA), and peptidoglycan (PG) was compared between dogs with T1DM and healthy dogs. Additionally, the effect of acute *in vitro* glucose exposure on leukocyte tumor necrosis factor (TNF) production from healthy dogs was measured.

Results:

Leukocytes from dogs with T1DM had significantly greater TNF production after LTA and PG stimulation compared with leukocytes from healthy dogs. Leukocyte interleukin (IL)-6 production was greater after stimulation with LPS, LTA, PG, and phosphate-buffered saline in the T1DM group. No such difference was noted when evaluating IL-10 production between groups regardless of stimulant. Dogs with T1DM had significantly greater IL-6 to IL-10 production ratios than healthy dogs. Acute exposure to dextrose did not augment cytokine production from healthy canine leukocytes.

Conclusions:

Dogs with T1DM have altered innate immunity characterized by upregulation of proinflammatory cytokine production without a concurrent change in anti-inflammatory cytokine production. This may be one explanation for the common infectious and inflammatory complications associated with T1DM in dogs.

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Abbreviations: (IL) interleukin, (LPS) lipopolysaccharide, (LTA) lipoteichoic acid, (PAMP) pathogen-associated molecular pattern, (PBMC) peripheral blood mononuclear cell, (PBS) phosphate-buffered saline, (PG) peptidoglycan, (RPMI) Roswell Park Memorial Institute, (T1DM) type 1 diabetes mellitus, (TNF) tumor necrosis factor

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Introduction

Diabetes mellitus is the most common spontaneous endocrinopathy in dogs, affecting up to 1.5% of the canine population.[1] Diabetes mellitus is generally classified as either insulin-resistant diabetes mellitus or insulin-deficiency diabetes mellitus in companion animals.[1] Insulin-resistant diabetes mellitus results from hormonally induced insulin antagonism and is uncommon in dogs.[1,2] Insulin-deficiency diabetes mellitus is characterized by progressive loss of pancreatic beta cells or beta-cell hypoplasia/abiotrophy and an absolute deficiency of insulin and is synonymous with type 1 diabetes mellitus (T1DM) in people with features of latent autoimmune diabetes of adults (type 1.5 diabetes mellitus).[1–4] With dogs, there are genetic, sex (female phenotype), as well as breed predilections, and age distribution is predominately middle age to early geriatric.[2,5] Standard medical management for T1DM in dogs consists of administration of intermediate or long-acting insulin and dietary alterations.[6,7]

Hyperglycemia and the accompanying metabolic changes associated with insulin resistance or insulin lack modulate the immune system.[8–10] Chronic hyperglycemia alters neutrophil cell function, cytokine production, and bacterial clearance.[11] Infectious and inflammatory disorders are common sequelae of T1DM in dogs, yet little is known about the effects of diabetes mellitus on the immune system in this species.[12–16] The purpose of this study was to evaluate the impact of the chronic diabetic state on whole blood production of tumor necrosis factor (TNF), interleukin (IL)-6, and IL-10 using a spontaneous canine model of T1DM and to determine the effect of acute exposure to glucose on whole blood production of TNF *in vitro* to determine if diabetes mellitus or glucose exposure resulted in a proinflammatory shift. We hypothesized that whole blood from dogs with diabetes mellitus would have an exaggerated proinflammatory (TNF, IL-6) response and blunted anti-inflammatory (IL-10) response to pathogen-associated molecular patterns (PAMPs), lipopolysaccharide (LPS), lipoteichoic acid (LTA), and peptidoglycan (PG).

Methods

Animals

This study was reviewed and approved by the University of Missouri Animal Care and Use Committee. Dogs with T1DM presenting to the University of Missouri Veterinary Medical Teaching Hospital from March 2009 to June 2010 were eligible for enrollment. Owners provided written consent for dogs to participate. Inclusion criteria for the T1DM group included evidence of persistent hyperglycemia and glucosuria that could not be attributed to hormone or medication-induced insulin resistance, recommendation to treat with intermediate or long-acting insulin, and a lack of clinical evidence of infection. Signalment, clinical, or clinicopathologic evidence of diabetes mellitus and medication administration were recorded for each dog.

Healthy 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. Enrollment criteria included sexually altered dogs greater than 0.5 years of age that were apparently healthy based on complete physical examination and blood glucose concentration performed at enrollment. Dogs were excluded from study if they were less than 0.5 years of age; had a history of illness, including hyperglycemia, vaccination, or medication other than parasite preventatives given within 2 months prior to enrollment; or were of a breed reported to have a breed-wide immunodysfunction syndrome related to innate immune response to pathogens (e.g., Rottweiler and Doberman pinscher).[17]

Blood Collection and Processing

Blood was collected aseptically from the jugular vein into sodium heparin Vacutainer tubes or serum collection Vacutainer tubes for whole blood culture or fructosamine concentration analysis, respectively. Serum was collected and frozen at -80 °C for fructosamine evaluation.

Whole Blood Culture

Heparinized whole blood from dogs with T1DM and healthy dogs was diluted 1:2 with modified Roswell Park Memorial Institute (RPMI) media (RPMI, 200 U Penicillin/ml, 200 μg streptomycin/ml, and 200 mM l-glutamine; Invitrogen, Carlsbad, CA) and added to 12-well plates.[18–20] Lipopolysaccharide (100 ng/ml) from *Escherichia coli* 0127:B8 (Sigma-Aldrich, St. Louis, MO), LTA (1000 ng/ml) from *Streptococcus faecalis* (Sigma-Aldrich), and PG (1,000 ng/ml) from *Staphylococcus aureus* (Sigma-Aldrich), or control [phosphate-buffered saline (PBS)] were added to the wells as described previously.[18,20] The wells were mixed thoroughly, and the plates were incubated for 24 h at 37 °C in 5% CO_2. After incubation, the plates were centrifuged (300 g × 7 min, 4 °C) and the supernatant was collected and frozen at -80 °C until batch analyses.

In Vitro *Exposure to Glucose*

To evaluate the direct effects of glucose on TNF production, heparinized whole blood from healthy dogs was mixed with modified RPMI in a 1:2 dilution and either dextrose (27.7 mM; Hospira Inc., Lake Forest, IL), mannitol (27.7 mM; osmotic control; Hospira Inc.), or PBS (non-osmotic control) was added. The blood was then stimulated with LPS, LTA, PG, or PBS and processed as indicated for the whole blood culture procedure.[18–20]

Fructosamine

Serum fructosamine concentrations were used as a biochemical marker of glycemic control and were evaluated by a commercial laboratory (Antech Diagnostics, Irvine, CA).[21]

Tumor Necrosis Factor

Whole blood culture supernatant TNF activity was evaluated using a cell-killing bioassay.[18,20,22] Briefly, murine fibroblasts (L929) were cultured on 96-well plates for 12 h, and then the samples were added. After a 20 h incubation with minimum essential medium (Invitrogen, Carlsbad, CA) plus horse serum and actinomycin D (Sigma-Aldrich), a 3-[4,5-dimethylthiazol-2-yl]-2,5,-di-phenyl tetrazolium bromide (Sigma-Aldrich) colorimetric assay was used to quantify the number of live cells per well. Absorption was measured at 630 nm, and optical density of test wells were compared with that of wells with known concentrations of recombinant canine TNF (R&D Systems, Minneapolis, MN) for quantification. The lower limit of detection for this assay is 0.5 ng/ml.

Interleukin-6 and Interleukin-10

Whole canine-specific enzyme-linked immunosorbent assays for IL-6 and IL-10 (Quantikine, R&D Systems) were used to determine the concentration of IL-6 and IL-10, respectively, in whole blood culture supernatant.[18,20] 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 (SigmaPlot, Systat Software Inc, San Jose, CA). An analysis of variance on ranks was used to compare TNF bioactivity among dextrose-, mannitol-, and PBS-treated blood for each stimulant. A Tukey multiple comparison procedure was used for *post hoc* analysis, when appropriate. A Mann–Whitney rank-sum test was used to compare whole blood TNF, IL-6, and IL-10 production and fructosamine concentrations between dogs with T1DM and healthy dogs. To evaluate the IL-6-to-IL-10 ratio, the concentration of IL-6 was divided by the concentration of IL-10 for each sample. For analysis, only LPS, LTA, and PG (i.e., PAMP-stimulated) IL-6-to-IL-10 ratios were evaluated. The PAMP-stimulated IL-6-to-IL-10 ratios were compared between T1DM and healthy groups using a Mann–Whitney rank-sum test. Outliers were defined as values 1.5 times the interquartile range greater or less than the third or first quartile, respectively, while extreme outliers were defined as values three times the interquartile range greater or less than the third or first quartile, respectively. A p value of < 0.05 was considered statistically significant.

Results

Patient Population

Ten dogs with diabetes mellitus were enrolled. Blood was collected on two separate occasions for 3 dogs, resulting in a total of 13 blood samples. Median age was 8 years (range 2–12). There were 5 sexually altered female and 5 sexually altered male dogs. Breeds represented were mixed breed ($n = 3$), Labrador retriever ($n = 2$), Bichon Frise ($n = 1$), Chihuahua ($n = 1$), miniature poodle ($n = 1$), schnauzer ($n = 1$), and Shih Tzu ($n = 1$). One dog was a newly diagnosed with diabetes; 9 dogs were currently receiving insulin therapy [human insulin (recombinant DNA origin) neutral protamine Hagedorn insulin $n = 6$, porcine insulin zinc suspension $n = 2$, insulin glargine (recombinant DNA origin) $n = 1$] with a median dose of 0.7 U/kg (range 0.275–0.88) every 12 h. Dogs had clinical signs attributable to poor glycemic control like polyuria/polydipsia ($n = 5$), polyphagia ($n = 1$), weight loss ($n = 4$), and/or cataract formation ($n = 7$). The healthy control group consisted of 6 dogs with a median age of 4 years (range 2–5). There were 2 sexually altered females and 4 sexually altered male dogs. Breeds represented were mixed (4) and Belgian Malinois (2). None of the dogs had clinical findings suggesting diabetes mellitus at the time of the study or in the 2 months after samples were collected. Serum fructosamine concentration was significantly greater for the dogs with diabetes (median 581; Q1 481; Q3 680 mmol/liter) compared with the healthy dogs (median 321; Q1 279; Q3 336 mmol/liter; $p = .001$).

Comparison of Tumor Necrosis Factor, Interleukin-6, and Interleukin-10 Production between Dogs with Type 1 Diabetes Mellitus and Healthy Dogs

Whole blood from dogs with diabetes mellitus had significantly greater supernatant TNF bioactivity after LTA ($p = .018$) and PG ($p = .032$) stimulation compared

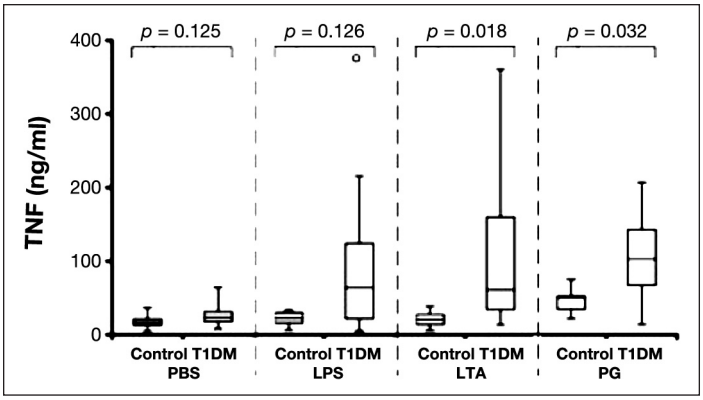

Figure 1. Comparison of LPS-, LTA-, PG-, and PBS (control)-induced whole blood TNF bioactivity from dogs with T1DM and healthy controls (control). The box represents the interquartile range, while the line within the box indicates the median. Whiskers include the largest and smallest values. Outliers are indicated with an open circle. Extreme outliers are not included. *P* values are indicated for each comparison.

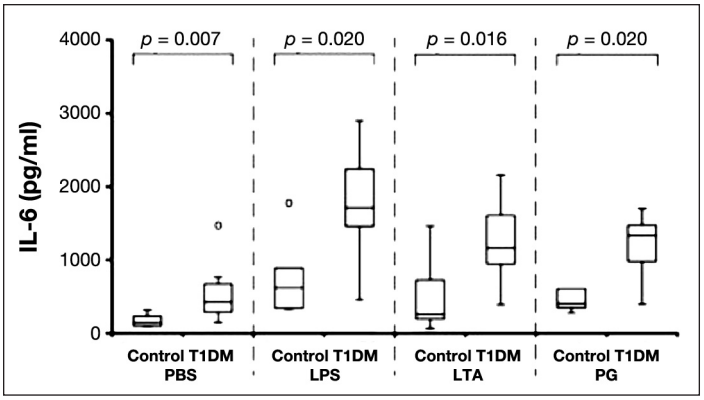

Figure 2. Comparison of LPS-, LTA-, PG-, and PBS (control)-induced whole blood IL-6 production from dogs with T1DM and healthy controls (control). The box represents the interquartile range, while the line within the box indicates the median. Whiskers include the largest and smallest values. Outliers are indicated with an open circle. Extreme outliers are not included. *P* values are indicated for each comparison.

with whole blood from healthy dogs (**Figure 1**). There was a significant difference in whole blood IL-6 production after stimulation with LPS (p = .020), LTA (p = .016), PG (p = .020), and PBS (p = .007) between the diabetes mellitus group and the healthy group (**Figure 2**). No such difference was noted when evaluating IL-10 production between groups, regardless of stimulant (**Figure 3**). Dogs in the T1DM group (median, Q1, Q3; 4.0, 2.5, 9.2) had a significantly greater IL-6-to-IL-10 ratio after PAMP stimulation than the control group (2.1, 1.3, 3.5; $p < .001$). This indicates that the relative production of IL-6 exceeded that of IL-10 in the T1DM group.

In Vitro *Effects of Glucose on Tumor Necrosis Factor Production*

Exposing whole blood to high concentrations of dextrose, mannitol, or the control solution *in vitro* failed to alter PBS-, LPS-, LTA-, or PG-stimulated TNF production (**Figure 4**).

Discussion

In this study, we have discovered initial evidence that leukocytes from dogs with T1DM have a proinflammatory shift. Leukocytes from dogs with T1DM produced significantly more TNF and IL-6 in response to PAMP motifs compared with leukocytes from healthy dogs. This was not the case with IL-10 production. The ratio of PAMP-stimulated IL-6-to-IL-10 production was greater in dogs with T1DM, further supporting a proinflammatory shift.[23] However, acute exposure to a high glucose concentration environment did not alter TNF production from leukocytes from healthy dogs. Taken together, these data suggest that dogs with diabetes mellitus have altered innate immunity characterized by upregulation of

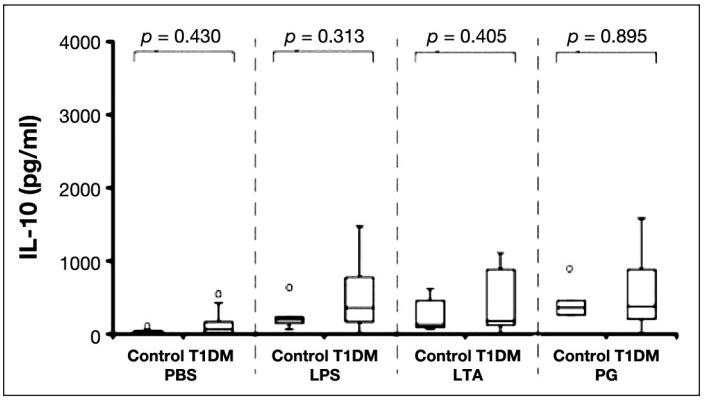

Figure 3. Comparison of LPS-, LTA-, PG-, and PBS (control)-induced whole blood IL-10 production from dogs with T1DM and healthy controls (control). The box represents the interquartile range, while the line within the box indicates the median. Whiskers include the largest and smallest values. Outliers are indicated with an open circle. Extreme outliers are not included. *P* values are indicated for each comparison.

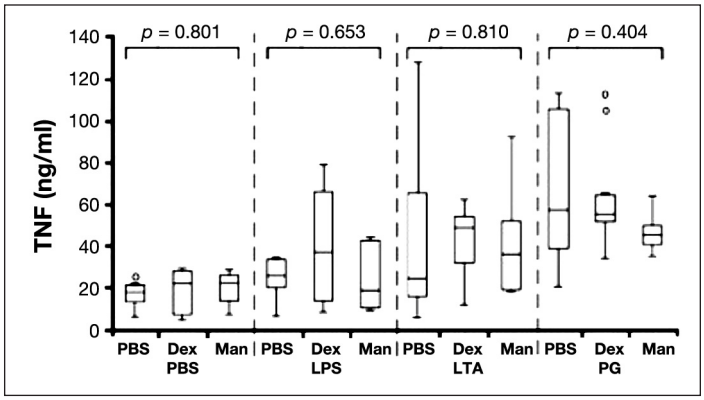

Figure 4. Comparison of LPS-, LTA-, PG-, and PBS (control)-induced whole blood TNF production from healthy dogs after acute exposure to dextrose, mannitol, or control (PBS). The box represents the interquartile range, while the line within the box indicates the median. Whiskers include the largest and smallest values. Outliers are indicated with an open circle. Extreme outliers are not included. *P* values are indicated for each comparison. Dex, dextrose; Man, mannitol.

proinflammatory cytokine production without a concurrent change in anti-inflammatory cytokine production, which cannot be contributed to acute effects of hyperglycemia alone. This may be one explanation for the infectious and inflammatory sequelae associated with diabetes mellitus in this species.

There is little debate that T1DM is associated with alterations in innate immunity in people and animal models, which may result in infectious and inflammatory sequelae. The exact manifestations of these perturbations, however, are unclear, in large part, because of conflicting reports in the literature. Focusing on cytokines, investigators have documented both upregulation and downregulation of leukocyte cytokine production in response to LPS.[8,9,24–29] Variation in these data could relate to several confounding variables, including the severity of metabolic derangement. For example, LPS-induced TNF production was upregulated in people with poor metabolic control compared with people with T1DM with good metabolic control.[25] Similarly, Kulseng and colleagues[30] demonstrated that LPS-induced peripheral blood mononuclear cell (PBMC) TNF production was greater in newly diagnosed T1DM compared with after initiation of treatment in people. In our study, 9/10 of the dogs were currently being treated with insulin, yet they all had relatively poor metabolic control, as evidenced by continued clinical signs of diabetes mellitus and high serum fructosamine concentrations, which are indicative of poor glycemic control.[21] Dogs and some people appear to have similar upregulation of cytokine production in response to poor metabolic control of T1DM, which might lead to the inflammatory sequelae often observed in these patient populations.

People and dogs with T1DM have an increased incidence of infection.[8,12–16,31,32] One proposed mechanism of increased infection risk involves inappropriate neutrophil activation and tissue damage, leading to breakdown in host mucosal defense to microbial invasion.[29] The higher IL-6-to-IL-10 ratio in the T1DM group suggests an imbalance between the proinflammatory and anti-inflammatory cytokine production, which could lead to neutrophil priming and activation. Upregulation of cytokine and chemokine production tends to be more dramatic in neutrophils compared with monocytes in people with T1DM.[29] Since neutrophils are the predominate leukocyte found in canine whole blood, the increased proinflammatory cytokine production in the T1DM group could be the result of neutrophil priming. The second theory explaining infection risk is reduced mononuclear cell activation, resulting in impaired production of TNF and IL-6 secondary to upregulation of IL-10.[24] We did not find this to be true in this cohort of dogs with diabetes mellitus.

In vitro, the addition of glucose to PBMC culture systems results in upregulation of LPS-induced TNF production from human PMBCs, indicating that glucose itself may have a proinflammatory effect.[33] To investigate this phenomenon, we incubated whole blood from healthy dogs with a high concentration of dextrose and compared baseline and stimulated TNF production. Mannitol was used as a control for osmotically induced alterations in TNF production.[34,35] Although we did not find a difference in TNF production when a high-glucose media was applied to the whole blood, varying the glucose concentration or incubation time could have yielded different results. We elected to evaluate TNF since it is a prototypical, early cytokine produced in response to danger signals (e.g., PAMPs). However, alterations to innate immunity differ based on the pathway involved, indicating that some signaling pathways remain unadulterated.[28,36] Future investigations of other inflammatory pathways is warranted prior to concluding that glucose has no direct immunomodulatory properties related to leukocyte cytokine production in dogs.

Components of gram positive (LTA, PG) and gram negative (LPS) bacteria were used to stimulate leukocyte response in our study. These PAMPs activate leukocytes through pattern recognition receptors on immune cells such as toll-like receptor-2, toll-like receptor-4, and nucleotide oligomerization domain-like receptors, resulting in the production of a number of inflammatory mediators important in response to infection, including TNF, IL-6, and IL-10. Whole blood culture was used to evaluate leukocyte response to these stimuli under varying metabolic conditions. We elected to use whole blood culture, compared with isolated PBMCs, since whole blood culture more accurately mimics physiological conditions *in vivo* by maintaining cell populations and plasma factors normally found in blood. This is particularly important because certain plasma proteins promote LPS-induced activation of leukocytes.[37] Whole blood culture also minimizes leukocyte stress and activation, a common problem during PBMC separation, while maintaining consistent cytokine production despite variations in leukocyte counts in dogs.[20] Nevertheless, evaluation of immunologic prowess *ex vivo* fails to completely recapitulate the complex and dynamic nature of the immune system, and therefore, these data should be interpreted with caution.

Conclusion

The impact of the diabetic state on innate immunity is an area of great concern in dogs and people. Conflicting data have been published indicating a need for further evaluation of the dynamic neurohormonal, metabolic, and immunologic impact of diabetes mellitus. Our findings provide some initial evidence that the metabolic dysfunction associated with T1DM might alter innate immunity in dogs, specifically PAMP-induced leukocyte cytokine production. Future work evaluating innate immunity in dogs with T1DM could help veterinarians develop novel interventions to reduce the incidence of infection and inflammation and further clarify the role of spontaneous diabetes mellitus in dogs as an immunologic model of T1DM for people.

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