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Transportation and routine veterinary interventions alter immune function in the dog

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Abstract

Rapid activation of the hypothalamic pituitary adrenal axis and the sympathetic nervous system are hallmarks of the acute stress response and these systems interact with the immune system by signaling though glucocorticoid and adrenergic receptors on immune cells. There is limited information about the effect of these physiologic responses on immunologic parameters of pet dogs enrolled in clinical studies. The objective of this study was to evaluate how travel, instrumentation and hospitalization alter immunologic parameters in pet dogs. Blood was collected from healthy dogs in a home environment and from healthy dogs at the time of presentation to the hospital and after instrumentation and 24 hours of hospitalization. We found that LPS-induced down regulation of TLR4 was blunted in dogs exposed to stress. Neutrophil and monocyte MHCII expression increased after transportation to the veterinary hospital but then became similar to that of the control dogs at the end of hospitalization. Peripheral blood mononuclear cell cytotoxicity function was blunted in dogs exposed to the stress of transportation as well as hospitalization. Neutrophil apoptosis was greater in dogs exposed to stress compared to controls although this effect significantly decreased after hospitalization stress. Conversely, stress did not alter induced or spontaneous cytokine production from leukocytes, neutrophil or monocyte expression of TLR4, LPS-induced down-regulation of monocyte TLR4, LPS-

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induced neutrophil and monocyte expression of MHCII or peripheral blood lymphocyte phenotype. Transportation and instrumentation/ hospitalization stress should be considered when interpreting immunologic studies in pet dogs.

Key Words: Animals, Apoptosis, Cytokines, Glucocorticoids, Leukocytes, Sympathetic Nervous System

Introduction

Exposure to short-term stressors results in activation of multiple physiological systems. This psychophysiological stress response is a fundamental survival mechanism which promotes the fight or flight response by preparing the cardiovascular, musculoskeletal, neuroendocrine and immune systems.[1, 2] Short-term stress modulates immune function in many species; an effect theorized to have evolved for preparing physiological responses to cope with trauma or infection as sequelae to injury from a predator, conspecific, or other threat. Glucocorticoids and catecholamines are the major hormones implicated in the mechanism of stress-induced immunomodulation. Immunomodulation induced by stress is multifaceted and includes alterations of immune cell trafficking, cytokine production, cytotoxic function, cell surface receptor expression, and induction of apoptotic pathways as part of inflammation resolution.[2]

However, there are limited data defining the impact of housing and clinical manipulation on the immunophenotype of dogs. The goal of this pilot study is to characterize immune function of client-owned dogs housed within the home environment *versus* the hospital and evaluate the impact of

transportation to a veterinary facility, examination, and instrumentation on multiple quantitative and functional immunologic parameters. Understanding if and how the stress of transportation, routine veterinary procedures, and hospitalization impact immune responses in dogs would refine clinical interpretation of immunologic studies in pet dogs presenting to veterinary health care facilities for care. Outcome measures included comparisons of the following immunologic parameters: natural killer cell cytotoxicity, peripheral blood lymphocyte phenotype, Toll like receptor 4 and major histocompatibility complex class II expression, neutrophil and lymphocyte apoptosis, and leukocyte cytokine production capacity. Samples were collected and measurements recorded for two time points, the time of presentation to the hospital and after instrumentation and 24 hours of hospitalization. A secondary evaluation compared immunologic parameters in dogs in a home environment to the dogs that had been hospitalized.

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Methods

Animals

All procedures were approved by the XXXXXXXX Animal Care and Use committee. Nine healthy, adult dogs ranging in age from 2 to 9 years (mean 5.1 years) weighing between 12.9 and 38 kg (mean 23.8 kg) owned by the students and faculty of the XXXXXXXX, Veterinary Health Center were recruited for this study. There were 4 neutered male, 2 spayed female and 3 intact female dogs included. Breeds represented were mixed breed (n=5), and one each Labrador retriever, Brittany spaniel, beagle and golden retriever. The dogs had to reside exclusively in a home environment and could not have any health problems, receive any medications other than routine parasitic preventatives or be a breed known to have an immunodysfunction syndrome. One group of dogs were used as a

control (n=3). These dogs had blood collected in their home environment without any other procedures (control). The other group of dogs (n=6), were assigned to the hospital group. Those dogs traveled with their owner to the Veterinary Health Center for evaluation. Immediately upon arrival to the hospital, blood was collected (transportation stress). Then, the dog received a complete physical examination, and an intravenous catheter was placed in the cephalic vein by first clipping and sterilely preparing the antebrachium. A 20g over the needle intravenous catheter was introduced into the cephalic vein and secured with tape. A soft padded bandage was then applied to the length of the antebrachium. An Elizabethan collar was applied to each dog. The dogs were placed in standard dog runs with free access to water for 24 hours. The dogs were walked on a leash outside four times during the hospitalization period. The dog's routine diet was supplied as an evening and then morning feeding. After 24 hours of hospitalization blood was collected again (hospitalization stress).

Blood was collected from the jugular vein into potassium EDTA, sodium heparin, and evacuated tubes for CBC and immunologic evaluation, respectively, at baseline for the control dogs and then at baseline (prior to all procedures; travel stress) and again after completion of the 24 hours of hospitalization (hospitalization stress) for the hospitalized dogs.

Immunologic evaluation

Peripheral blood mononuclear cells were purified from heparinized blood with Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). Plasma was removed and blood were diluted with PBS. 5ml diluted blood was then laid on top of 3ml Histopaque 1077 in 15ml tube. After centrifugation at 400 x g for 30 minutes at 25° C, PMBC were collected and transferred into a new 15ml tube. Cells were then washed twice in PBS and resuspended to a final concentration of 10^6 cells per mL.

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PBMC cytotoxicity was evaluated as a marker of NK cell-like activity using a previously described cytotoxicity assay.[3] Canine thyroid adenocarcinoma (CTAC) cells were maintained in RPMI 1640 supplemented with 10% FBS at 37°C in 5% CO_2. For the 3 days prior to the assay, cells were subcultured every 24 h to ensure that cells are in the log phase of growth. Prior to the assay, CTAC cells were labeled with 10ul of 3mM green fluorescent 3,3′-Dioctadecyloxacarbocyanine (DiO; Sigma-Aldrich, St. Louis, MO) per ml for 20 minutes at 37°C in 5% CO_2. Cells were then washed twice in PBS and resuspended to a final concentration of 10^5 cells per mL. Purified PBMC were comingled with DiO-labeled CTAC cells at ratios of 1:1, 10:1, 25:1 or 50:1 in a round bottom 96 well plate. Single cell populations of PBMC or DiO labeled CTAC cells were used as controls. After 24 h at 37°C with 5% CO_2, propidium iodide (PI) was added. Plates were then incubated for 15 minutes in dark and samples transferred into a FACS tube on ice. Samples were immediately analyzed at the XXXXXXXX Cell and Immunology Core Facility using Beckman Coulter's CyAn ADP and Summit software. A minimum of 15,000 events were recorded for each sample. Events were analyzed as previously described.[3] Briefly, CTAC cells were gated on a forward/side scatter plot and then applied to a plot comparing DiO and PI. DiO and PI positivity were determined using unstained cells as controls. DiO^+/PI^+ cells were defined as dead CTAC cells and DiO^+/PI^- cells were defined as live CTAC cells. Baseline cell death was established using DiO/PI stained CTAC cells alone.

Lymphocyte phenotyping was performed using the following antibodies: anti-rat CD3-PE (abcam; clone: KT3), anti-canine CD21-Alexa Fluor 647 (AbD Serotec; clone: CA2.1D6), anti-dog CD5-FITC (AbD Serotec; Clone: YKIX322.3), anti-mouse/rat FoxP3-allophycocyanin (APC) (eBioscience; Clone: FJK-16s), anti-dog CD4-FITC (AbD Serotec; Clone: YKIX302.9), anti-canine CD25-PE (eBioscience; Clone: ACT-1), anti-human CD3-Alexa Fluor 647 (AbD Serotec; Clone: CD3-12), anti-canine CD8-APC (eBioscience; Clone: YCATE55.9), anti-human CD56-PE (Dako; Clone:

MOC-1), rat IgG2a APC isotype control (eBioscience), mouse IgG1 PE isotype control (eBioscience) and rat IgG2a FITC isotype control as described previously.[4] For the assay, 200µl of 10^6 PBMC per sample were added to the wells of a 96 well round bottom plate. Plates were centrifuged at 300g for 6 minutes and the supernatant removed. After addition of 50µl of FACS buffer, cells were stained for CD4 T cell ($CD4^+/CD3^+$), CD8 T cell ($CD8^+/CD3^+$), NK cell ($CD5^+/CD56^+/CD3^-$) and B cell ($CD21^+$) markers. After addition of antibodies, cells were incubated for 30 minutes on ice protected from light. At the end of incubation time, cells were then washed twice in PBS and fixed with 2% paraformaldehyde. Samples were analyzed at the XXXXXXXX Cell and Immunology Core Facility using Beckman Coulter's CyAn ADP and Summit software. A minimum of 15,000 events were recorded for each sample. Unstained cells and matched isotype controls from the same manufacturer were used for negative antibody controls. Cells were gated as outlined previously.[4] First, lymphocytes were identified on forward versus side scatter plots. Then CD3 versus CD4 and CD3 versus CD8 plots were constructed. Lymphocytes that were double positive for CD3 and CD4 or CD3 and CD8 were identified. Lymphocytes that were positive for CD21 on side scatter versus CD21 plots were identified as B cells. NK-like cells were identified by first selecting for lymphocytes on a forward versus side scatter plot and then selecting lymphocytes that were CD3 negative on a histogram plot. The CD3 negative cells were then applied to a CD56 plot and cells that were CD56 positive were identified.

To evaluate TLR4 and MHCII expression, PMNs were stimulated with lipopolysaccharide from *Escherichia coli* 0127:B8 or PBS and then incubated for 2 h at 37°C in 5% CO2. At the end of incubation, cells were washed and then incubated with PE-conjugated Anti-Human CD284 (TLR4, HTA125 clone, eBioscience Inc.) or FITC-conjugated Mouse Anti- Human Leukocyte Antigen – antigen D Related (BD Biosciences; Clone: G46-6) for 30 minutes in dark for identification of TLR4 or

MHCII expressing cells, respectively.[3, 5, 6] After incubation, cells were washed and fixed. Samples were analyzed using flow cytometry as previously stated. Unstained cells and matched isotype controls from the same manufacturer were used for negative antibody controls. PMNs were identified on forward versus side scatter plots. Then the PMNs were gated and applied to a PE (TLR4) vs FITC (MHCII) plot. Cells positive for TLR4, MHCII and double positive cells were identified as previously described.[3] The LPS-induced percent change in cell surface marker expression was calculated by dividing the difference between LPS and PBS cell surface marker expression by the PBS cell surface marker expression.

Lymphocyte and neutrophil apoptosis was measured using an Annexin-V/propidium iodide (PI) detection kit (FITC Annexin V Apoptosis Detection Kit II, BD Pharmingen).[5, 7] First, the red blood cells in 100 ul of whole blood were lysed using cold distilled water and 10× PBS, and then incubated with FITC labeled Annexin-V and PI for 15 minutes. Flow cytometry analysis was performed immediately after adding 400 µL of binding buffer. Samples blocked with recombinant Annexin-V were used as a negative control. Flow cytometry was performed as previously stated. Gaiting was performed by first identifying neutrophils and lymphocytes based on forward vs side scatter plots as previously described.[7] Each cell type was then applied to a FITC vs PI plot. Cells that were PI-/annexin+ were identified as undergoing apoptosis while necrotic or dead cells were identified as PI+.

Lipopolysaccharide from *Escherichia coli* 0127:B8 was used to stimulate the whole blood for evaluation of cytokine production as previously described [8-13]. Blood was diluted 1:2 with media and samples were cultured on 12 well plates with LPS, LTA or PBS and then incubated for 24 h at 37°C in 5% CO2. Cell supernatant was collected at end of incubation and stored in -80°C for analysis. Canine specific magnetic bead-based multi-analyte immunoassay were used to evaluate IL-6, IL-10 and

TNF-α production according to the manufacturer's instructions. Cytokine concentrations were quantified using MILLPLEX Analyst 5.1 software and standard curves.

Statistical analysis

Statistical analysis was performed using commercially available software (Sigmaplot 13.00, Systat, San Jose, CA). Normality was assessed using histogram plots and a Shapiro-Wilk test. Variance was evaluated using a Brown-Forsythe test. Data were compared among groups using an ANOVA with a post-hoc Holm-Sidak method for all pairwise multiple comparison procedures as appropriate. A p-value < 0.05 was considered statistically significant.

Results

Using a target cell to PBMC ratio of 1:1, dogs exposed to transportation stress and hospital stress had reduced cytotoxicity compared to control dogs (Figure 1). Using a target cell to PBMC ratio of 10:1 and 25:1, only the dogs undergoing hospital stress had significantly lower PBMC cytotoxicity compared to the control group. PBMC cytotoxicity was not significantly different between dogs that experienced stress and the controls.

Neutrophil TLR4 expression was similar between dogs in the control group and dogs that experienced transportation or hospital stress (Figure 2). Neutrophils exposed to LPS had a reduced percentage of cells with TLR4 expression as expected. This phenomenon was noted in the control dogs and hospitalized dogs at baseline. However, in the hospitalized dogs, the LPS-induced percent change in cells expressing TLR4 surface marker was significantly less after hospital stress than for the control group (control, 14.1±4.9 %; transportation stress 5.8±8.4 %; hospital stress 0.7±3.5 %; p=0.031). There

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was no difference in monocyte TLR4 expression or LPS-induced percent change in monocyte cells expressing TLR4 surface among groups.

Dogs exposed to transportation stress had a significantly greater percentage of neutrophils expressing MHCII than the control dogs or dogs exposed to hospital stress, however neutrophil MHCII expression was not different between dogs undergoing hospital stress and controls (Figure 3). There was no difference in LPS-stimulated neutrophil MHCII expression among groups. The percentage of monocytes constitutively expressing MHCII was greater in dogs exposed to transportation stress compared to dogs undergoing hospitalization stress and the control group. After LPS-stimulation, percentage of monocytes expressing MHCII from dogs exposed to transportation stress was significantly greater than the dogs exposed to hospital stress or the control group. The LPS-induced percent change in neutrophils or monocytes expressing MHCII was not different among dogs exposed to transportation stress, hospitalization stress or controls.

Spontaneous neutrophil apoptosis was significantly greater in dogs undergoing transportation stress or hospitalization stress compared to control (Figure 4). Additionally, dogs undergoing transportation stress had significantly greater neutrophil apoptosis than dogs undergoing the stress of hospitalization.

The percentage of peripheral blood CD4+, CD8+, CD21+ and NK cells did not differ among dogs exposed to transportation stress, hospitalization stress or controls (Figure 5). LPS stimulation resulted in production of TNF, IL-6 and IL-10 from the leukocytes of all dogs (Figure 6). There were no differences in LPS or unstimulated TNF, IL-6 or IL-10 production among dogs that experienced transportation stress, hospitalization stress or the controls.

Discussion

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Rapid activation of the HPA axis and the sympathetic nervous system are hallmarks of the acute stress response and these systems interact with the immune system by signaling though glucocorticoid and adrenergic receptors on immune cells. In this study we found that the stress of transportation and hospitalization alters the immune system in healthy dogs. Specifically, LPS-induced down regulation of TLR4 was blunted in dogs exposed to stress. Neutrophil and monocyte MHCII expression increased after transportation to the veterinary hospital but then became similar to that of the control dogs at the end of hospitalization. PBMC cytotoxicity function was blunted in dogs exposed to the stress of transportation as well as hospitalization. Neutrophil apoptosis was greater in dogs exposed to stress compared to controls although this effect significantly decreased after hospitalization stress. Conversely, stress did not alter induced or spontaneous cytokine production from leukocytes, neutrophil or monocyte expression of TLR, LPS-induced down-regulation of monocyte TLR, LPS-induced neutrophil and monocyte expression of MHCII or peripheral blood lymphocyte phenotype.

TLR4 is a cellular receptor involved in recognition of LPS which allows activation of the innate immune system during infection. Binding of LPS to TLR4 on the neutrophil results increased inflammatory cytokine and chemokine production, resulting in immune stimulation and a decrease in neutrophil apoptosis. TLR4 receptor density on the cell surface can be altered by LPS though several mechanisms including endocytosis of the receptor and downregulation of receptor expression. Generally, LPS exposure results in a reduction of TLR4 on the cell surface of neutrophils, as part of the pathway for endotoxin tolerance.[14] Indeed this is typically observed in neutrophils from healthy dogs.[3] While we did not observe a difference in constitutive expression of TLR4 on either neutrophils or monocytes, we did find that LPS-mediated neutrophil TLR4 cell surface expression differed in dogs exposed to stress. In people, the chronic stress of arduous military training results in upregulation of TLR4 expression on monocytes.[15] In dogs with steroid-responsive meningitis-

arteritis, peripheral blood monocyte expression of TLR4 is reduced by glucocorticoid administration, but a similar effect is not observed with neutrophils.[16] In dogs exposed to the stress of hospitalization in our study, LPS-mediated neutrophil TLR4 cell surface expression was blunted implying that expected compensatory mechanisms associated with endotoxin tolerance are altered by stress.

MHCII is a receptor expressed on neutrophils and monocytes and is important for antigen recognition and cross talk between the innate and adaptive immune systems. Downregulation of MHCII is a hallmark of altered immune function in people following stressful events. Both cortisol and IL-10 contribute to downregulation of MHCII on monocytes.[17, 18] Interestingly, dogs in this study had increased neutrophil and monocyte MHCII expression following transportation stress. This increased expression was not present in hospitalized dogs, suggesting the effect was transient. Interferons upregulate MHCII expression in human monocytes in vivo although interferon was not measured in dogs in this study. Hydrocortisone has also been shown to up-regulate MHCII expression on human monocytes in vitro, but similar studies have not been carried out in dogs.[19, 20] Further investigation into the mechanism of MHCII upregulation and the effect of other immunologic parameters on MHCII expression in the dog are needed.

PBMC cytotoxicity is a marker of NK cell function and is an important aspect of cell mediated immunity especially in the context of clearance of infected and neoplastic cells. PBMC cytotoxicity function was blunted in dogs exposed to transportation and hospitalization stress. This is consistent with studies in other species. In rats, sleep deprivation reduces splenic NK cell cancer cytotoxicity through B2 adrenergic signaling.[21] In people, psychological stress prior to a planned surgical procedure results in reduced NK cell cytotoxicity, which is then further exacerbated with anesthesia and surgery.[22] Furthermore, when people are exposed to a series of stressful events (chronic stress)

changes in NK cell cytotoxicity are more variable. Death of a spouse results in NK cell dysfunction while experiencing a natural disaster upregulated NK cell function.[23] Some of the differences in these responses could be due production of glucocorticoids; grieving is generally associated with increased cortisol while trauma is typically associated with decreased cortisol.

Apoptosis is an important inflammation resolution pathway. Neutrophil clearance from the site of inflammation happens predominately though this pathway. Inhibition of apoptosis leads to neutrophil immortality and, in many cases, tissue damage. Conversely, early neutrophil apoptosis could result in unbridled infection. Glucocorticoids are potent inhibiters of neutrophil apoptosis for up to 48 hours ex vivo. [24, 25] Likewise, beta adrenergic agonists have a similar effect.[26] Thus, it would be expected that stress would inhibit neutrophil apoptosis. Surprisingly, we found that neutrophil apoptosis was greater in dogs exposed to the stress of transportation compared to controls although this effect significantly decreased after the stress of hospitalization. This is similar to conflicting data in people following exercise – while acute bouts of exercise accelerates neutrophil apoptosis, moderate chronic exercise results in decreased neutrophil apoptosis.[27] In bulls exposed to acute transportation stress, there is down regulation of genes associated with neutrophil apoptosis suggesting there could be differences in endogenous stress and in vitro exposure to glucocorticoids or beta adrenergic agonists.[28]

In most species, catecholamines alter activation of NF-kB which, in turn, alters production of cytokines.[29, 30] However, the response is dependent on the type of catecholamine involved. Signaling through alpha adrenergic receptors on immune cells promotes production of proinflammatory cytokines like TNF and IL-6 while signaling though beta adrenergic receptors results in inhibition of proinflammatory cytokine production.[31-34] Glucocorticoids also inhibit cytokine production and canine leukocytes contain glucocorticoid receptors. [35, 36] We did not find a difference in

spontaneous or LPS-induced TNF, IL-6 or IL-10 production between the dogs undergoing stress and the control dogs. In people experiencing stress, LPS-induced TNF and IL-6 production is downregulated at 20 minutes post stressor, but returns to baseline values by one hour. Blunting of cytokine production is associated with a spike in epinephrine and norepinephrine in the blood, but proceeds the spike in cortisol.[37] The rapid return of cytokine response post-acute, transient stress has been demonstrated multiple times in people.[38] In people certain stressors result in promotion of LPS-induced TNF production from whole blood at 48 hours.[39] Therefore, it is possible that the timing of our sample collection explains why our results are different.

Peripheral blood lymphocyte subsets are altered with stress, however, the type of change is variable based on the species and type of stress involved. In people undergoing psychological stress, there is an increase in the percentage of CD8+ T cells but no concurrent change in CD4+ T cells.[40] In rats, 48 hours after a psychological stress there is an increase in the percentage of CD4+ T cells and NK cells while CD8+ T cells and B cells increase in the peripheral blood although there is some variability in these responses with certain subgroups of rats having no change in their peripheral blood lymphocyte phenotype.[41] In dogs there is limited information available about changes to lymphocytes during stress. Stress induced by air transport results in lymphopenia and this change in lymphocyte count is thought to be due, in part, to lymphocyte apoptosis.[42, 43] Acute administration of glucocorticoids results in a decrease in CD4+ T cells and B cells at 24 hours in dogs, however, chronic administration of prednisone for three weeks does not alter peripheral blood lymphocyte phenotype composition.[44, 45] In our study, transportation, routine examination, phlebotomy and instrumentation have minimal impact on lymphocyte phenotype distribution. This suggests that short term stress has minimal impact on lymphocyte phenotype distribution in the dog.

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The effects of stress on the immune system in people are variable, contrasting, and likely dependent on the duration of stress, leukocytes affected, physiologic or pharmacologic glucocorticoids, and the timing of stress relative to the time of a particular immune response to concurrent injury.[46] Similarly, in this study, the immune response of dog to acute stress of short duration (transportation to the hospital) and acute stress of longer duration (24 hours) was variable. While NK cytotoxicity, LPS induced neutrophil TLR4 expression, and neutrophil apoptosis were decreased, neutrophil MHCII expression was increased. Neutrophil function was enhanced and expression of MHCII increased following acute stress, but did not differ from control following hospitalization. The return to control levels following the 24 hours in the hospital may represent habituation and desensitization to the hospital environment. Such acclimation effects may be selective, as not all parameters returned to control levels following 24 hours of hospitalization. In comparison to controls, hospitalized dogs demonstrated blunted immune function, with decreased PBMC cytotoxicity, enhanced apoptosis of neutrophils, and decreased neutrophil TLR4 expression following both travel and hospitalization. Lastly, circulating lymphocyte phenotype and stimulated leukocyte cytokine production was not different among the different groups. These data underscore the complexity of stress and its effect on the immune system and raise compelling questions about how clinical outcomes might be influenced by environmental stress associated with hospital visits in veterinary patients. Future work should address how stressor chronicity affects immunologic changes and whether these responses are predictive of clinical outcomes, as there may exist an unmet need for biomarkers to identify stress-vulnerable patient populations. Additionally, investigating the effects of stress on dogs with genetically mediated or other alterations in immune function was not evaluated in this study and might alter these results.

The stress of transportation and routine veterinary procedures alters the immune system in healthy dogs including cellular receptor expression, cytotoxic function and neutrophil apoptosis.

Conversely, stress did not alter cytokine production or peripheral blood lymphocyte phenotype. Overall, the acute stress associated with transportation appeared to have a greater impact on immune parameters compared to the longer-term stress of hospitalization. Investigators should consider the stress of transportation, routine veterinary procedures and hospitalization as confounding variables when interpreting immunologic data from dogs.

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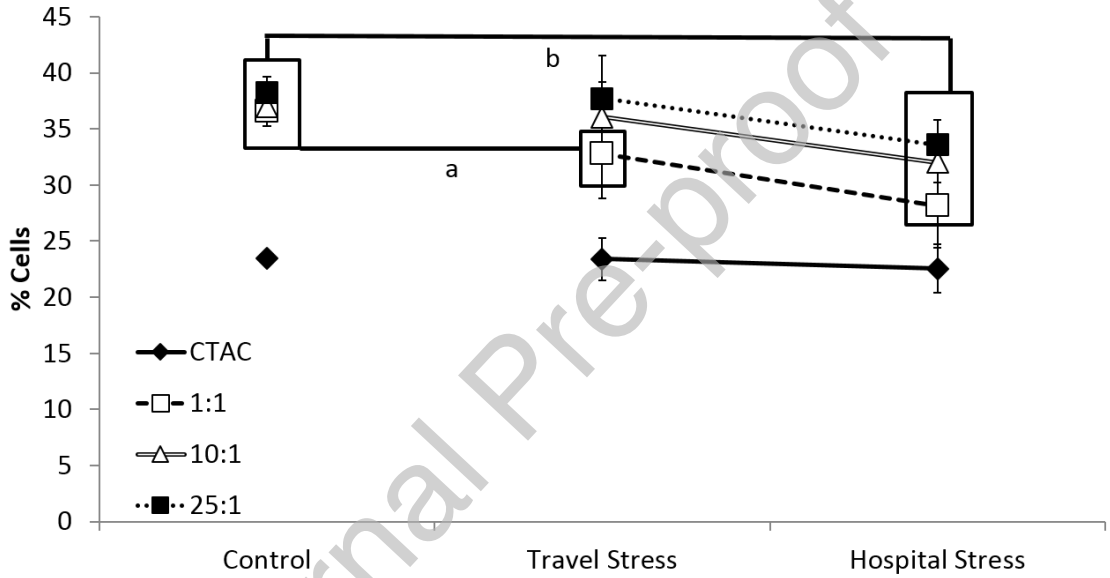
Figure 1: Comparison of the mean±SD percentage of CTAC cells undergoing spontaneous death (CTAC) or PBMC -mediated cytotoxicity (NK-cell like activity) at various PBMC to CTAC cell ratios (1:1, 10:1, 25:1) among the control dogs and dogs undergoing transportation or hospitalization stress. Dogs exposed to transportation stress (1:1 ratio) and hospital stress (1:1, 10:1, 25:1 ratios) had reduced cytotoxicity compared to control dogs. [a]P=0.013, [b]P<0.04.



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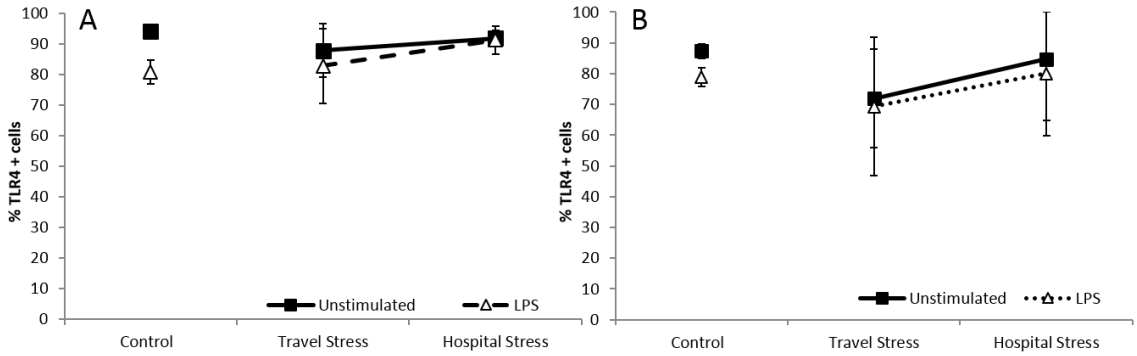

Figure 2: Comparison of the mean±SD of neutrophils (A) and monocytes (B) expressing TLR4 on their cell surface with (LPS) or without (unstimulated) LPS stimulation among the control dogs and dogs undergoing transportation or hospitalization stress.

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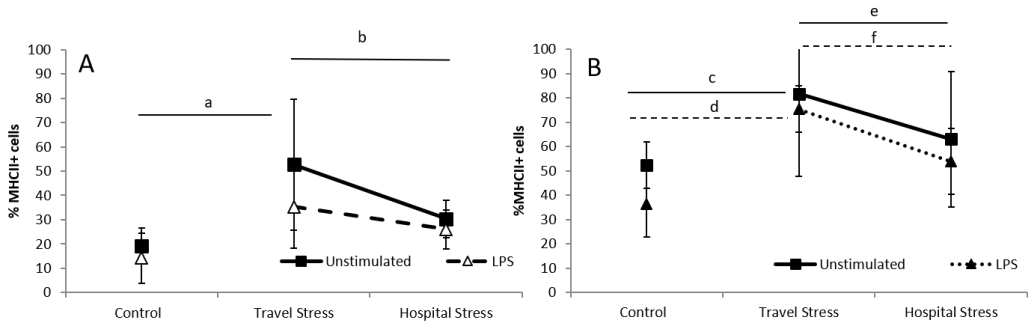

Figure 3: Comparison of the mean±SD of neutrophils (A) and monocytes (B) expressing MHCII on their cell surface with (LPS) or without (unstimulated) LPS stimulation among the control dogs and dogs undergoing transportation or hospitalization stress. Dogs exposed to transportation stress had a greater percentage of neutrophils (constitutive expression) and monocytes (constitutive and after LPS exposure)) expressing MHCII than the control dogs or dogs exposed to hospital stress. $^{a}P=0.015$, $^{b}P=0.044$. $^{c}P=0.006$, $^{d}P=0.005$. $^{e}P=0.024$, $^{f}P=0.046$.

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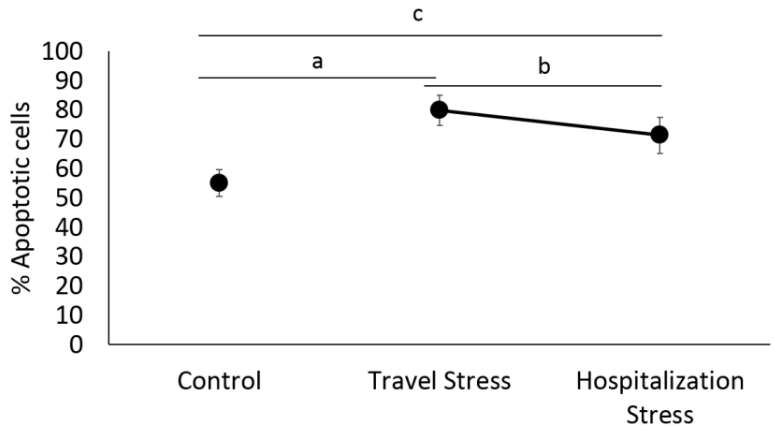

Figure 4: Comparison of the mean±SD of the percentage of neutrophils undergoing spontaneous apoptosis between the control dogs and dogs undergoing transportation or hospitalization stress. Spontaneous neutrophil apoptosis was greater in dogs undergoing transportation or hospitalization stress compared to control and dogs undergoing transportation stress had greater neutrophil apoptosis than dogs undergoing the stress of hospitalization. $^{a}P<0.001$, $^{b}P=0.022$, $^{c}P=0.009$.

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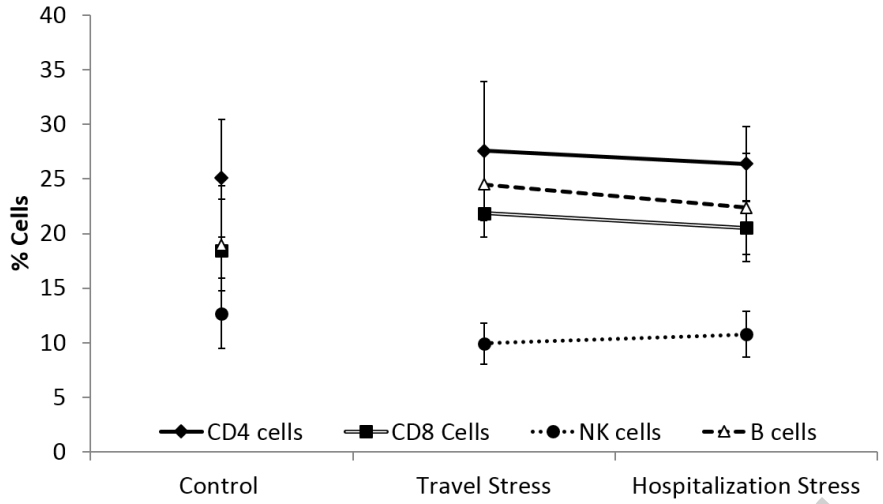

Figure 5: Comparison of the mean±SD of the peripheral blood lymphocyte phenotype between the control dogs and dogs undergoing transportation or hospitalization stress.

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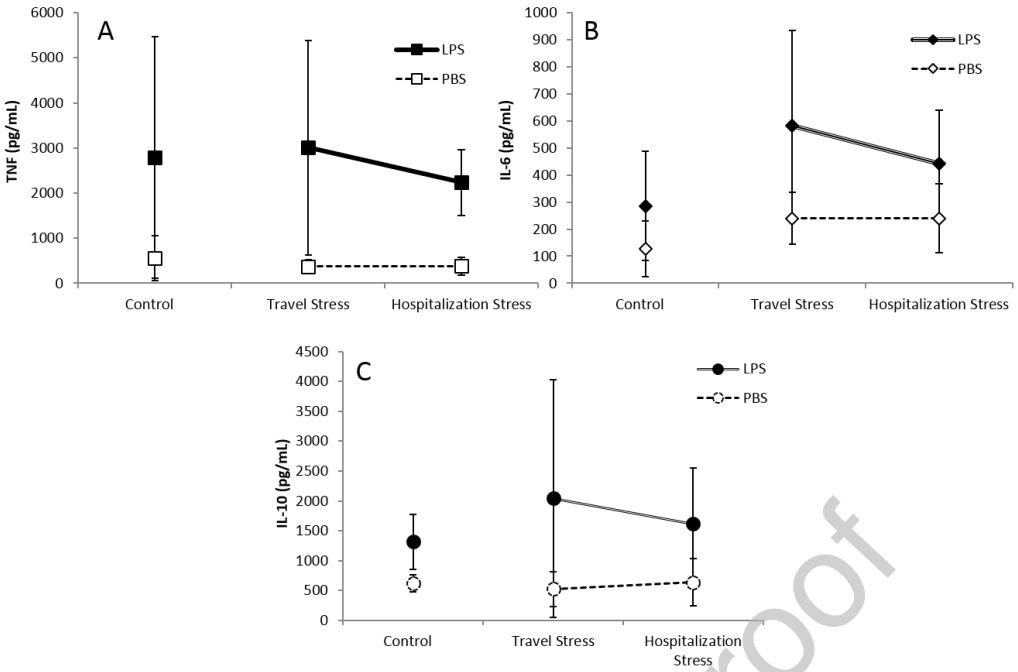

Figure 6: Comparison of the mean±SD of LPS and PBS-stimulated TNF (A), IL-6 (B) and IL-10 (C) production from whole blood between the control dogs and dogs undergoing transportation or hospitalization stress.

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