Effect of disrupted mitochondria as a source of damage-associated molecular patterns on the production of tumor necrosis factor α by splenocytes from dogs

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OBJECTIVE
To evaluate the effects of damage-associated molecular patterns (DAMPs) derived from disrupted mitochondria on canine splenocytes and other immune cells.

SAMPLES
Liver, spleen, and bone marrow samples obtained from 8 cadavers of healthy research Beagles that had been euthanized for other purposes.

PROCEDURES
Mitochondria were obtained from canine hepatocytes, and mitochondrial DAMPs (containing approx 75% mitochondrial proteins) were prepared. Mitochondrial DAMPs and the nuclear cytokine high-mobility group box protein 1 were applied to splenocytes, bone marrow–differentiated dendritic cells, and a canine myelomonocytic cell (DH82) line for 6 or 24 hours. Cell culture supernatants from splenocytes, dendritic cells, and DH82 cells were assayed for tumor necrosis factor α with an ELISA. Expression of tumor necrosis factor α mRNA in splenocytes was evaluated with a quantitative real-time PCR assay.

RESULTS
In all cell populations evaluated, production of tumor necrosis factor α was consistently increased by mitochondrial DAMPs at 6 hours (as measured by an ELISA). In contrast, high-mobility group box protein 1 did not have any independent proinflammatory effects in this experimental system.

CONCLUSIONS AND CLINICAL RELEVANCE
The study revealed an in vitro inflammatory effect of mitochondrial DAMPs (containing approx 75% mitochondrial proteins) in canine cells and validated the use of an in vitro splenocyte model to assess DAMP-induced inflammation in dogs. This experimental system may aid in understanding the contribution of DAMPs to sepsis and the systemic inflammatory response syndrome in humans. Further studies in dogs are needed to validate the biological importance of these findings and to evaluate the in vivo role of mitochondrial DAMPs in triggering and perpetuating systemic inflammatory states. (Am J Vet Res 2016;77:604–612)

Systemic inflammatory response syndrome is a potentially life-threatening condition that develops secondary to massive immune system activation or dysregulation of a localized inflammatory response.1,2 Clinically, SIRS involves abnormalities in 2 or more of the following variables: core temperature, heart rate, respiratory rate, and WBC count or morphology.1 Systemic inflammatory states can have both infectious and noninfectious etiologies, with the former commonly referred to as sepsis. Noninfectious SIRS can be caused by conditions such as severe ischemia, hemorrhagic shock, or multiple trauma-associated tissue injuries and frequently results in dysfunction of multiple organs such as renal failure and acute lung injury.2,3

Damage-associated molecular patterns are triggers of noninfectious SIRS.3,4 These patterns are found on molecules released from endogenous sources during cell necrosis. Recognition of these patterns by membrane-bound or cytosolic pattern-recognition receptors, such as TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-inducible gene 1 (RIG-I-like) receptors, results in the activation of intracellular cascades that lead to inflammatory responses similar to those that develop follow-

ABBREVIATIONS
DAMP Damage-associated molecular pattern
emPAI Exponentially modified protein abundance index
FPR Formyl peptide receptor
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
HMGB1 High-mobility group box protein 1
LC-MS/MS Liquid chromatography tandem mass spectrometry
mtD Mitochondrial DAMP (soluble protein fraction)
mtF Bulk fractionated mitochondria
SIRS Systemic inflammatory response syndrome
TLR Toll-like receptor
TNFα Tumor necrosis factor α

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ing activation by pathogens such as bacteria.\textsuperscript{3,5} In this manner, DAMPs modulate the immune system by affecting the function of antigen-presenting cells (dendritic cells and macrophages), eosinophils, mast cells, and neutrophils.\textsuperscript{6-10} Damage-associated molecular patterns that have been well studied in humans and mice include HMGB1, heat shock proteins, nucleic acids, and S100 proteins.\textsuperscript{3,5,11}

The importance of DAMPs derived from the breakdown of mitochondria has more recently been recognized. Mitochondria are evolutionary endosymbionts derived from bacteria; when exposed to the immune system, mitochondria elicit a response similar to that associated with bacteria.\textsuperscript{12,13} Mitochondrial DAMPs include mitochondrial DNA, N-formyl peptides, and transcription factor A, mitochondrial.\textsuperscript{14} These DAMPs are highly inflammatory and, among other effects, cause upregulation of TNF\textalpha in splenocytes, production of type I interferons by plasmacytoid dendritic cells, and marked neutrophil chemotaxis in mice and humans.\textsuperscript{5,15-21} To date, the role of mtDs as a trigger of SIRS in dogs has not been explored, to our knowledge.

Given that dogs may provide a more relevant animal model with which to study sepsis and SIRS in humans than rodents and that mice may fail to mimic responses in human inflammatory states, dogs may be a useful alternative to rodents in studies of the immunopathogenesis of these conditions.\textsuperscript{22,23} The objective of the study reported here was to evaluate the effects of DAMPs derived from disrupted mitochondria on canine splenocytes and other immune cells. By characterizing the response of canine splenocytes to mtDs, the intent was to develop an in vitro system for investigation of SIRS-induced immune dysregulation in dogs. We hypothesized that mtDs would cause upregulation of expression of the cytokine TNF\textalpha in several canine cell types involved in the inflammatory cascade.

**Materials and Methods**

**Dogs**

The 8 dogs used in the study had been part of a research colony and were euthanized for other purposes by IV overdose of pentobarbital. The study was approved by the institutional animal care and use committee, and all dogs had been maintained in university laboratory animal resources facilities and housed according to the institutional animal care and use committee guidelines.

**mtDs**

Approximately 60 g of freshly harvested liver tissue was collected from each of 2 healthy Beagle cadavers. Mitochondria were isolated from the hepatocytes in the tissue samples with the high-purity protocol of a commercially available kit. Mitochondrial preparations were examined with a transmission electron microscope\textsuperscript{b} at an accelerating voltage of 80 kV.

Bulk fractionated mitochondria were prepared by freeze-thaw cycling of intact mitochondrial preparations. Mitochondria were placed alternately in a dry ice-ethanol bath and a water bath at 37°C for 5 minutes each with 3 repetitions. A protease inhibitor mixture was added at a ratio of 1:100 (vol:vol) prior to freeze-thaw cycling.\textsuperscript{c} Concentrations of mtF were determined by means of a fluorometer with a bovine serum albumin standard.\textsuperscript{d}

Soluble mitochondrial proteins were prepared from freeze-thaw-cycle–disrupted mitochondria that had been derived from freshly harvested hepatocytes. After 3 freeze-thaw cycles, the preparation was centrifuged at 14,000 \( \times \) g at 4°C for 20 minutes to pellet the mitochondrial membranes and membrane-bound proteins and leave the soluble proteins in the supernatant. Pellets were discarded, and supernatants were stored for further use. In keeping with prior experiments,\textsuperscript{6} these fractions are referred to as mtDs. Samples were quantified fluorometrically as described, and protein composition was determined by capillary LC-MS/MS.\textsuperscript{e} We also quantified the amount of each specific protein in each mtD preparation with the emPAI, which provides a relative quantitation on the basis of the number of peptide fragments detected by mass spectroscopy.\textsuperscript{24}

**Other DAMPs**

Nonmitochondrial DAMPs were purchased from commercial sources. Recombinant human HMGB1, which has 100% amino acid homology with canine HMGB1 and was grown in a murine myeloma cell line, was used; it contained < 0.1 U of endotoxin/\( \mu \)g of protein and was > 95% pure.\textsuperscript{f} Lipopolysaccharide from *Salmonella enterica* serotype Typhimurium was used and was > 99% pure.\textsuperscript{5}

**Splenocytes**

Immediately following euthanasia, an approximately 5-cm\textsuperscript{3} portion of fresh spleen was harvested from each of the 8 dogs. The spleen was stored in PBS solution on ice for transport to the laboratory (duration of storage and transport, \(< 5\) minutes) and then quickly pressed through a metal strainer to break up the connective tissue. The strained splenocytes were homogenized further with a 5-mL syringe and concentrated by centrifugation. Erythrocytes were lysed with an ammonium chloride lysis buffer, and the splenocytes were washed twice with sterile PBS solution. Counted cells were then resuspended in supplemented RPMI medium containing 10% fetal bovine serum, 25mM HEPES, 2mM L-glutamine, penicillin (50 U/mL), streptomycin (50 mg/mL), and 5 \( \times \) 10\(^{-6}\) M \( \beta \)-2-mercaptoethanol and used immediately in the experimental assays.

**Macrophage cell line**

Canine DH82 cells\textsuperscript{h} were used to approximate macrophage function; these cells are well described as representative macrophages and monocytes and are...
derived from a canine histiocytic sarcoma line.\textsuperscript{25} The cells were quickly defrosted from liquid nitrogen in a water bath at 37°C, collected by centrifugation (1,500 \( \times \) g at 4°C for 7 minutes), and cultured in RPMI medium with 10% fetal bovine serum. Cells were recovered for 24 hours at 37°C in an atmosphere containing 5% \( \text{CO}_2 \) prior to use in functional assays.

**Dendritic cells**

Dendritic cells were differentiated from bone marrow cells as previously described.\textsuperscript{26} Briefly, bone marrow cells were harvested from the femurs of 3 study dogs immediately following euthanasia. Cells were placed in sterile PBS solution containing penicillin (50 U/mL) and streptomycin (50 mg/mL) and concentrated by centrifugation. Erythrocytes were lysed with an ammonium chloride lysis buffer, and the remaining cells were washed twice with sterile PBS solution, counted on a hemocytometer, and plated in RPMI medium with 10% fetal bovine serum supplemented with recombinant canine granulocyte–monocyte colony-stimulating factor (20 ng/mL). Cells were incubated at 37°C in an atmosphere containing 5% \( \text{CO}_2 \) for 6 to 10 days to allow differentiation into dendritic cells; the dendritic cells were then collected by trituration and washed twice in sterile PBS solution prior to use in functional assays.

**Cell activation assays**

For the splenocytes, DH82 cells, and dendritic cells, approximately 1 \( \times \) 10\(^6\) cells of each type were aliquoted into separate wells, and the previously described DAMPs were applied to the cells at the following concentrations: mtD, 80 \( \mu \)g/mL; mtF, 80 \( \mu \)g/mL; HMGB1, 5 \( \mu \)g/mL; and lipopolysaccharide, 1 \( \mu \)g/mL (positive control). Relevant concentrations of DAMPs were determined from published reports\textsuperscript{6,15,16,27,28} and were tailored on the basis of results of preliminary studies performed prior to the study reported here. A negative control in which 10% RPMI medium was substituted for DAMPs was included in all experiments. After DAMPs were applied, cells were incubated at 37°C in an atmosphere containing 5% \( \text{CO}_2 \) for either 6 or 24 hours. Splenocyte assays were repeated with cells obtained from 8 dogs; ELISAs were performed on supernatants from all 8 experiments, but the quantitative real-time PCR assay was performed on cells from only 4 dogs. Dendritic cell assays were repeated with cells obtained from 3 dogs. Bulk fractioned mitochondria were not applied to dendritic cells because of limited DAMP availability.

**TNF\( \alpha \) ELISA**

The TNF\( \alpha \) ELISA was performed on cell culture supernatants from splenocytes, DH82 cells, and dendritic cells. The ELISA was obtained from a commercial supplier, and manufacturer protocols were followed.\textsuperscript{1} Each sample was run in triplicate, and a mean concentration was calculated.

**Quantitative real-time PCR assay for TNF\( \alpha \)**

A quantitative real-time PCR assay for TNF\( \alpha \) was performed on RNA collected from splenocytes. Cells were prepared by trituration, and the RNA was immediately isolated with a commercially available kit\textsuperscript{1} according to the manufacturer’s protocol in an RNase-free hood. Concentrations of mRNA in samples were determined by spectrophotometry, and purity was assessed by measuring \( A_{260/280} \) (the ratio of absorption of UV light at a wavelength of 260 nm vs 280 nm); values \( > 1.9 \) were considered acceptable.\textsuperscript{8} The cDNA was prepared with a reverse transcription kit\textsuperscript{1} according to the manufacturer’s recommendations. For real-time experiments, a canine GAPDH primer was used as an endogenous control,\textsuperscript{29} and negative control wells were used as the zero calibrator. Exon-spanning primer sequences for TNF\( \alpha \) were obtained from a published report\textsuperscript{29} and purchased from a commercial supplier.\textsuperscript{8} Relative quantitation was performed by the compara-
tive threshold cycle method (ΔΔCt) method. Reaction conditions for the real-time PCR assay were as follows: 50 ng of cDNA template (assuming 90% yield from reverse transcription), 0.5 μM of each primer, and 1X master mix containing DNA polymerase, deoxynucleotides, SYBR Green, and reaction buffers. Thermal cycler conditions were 95°C for 10 minutes (activation), 95°C for 15 seconds (melting), and 58°C for 1 minute (annealing and extension) for 40 cycles; melting curve analysis was performed between 55° and 95°C to rule out primer-dimer or spurious product formation. All reactions were performed in duplicate, and a mean TNFα concentration was calculated for analysis. The real-time PCR assay was performed on a thermal cycler and analyzed with software.

### Statistical analysis

Data from each experiment were tested for outliers by calculating the interquartile range (25th to 75th percentile) for each data set, and extreme outliers > 3 times the interquartile range were eliminated. Data were tested for normality with the Shapiro-Wilk test; as the data were not normally distributed, differences in cell stimulation for each DAMP were assessed with a Kruskal-Wallis test. For experiments that yielded a significant result, post hoc pairwise comparisons were performed with the Dwass-Steel test in which medium was used as the negative control. A value of P < 0.05 was considered significant for all comparisons. All statistical analyses were performed with a commercially available statistical software package.

### Results

#### Characterization of mtDs

Electron microscopy was used to examine purified mitochondria for verification of cell separation methods (Figure 1). To confirm that the mtD preparations were enriched for mitochondrial proteins, each preparation of mtD (preparations from 2 dogs) was analyzed by LC-MS/MS, and the relative quantities of each protein were determined on the basis of the emPAI Table 1. Approximately 75% of the proteins identified were of mitochondrial origin, and this finding was consistent for both mtD preparations.

#### TNFα production by DAMP-stimulated splenocytes

The concentration of TNFα in splenocytes was significantly greater in cells that were stimulated for 6 hours with either mtD (P = 0.023) or mtF (P = 0.039), compared with that of cells in RPMI medium alone (Figure 2). The TNFα ELISAs were also performed after stimulation of splenocytes for 24 hours; however, TNFα concentration in any of the DAMP-stimulated cell samples did not differ significantly from that of cells in RPMI medium alone at this time point. Treatment with the positive control (lipopolysaccharide)

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**Table 1**—Representative relative quantification of mitochondrial proteins (determined on the basis of the emPAI) in one of the mtD preparations obtained from hepatocytes harvested from 2 Beagle cadavers.

<table>
<thead>
<tr>
<th>Protein</th>
<th>emPAI</th>
<th>Molar (%)</th>
<th>Weight (%)</th>
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<tbody>
<tr>
<td>Mitochondrial proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ornithine carbamoyltransferase</td>
<td>83.9</td>
<td>26.8</td>
<td>20.6</td>
</tr>
<tr>
<td>Glutamate dehydrogenase 1</td>
<td>28.3</td>
<td>9.1</td>
<td>11.4</td>
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<td>δ-1-pyrroline-5-carboxylate dehydrogenase</td>
<td>21.8</td>
<td>7.0</td>
<td>8.3</td>
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<tr>
<td>60-kDa heat shock protein</td>
<td>18.7</td>
<td>6.0</td>
<td>6.9</td>
</tr>
<tr>
<td>Alddehyde dehydrogenase</td>
<td>10.1</td>
<td>3.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>11.6</td>
<td>3.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Serine-pyruvate aminotransferase</td>
<td>9.7</td>
<td>3.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Acyl-CoA synthetase family member 2</td>
<td>5.6</td>
<td>1.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Adenosine triphosphate synthase subunit beta</td>
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<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Methylmalonate-semialdehyde dehydrogenase</td>
<td>3.6</td>
<td>1.1</td>
<td>1.3</td>
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<tr>
<td>Alddehyde dehydrogenase X</td>
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</tr>
<tr>
<td>Isovaleryl-CoA dehydrogenase</td>
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<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Others</td>
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<td>10.8</td>
<td>10.0</td>
</tr>
<tr>
<td>Total mitochondrial proteins</td>
<td>76.0</td>
<td>74.1</td>
<td></td>
</tr>
</tbody>
</table>

| Nonmitochondrial proteins                            |       |           |            |
| Catalase                                             | 36.6  | 12.0      | 13.0       |
| α-Aminoadipic semialdehyde dehydrogenase             | 9.8   | 3.1       | 3.6        |
| Nonspecific lipid-transfer protein isofrom 3         | 3.3   | 1.1       | 1.2        |
| Serum albumin precursor                              | 2.6   | 0.8       | 1.1        |
| Quinone oxidoreductase                               | 3.5   | 1.1       | 0.8        |
| Others                                               | NA    | 5.9       | 6.2        |
| Total nonmitochondrial proteins                      | 24.0  |           | 25.9       |

Soluble mitochondrial proteins were prepared from freeze-thaw-cycle disrupted mitochondria that had been derived from freshly harvested hepatocytes. Samples were quantified fluorometrically, and protein composition was determined by capillary LC-MS/MS. The amount of each specific protein in each mtD preparation was quantified with the emPAI, which provides a relative quantitation on the basis of the number of peptide fragments detected by mass spectrometry. The third and fourth columns represent the percentage protein composition determined on a molar and weight ratio, respectively. NA = Not applicable.
increased the amount of TNFα at both 6 and 24 hours. A quantitative real-time PCR assay was subsequently performed on mRNA isolated from DAMP-treated splenocytes to evaluate whether TNFα mRNA expression was upregulated by mtDs. Compared with findings for the control sample (medium with GAPDH as an endogenous control), no significant increase in TNFα mRNA expression was evident in splenocytes stimulated by mtD or mtF at 6 hours, compared with findings for cells treated with medium alone. Treatment with LPS increased splenocyte production of TNFα at both 6 and 24 hours. Treatment of splenocytes with HMGB1 did not result in a significant increase in TNFα concentration. 

![Figure 2](image)

**Figure 2**—Mean ± SEM TNFα concentration (A) and change in TNFα mRNA expression (B) in splenocytes obtained from Beagle cadavers after exposure to various DAMPs. A—Fresh canine splenocytes (1 X 10⁶ cells/experiment) were exposed to mtD (80 µg/mL), mtF (80 µg/mL), HMGB1 (5 µg/mL), lipopolysaccharide (1 µg/mL; LPS [positive control]), or 10% RPMI medium (negative control) for 6 (black bars) or 24 (gray bars) hours. The TNFα concentrations in cell culture supernatants were measured by an ELISA. Experiments were repeated 8 times (1 experiment/dog). Production of TNFα was increased in splenocytes stimulated with mtD or mtF at 6 hours, compared with findings for cells treated with medium alone. Treatment with LPS increased splenocyte production of TNFα at both 6 and 24 hours. Treatment of splenocytes with HMGB1 did not result in a significant increase in TNFα concentration. B—Canine splenocytes were incubated with mtD, mtF, HMGB1, or LPS for 6 hours at the aforementioned concentrations, and the amount of TNFα mRNA was quantified by a quantitative real-time PCR assay. The mRNA concentrations are expressed as the log fold change from findings for cells treated with RPMI medium alone; GAPDH was used as an endogenous control. Experiments were repeated 4 times (1 experiment/dog). Pairwise comparisons against the medium control data were not significant for any DAMP. *Value was significantly different (P < 0.05) from that for cells exposed to medium alone at the same time point.

![Figure 3](image)

**Figure 3**—Mean ± SEM TNFα production in DH82 cells (derived from a canine histiocytic sarcoma line; A) and dendritic cells (B) obtained from 3 Beagle cadavers after exposure to various DAMPs. The DH82 cells (1 X 10⁶ cells/experiment) were exposed to mtD (80 µg/mL), mtF (80 µg/mL), HMGB1 (5 µg/mL), lipopolysaccharide (1 µg/mL; LPS [positive control]), or 10% RPMI medium (negative control) for 6 hours. Dendritic cells were not exposed to mtF owing to limited DAMP availability. The TNFα concentrations in cell culture supernatants were measured by an ELISA. For each cell type, experiments were repeated 3 times (1 experiment/dog). Compared with findings for cells in medium alone, production of TNFα was increased in both DH82 and DCs stimulated with mtD. Treatment with LPS increased production of TNFα in both cell types at 6 hours. Treatment of either cell type with HMGB1 did not result in a significant increase in TNFα concentration. *Value was significantly different (P < 0.05) from that for cells exposed to medium alone at the same time point.

**TNFα production by DH82 cells and bone marrow–derived dendritic cells**

The DH82 cells (macrophage-like cells) and dendritic cells were stimulated with DAMPs or lipopolysaccharide (positive control) for 6 hours; cell culture supernatants were collected and analyzed for TNFα. With regard to DH82 cells, treatment with mtD, but not mtF, significantly (P = 0.036) increased TNFα production, compared with the finding for cells exposed to RPMI medium alone (Figure 3). The supernatant...
from cultured dendritic cells stimulated by mtD for 6 hours also had a significantly ($P = 0.040$) greater TNF$\alpha$ concentration, compared with the RPMI medium control. No significant increase in TNF$\alpha$ concentration was evident for dendritic or DH82 cells treated with HMGB1; changes in TNF$\alpha$ concentration induced by LPS are shown as a reference.

**Discussion**

In the study reported here, the activation of canine immune cells by mitochondrial fractions was investigated, and the results indicated that mtDs (containing approx 75% mitochondrial proteins) had a significant impact on stimulating the production of TNF$\alpha$ in splenocytes, dendritic cells, and DH82 cells after 6 hours of exposure, as assessed by an ELISA. These findings are in agreement with studies$^{14,31,32}$ in mouse splenocytes, wherein mtDs (eg, mitochondrial transcription factor A or mitochondrial DNA) were shown to promote TNF$\alpha$ release, suggesting that the in vitro experimental system used in the present study would be useful in the study of the impact of mtDs in dogs.

Splenocytes include a diverse population of inflammatory cells and are frequently used in immunologic studies because their heterogeneity with both innate and adaptive immune functions may mimic in vivo responses.$^{15,21,33-35}$ More importantly, this cell population has been shown to have functional impairment in sepsis.$^{36,37}$ Dendritic cells and macrophages are important immune cell constituents of splenocytes that contribute to cytokine production and are modulators of both the innate and adaptive immune responses. In sepsis and SIRS, dendritic cells and macrophages are some of the first immune cells to respond to DAMPs and are critical cells in controlling subsequent innate and adaptive immune responses.$^{38-40}$ Therefore, in the present study, we investigated whether these cell populations contributed to the DAMP-induced increase in TNF$\alpha$ production by splenocytes (as detected in cell culture supernatants by ELISA). Unlike for humans and mice, there are few commercially available reagents available for purifying myeloid subpopulations (eg, dendritic cells) from canine splenocytes. For that reason, we generated dendritic cells from canine bone marrow samples and used a canine macrophage-like cell line (DH82 cells) to approximate these splenocyte subpopulations.

Two distinct mitochondrial fractions were used in the present investigation to activate immune cells: mtF (containing mitochondrial proteins, membranes, and DNA) and mtD (soluble mitochondrial proteins and DNA without membranes and membrane-bound proteins). The data obtained suggested that both mtD and mtF have inflammatory effects in dogs because both fractions stimulated splenocyte production of TNF$\alpha$ after 6 hours of exposure, compared with findings for splenocytes after exposure to RPMI medium alone; mtD stimulated dendritic cells and DH82 cells to a similar extent. These results provided evidence that mitochondrial components are important triggers of inflammation in dogs, which is consistent with recent reports of the inflammatory potential of mtDs in other model systems.$^{5,15,21}$

The cytokine TNF$\alpha$ has a key role in the pathogenesis of SIRS.$^{41,42}$ When administered exogenously to experimental animals, TNF$\alpha$ causes many classic clinical hallmarks of SIRS including hypotension, shock, acidemia, and thrombosis.$^{33-45}$ Mechanistically, TNF$\alpha$ causes endothelial dysfunction and vasodilation by stimulating inducible nitric oxide synthase and cyclooxygenase-2, as well as increased expression of various leukocyte adhesion molecules.$^{46,47}$ In humans and dogs with sepsis, bloodstream concentration of TNF$\alpha$ is markedly high, particularly early in the course of the disease.$^{48-50}$

In the present study, we focused on TNF$\alpha$ expression following mtD exposure because TNF$\alpha$ expression has been recently shown to be upregulated by certain mtDs.$^{51}$ The precise pathways by which mtDs induce expression of TNF$\alpha$ are unknown and likely diverse. Results of some studies$^{6,52}$ have indicated that mtDs act through TLR-9, FPRs, or NLRP3 (a NOD-like receptor). Given that the mtD fraction used in the present study was highly protein rich, it is possible that an abundance of N-formyl peptides acted on immune cells primarily through FPRs. The FPRs are present on both neutrophils and monocytes, which are enriched in the spleen.$^{20,53}$ The FPRs act via the nuclear factor-xB pathway, which leads to TNF$\alpha$ production.$^{52}$ Although evaluating the role of FPRs was beyond the scope of this study, it would be interesting to use N-formylmethionyl-leucyl-phenylalanine (a known FPR agonist) or an FPR antagonist to determine whether mtD stimulation of TNF$\alpha$ production occurs through this key receptor. Investigations studying the interactions of mtDs with other receptors (eg, TLR-9) would be similarly valuable.

Although splenocyte concentration of TNF$\alpha$ was increased following treatment with mtD, an increase in the amount of TNF$\alpha$ mRNA was not detected at the time points evaluated. We initially chose to evaluate amounts of TNF$\alpha$ mRNA after 6 hours of splenocyte exposure on the basis of published reports.$^{53-55}$ However, it is likely that a detectable increase in TNF$\alpha$ mRNA was present only at time points earlier than 6 hours. Recently, it has been shown that serum TNF$\alpha$ concentration peaks within 1 hour following lipopolysaccharide administration in dogs,$^{56}$ and therefore, further studies of mRNA expression in our experimental system at exposure time points < 6 hours are warranted. Regardless, the presence of TNF$\alpha$ protein detected by ELISA is a biologically and clinically relevant marker in this experimental system. Given that TNF$\alpha$ is an important cytokine involved in SIRS, we believe that TNF$\alpha$ protein detection in this experimental system is useful as a marker to assess the inflammatory potential of mitochondrial stimulation. Induction of TNF$\alpha$ following mtD stimulation appears to be common across species, and although numerous other stimuli can induce TNF$\alpha$ production, TNF$\alpha$ is a useful cytokine for...
assessment of inflammatory responses, and its expression appears to be uniquely upregulated following mtD exposure.\textsuperscript{57}

In the present study, HMGB1 did not stimulate an inflammatory response in any of the cell types assessed. A high plasma concentration of HMGB1 has been shown to be an important biomarker of SIRS in dogs; however, whether HMGB1 actually triggers an inflammatory response in dogs similar to that seen in mice and humans has not been determined.\textsuperscript{58–61} The actual contribution of HMGB1 to inflammation is somewhat controversial. Although early experiments\textsuperscript{11} revealed the proinflammatory abilities of HMGB1, results of recent studies\textsuperscript{53,61} have suggested that purified HMGB1 produced in a non-prokaryotic system may not be a proinflammatory agent by itself. Studies\textsuperscript{62–65} have also shown that the mitochondrial action of HMGB1 is significantly enhanced when it is combined with factors such as single-stranded DNA, interleukin-1β, or lipopolysaccharide, and HMGB1 is known to strongly bind some of these components. The data obtained in the present study have suggested that HMGB1 may need to be combined with a costimulatory DAMP to trigger an inflammatory response in canine splenocytes.

There are several limitations to the present study. Most notably, the mtDs were not completely pure, but rather only enriched (approx 75%) for mitochondrial proteins. On the basis of the electron microscopy findings, the primary contaminant in the mtD preparations appeared to be peroxisomes. However, unlike mitochondria, there are to our knowledge no published data indicating that immune cells develop an inflammatory response when exposed to peroxisomes. To the contrary, healthy peroxisomes are, on balance, anti-inflammatory and are the primary source of the reactive oxygen species-scavenging enzyme catalase.\textsuperscript{56,67} Catalase was also the most abundant (approx 12%) nonmitochondrial protein in the study mtDs. Therefore, although we cannot define the exact contribution of nonmitochondrial proteins to the observed inflammatory response, we believe that the contribution was likely minimal. Further studies involving more highly purified mitochondrial preparations should help resolve this question. Additionally, the increase in TNFα expression in response to mtD was small, especially compared with the response to lipopolysaccharide. For this reason, more studies are warranted to demonstrate the biological importance of the findings of the present study. Finally, the data obtained in this study would have been augmented by investigating the effects of mtDs on other inflammatory cytokines; this area also provides fertile ground for future investigations.

Results of the study reported here have provided information regarding the role of mtDs in dogs, and the in vitro experimental system used should prove useful in further investigating the role of these important inflammatory triggers in this species. Studies are warranted to elucidate the mechanisms underlying the cellular responses detected in the present study and to better characterize the specific mtDs that are most important in clinical patients; such information may ultimately improve outcomes for dogs with sepsis or SIRS.

Acknowledgments

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Footnotes

a. Qiagen Qproteome Mitochondria Isolation Kit, Qiagen USA, Valencia, Calif.
b. ThTOL USA Inc, Peabody, Mass.
c. Protease Inhibitor Cocktail P8340, Sigma-Aldrich, St Louis, Mo.
d. Qubit 2.0 Fluorometer, Qubit Protein Assay Kit, Life Technologies, Carlsbad, Calif.
f. R&D Systems, Minneapolis, Minn.
g. Sigma-Aldrich Corp, St Louis, Mo.
h. Provided by Dr. S. Krakowka, The Ohio State University, Columbus, Ohio.
i. Canine TNF-alpha Quantikine ELISA Kit, R&D Systems, Minneapolis, Minn.
j. Qiagen RNeasy Mini Kit, Qiagen USA, Valencia, Calif.
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