Project Title: Cytokines and acute phase proteins: Validation of a new rapid diagnostic assay to measure the health of stranded marine mammals

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The objectives of the study were:

1. Assess the cross-reactivity in pinnipeds of human reagents to quantify cytokines and acute phase proteins in the cell culture supernatant of un-stimulated and stimulated human and pinniped peripheral blood mononuclear cells.
2. Validate the specificity of the cross-reactivity by comparing the secretion patterns in pinnipeds to those that result in specific patterns of secretion in human cells.

The milestones and timelines were:

The collection, processing, and culturing of pinniped and human whole blood is expected to be completed within the first 6 months of the funding period. It is expected that we will receive blood samples from clinically healthy animals 1-2 times per month. Luminex experiments are expected to be completed within one month following the collection of all supernatant samples. The remaining time will be used to analyze data, prepare a final report, and prepare and submit a manuscript for publication.
Summary

Cytokines are important small proteins that help direct a proper immune response to pathogens. Acute phase proteins are a group of blood proteins that change in concentration in animals subjected to external or internal challenges, such as infection and inflammation. The present study was conducted to assess and validate the cross-reactivity of commercially-available multiplex human and canine kits coupled with the Luminex platform to measure cytokines and acute phase proteins in three pinniped species. The human cytokine kit allowed the detection of cytokines in the supernatant of mitogen-induced human peripheral blood mononuclear cells, but not in the three pinniped species studied, with the exception of TNFα. The human acute phase protein kit allowed the detection of acute phase proteins in the supernatant of human peripheral blood mononuclear cells, however, not surprisingly, stimulation with mitogens did not further increase the level of proteins. In contrast, the canine cytokine kit did cross-react with the majority of cytokines in all three pinniped species. Importantly, cytokines that were quantified in pinnipeds included three pro-inflammatory cytokines (IL-6, IL-8 and TNFα), the Th1 cytokine INFγ, and the Th2 cytokine IL-10. Overall, the Luminex platform and the canine multiplex cytokine kit allowed the successful measurement of potentially clinically important pinniped cytokines. This additional tool may provide veterinarians with additional information to detect sub-clinical signs of inflammation or evidence for immune response, which may not be revealed during regular medical evaluation, e.g. physical examination, hematology, and serum chemistry. Furthermore, in view of the considerable efforts expended in the treatment and rehabilitation of stranded marine mammals in terms of limited financial resources, personnel, and housing, as well as the limited state of the art diagnostic tools available, measurement of cytokines may provide veterinarians with an addition tool to help monitor the health status of pinnipeds during rehabilitation, as well as contribute to the health assessments of wild populations.
Introduction

Efforts expended in the treatment and rehabilitation of stranded marine mammals are considerable, especially in terms of limited financial resources, personnel, and housing. Importantly, limited state of the art diagnostic tools are available that may better assess the health of an animal, potentially resulting in reduced rehabilitation time and overall costs. Additional tools may reveal sub-clinical signs of inflammation or evidence for immune response, which may not be revealed during regular medical evaluation, e.g. physical examination, hematology, serum chemistry, and bacterial cultures.

Cytokines are small cell-signaling protein molecules that are secreted by numerous cells of the immune system. These include interferon, interleukin, and growth factors that are secreted by certain cells of the immune system and have an effect on other cells. Cytokine functions can be subdivided into several broad categories, including lymphocyte proliferation and differentiation, lymphoid development, cell trafficking, control of immune effectors and inflammation, and innate immunity and antigen presentation [1]. Importantly, cytokines can have functions that belong to more than one category.

Commonly, cytokines are divided into pro-inflammatory and anti-inflammatory groups. Pro-inflammatory cytokines, secreted in the beginning of an immune response, include interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor (TNF), and are produced predominantly by macrophages, monocytes and T helper 1 (Th1) lymphocytes. Anti-inflammatory cytokines, secreted to dampen an inflammatory response, include IL-4, IL-10, and IL-13 and are secreted predominately by T helper 2 (Th2) lymphocytes.

Cytokines can also be used to define the direction of an immune response orchestrated by T helper (Th) cells. Th1 cells secrete interferon gamma (INF\(\gamma\)), IL-2, and TNF, which stimulate cell-mediated immunity to help combat intracellular pathogens (e.g. viruses), whereas Th2 cells produce IL-4, IL-10, IL-6, and IL-13, which inhibit cell mediated (Th1)
immunity and promote humoral (i.e. antibody mediated) immune responses to help combat extracellular pathogens (e.g. extracellular bacteria, parasites) [2].

Acute phase proteins (APP) are a class of proteins, predominantly produced by the liver, whose plasma concentrations increase (positive acute-phase proteins) or decrease (negative acute-phase proteins) in response to inflammation. The proinflammatory cytokines IL-6, TNF$\alpha$ and IL-1$\beta$ are the major inducers of APP synthesis. APP are considered to be non-specific innate immune components involved in the restoration of homeostasis and the restraint of microbial growth before animals develop acquired immunity to a challenge [3]. The circulating concentrations of the APP can be related to the severity of the disorder and the extent of tissue damage in the affected animal.

C-reactive protein (CRP), haptoglobin (Hp), acute phase serum amyloid A (A-SAA), and serum amyloid P (SAP) are examples of APPs used as markers for inflammation and stress in veterinary medicine [3]. The physiological role of CRP is to bind to phosphocholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system via the C1Q complex [4]. The physiological role of Hp is to binds free hemoglobin (Hb), which is toxic and proinflammatory [5]. The physiological role of A-SSA includes the recruitment of immune cells to inflammatory sites and the induction of enzymes that degrade extracellular matrix [6]. The physiological role of SAP is thought to be similar to that of CRP [3].

Given the paucity of marine mammal specific reagents, specifically monoclonal antibodies directed against pinniped cytokines and APPs, this project evaluated the cross-reactivity of commercially available human and canine reagents with pinnipeds as the first step to validate a new diagnostic tool for these species. Limited data suggest antibodies directed against human cytokines will cross-react with marine mammal (specifically cetaceans) proteins, cells, and tissues. For example, commercially available anti-human IL-1$\alpha$, IL-1$\beta$, IL-
8, and TNFα antibodies were shown to label snap frozen cetacean lymph node sections in a pattern similar to that obtained with human tissue [15]. Cross-reactivity of antibodies against human cell surface proteins was tested on beluga whale (Delphinapterus leucas) and bottlenose dolphin (Turisops truncatus) peripheral blood lymphocytes using flow cytometry [17, 18]. Anti-MHC class I and II as well as anti-CD2 reacted with virtually all beluga peripheral blood lymphocytes [17]. Anti-MHC class II reacted with dolphin peripheral blood lymphocytes [18]. Given the success of using anti-human antibodies in labeling cetacean cytokines, cells, and tissues, it was reasonable to assume that anti-human antibodies would label pinniped cytokines and acute phase proteins. During the course of this project, a commercial kit to detect canine cytokines became available and was also evaluated. Pinnipeds and canines are both members of the Order Carnivora [2], thus increasing the likelihood of cross-reactive antibodies between canine and pinniped cytokines.

This project was designed to assess the cross-reactivity of human and canine reagents to quantify cytokines and acute phase proteins in the cell culture supernatant of un-stimulated and mitogen-stimulated human and pinniped peripheral blood mononuclear cells, and to validate the specificity of the cross-reactivity by comparing the secretion patterns in pinnipeds to those that result in specific patterns of secretion in human or canine cells. The Luminex© multiplex platform along with commercially available cytokine/APP kits, was chosen as it allows for the simultaneous and rapid quantification of large numbers of proteins (e.g. cytokines, APPs) using small volumes.

**Materials and Methods**

**Source of blood**

Human whole blood \( n=3 \) was purchased commercially from Biological Specialties Corporation (Colmar, PA). Canine whole blood \( n=3 \) was purchased commercially from
Lampire (Pipersville, PA). Pinniped whole blood was collected from stranded, clinically healthy pinniped species housed at the Mystic Aquarium (Mystic, CT) and the University of New England’s Marine Animal Rehabilitation Center (Biddeford, ME), both of which are part of the Northeast Region Marine Mammal Stranding Network. Blood from three individuals of each species was collected, harbor seal (*Phoca vitulina*), grey seal (*Halichoerus grypus*), and harp seal (*Phoca groenlandica*). All blood samples were collected into sodium heparin tubes, kept cool on ice packs in the dark, shipped to our laboratory, and processed within 24 hours of collection. The co-author, De Guise, is authorized by NOAA to receive blood samples from stranded marine mammals at the University of Connecticut.

**Isolation of PBMCs**

For all blood samples, whole blood was mixed 1:1 with Hanks Balanced Salt Solution (HBSS, Life Technology, Grand Island, NY) and gently mixed at room temperature. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Paque plus for 40 minutes at 990 g at 25°C. The PBMCs were be collected, washed twice with complete Dulbecco’s modified eagle medium (DMEM), and enumerated with their viability assessed using the exclusion dye trypan blue. Typically, cell viability was >90%. Complete DMEM was supplemented with (all from Life Technologies, Grand Island, NY) 1 mM sodium pyruvate, 100 μM non-essential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, along with 10 % fetal bovine serum (Hyclone, Logan, UT).

**Stimulation of PBMCs**

PBMC concentration was adjusted to 2 x 10^6 cells/ml and plated in 96 well flat bottom plates (Falcon, Becton Dickinson, Lincoln Park, NJ), in triplicate. Cells were incubated at 37°C
with 5% CO$_2$ for a total of 48 hours with the T lymphocyte mitogen concanavalin A (ConA; Sigma, St. Louis, MO) at a final concentration of 5 µg/ml, a concentration demonstrated to induce secretion of IL-2, IL-4, IL-5, IL-10, INF$_{\gamma}$, and TNF$\alpha$ from human PBMCs [19]. In a separate set of wells, cells were incubated with the B lymphocyte mitogen lipopolysaccharide (LPS) purified *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO) at a final concentration of 0.1 µg/ml, a concentration demonstrated to induce secretion of IL-1$\beta$, IL-6, IL-8, TNF$\alpha$ [20], as well as C-reactive protein [21], from human PBMCs. In a separate set of wells, cells were incubated with medium alone, i.e. unstimulated cells, to serve as a negative control. At the end of 48 hours, tissue culture supernatant was harvested by centrifuging the plates for 10 minutes at 220 g. The supernatant was collected from each well and stored at -80°C until analysis.

**Cytokine and APP measurement**

Cytokines were quantified using the Bio-Plex Pro™ Human Cytokine Th1/Th2 Panel (Bio-Rad, Hercules, CA) or the Millipore Canine Cytokine 13-plex (Millipore, Billerica, MA), and APPs were quantified using the Bio-Plex Pro™ Human Acute Phase 4-plex Panel Complete Kit (Bio-Rad, Hercules, CA), all according to the manufacturers’ instruction. The Th1/Th2 cytokine kit included antibodies to detect IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFN-$\gamma$, and TNF$\alpha$. The canine cytokine kit included antibodies to detect IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, IFN-$\gamma$, IP-10, KC-Like, MCP-1, and GM-CSF. The APP kit included antibodies to detect C-reactive protein, haptoglobin, serum amyloid P, and $\alpha$-2-macroglobulin. For all kits, standards were prepared as 1/4 serial dilutions to generate an eight standard concentration set. The Millipore canine kit and the Bio-Rad human APP kit included a set of positive controls.

Assays were performed in 96-well Bio-Rad Pro flat bottom plates (Bio-Rad, Hercules, CA). After the incubation and conjugation process, the plate were measured on the Luminex
xMAP 200 system (Luminex Corporation, Austin, TX), and analyzed using StarStation software version 2.0 (Applied Cytometry Systems, Sheffield, UK). Prior to each use of the xMAP 200 system, an instrument calibration and validation procedure, using the Bio-Rad Validation and Calibration (Bio-Rad, Hercules, CA), was performed to assure the instrument was performing properly, as per manufacturers' instruction. The raw data were measured as the observed concentration (pg/ml) of each analyte for each sample and were calculated using a curve fit generated for each analyte from the eight standards. If a sample concentration was below the lowest standard curve value, it was given a value of half of the lowest standard value. For each commercial kit, all supernatant samples from the appropriate species were run at the same time, e.g. supernatant from the canines (n=3) and the three pinniped species (n=3 per species) were processed on one plate using the same Millipore assay kit, thus reducing potential day to day and kit to kit variability.

Results

Luminex xMAP 200 system

Prior to each use of the system, the instrument passed both calibration and validation tests. The lowest standard value calculated from the standard curve for each analyte is listed in Table 1.

Human Cytokine Th1/Th2 Panel

When human PBMCs were stimulated with either the mitogen ConA or LPS, the concentrations of most cytokines were increased when compared to unstimulated cells (Figure 1). With the exception of TNFa, no other pinniped cytokine showed an increase in concentration when PBMCs were stimulated with mitogens compared to unstimulated (Figure
2). The majority of pinniped cytokine concentrations were near or below the lowest standard curve value.

*Human Acute Phase 4-plex Panel*

Stimulation of human PBMCs with either ConA or LPS did not further increase secretion of any acute phase proteins (Figure 3). No pinniped acute phase protein appeared to be detected using the human kits, as their concentrations were near or below the lowest standard curve value (Figure 3).

*Millipore Canine Cytokine 13-plex*

When canine PBMCs were stimulated with either the mitogen ConA or LPS, the concentrations of cytokines were increased when compared to unstimulated cells (Figure 4), with the exceptions of IL-2, IL-7, and IP-10. The canine kit appeared to cross-react with some of the pinniped cytokines as the concentrations of several cytokines were increased in PBMCs stimulated with ConA or LPS when compared to unstimulated cells (Figure 5) in a similar pattern as for canines, although not always to the same magnitude.

As noted above, ConA was demonstrated to induced the secretion of IL-10, INF\(\gamma\), and TNF\(\alpha\) from human PBMCs [19], while LPS was shown to induce the secretion of IL-6 from human PBMCs. This secretion pattern was similar in the present study, and Figure 6 shows the pattern of cytokine secretion in both canines and pinnipeds species induced by either ConA or LPS, with the exception of IL-6 in grey seals.

While not all measureable cytokines in canines were detected in pinnipeds, importantly, clinically relevant cytokines that were quantified in both canines and pinnipeds included three pro-inflammatory cytokines (IL-6, IL-8(except in harp seals) and TNF\(\alpha\)), the Th1 cytokine INF\(\gamma\), and the Th2 cytokine IL-10, an anti-inflammatory cytokine.
Discussion

Our results demonstrated that some canine antibodies for cytokines, as part of a commercially available kit, can cross-react with pinniped cytokines using the Luminex platform. This is important as species-specific antibodies to pinniped cytokines are lacking.

Numerous studies have assessed pinniped cytokine expression at the RNA level. For example, gene expression of IL-2 and IL-1 was detected in harbor seal PBMCs following exposure with ConA [7]. IL-1 like activity, as measured by a bioassay, was detected in harbor seal and grey seal leukocytes following a 48 hour stimulation with LPS [8]. The same authors also detected IL-6 like activity in harbor and grey seals following a 24 hour stimulation with LPS [9]. IL-2 was sequenced and characterized from a northern elephant seal (Mirounga angustirostris) [10] and IL-2 mRNA expression peaked in the first 8 hours following stimulation with ConA.

Assessing cytokine expression at the message level did reveal several interesting and important findings relevant to animal health. For example, changes in blood cytokine IL-10 mRNA levels were investigated between healthy and diseased harbor porpoises (Phocoena phocoena) [11]. Whole blood IL-10 mRNA was higher in severely diseases harbor porpoises with evidence of chronic bacterial infections.

Changes in cytokine expression were measured in blood samples from harbor seal pups taken at admission and after rehabilitation [12]. mRNA expressions of IL-1β, 2, 4, 6, 8, 10, 12, IFN-γ, and transforming growth factor (TGF) beta, as well as the APP haptoglobin (HP), heat shock protein (HSP) 70 and metallothionein (MT) were measured. Higher levels of the pro-inflammatory cytokines IL-1β, 6, 8, and 12 were found at admission, consistent with an activated immune system, whereas the anti-inflammatory cytokine IL-4 was increased after
rehabilitation, suggesting recovery from infections and maturation of the immune system during rehabilitation.

C-reactive protein was purified and characterized from harbor seal serum [13]. Specific monoclonal antibodies to CRP were generated to develop an immunoassay to quantify serum CRP and used to measure serum CRP during harbor seal pup rehabilitation. From 13 animals, four had undetectable CRP levels and also had no abnormal clinical signs other than dehydration and malnutrition. One other animal with undetectable CRP levels had an encapsulated abscess and an elevated monocyte count. However, eight animals had CRP levels above the detectable levels, of which seven had clinical signs consistent with concurrent inflammatory disease.

Plasma concentrations of the APP haptoglobin (Hp) from Steller sea lions (*Eumetopias jubatus*) and harbor seals were compared between areas where these populations were declining to areas where the populations were stable [14]. Significantly higher levels of Hp were found in the samples from the areas of decline compared to those from stable populations. Based on these findings, the authors proposed that one may be able to distinguish these compromised pinniped populations using Hp as a biomedical indicator [14].

Taken together, these data above highlight the potential important diagnostic value of measuring cytokines and APPs in marine mammal species, especially those in rehabilitation facilities. However, there are several disadvantages involving the methods described above. First, RNA must be quickly stabilized to prevent RNA degradation or changes in gene expression ex vivo, which may not reflect the true condition of an animal. Second, there are numerous reagents (RNA extraction kits, species specific primers, RT-PCR kits) and equipment (centrifuges, RT-PCR machines) to purchase and maintain, as well as clean bench space (i.e. to prevent contamination of samples) to perform the work. Third, each cytokine and APP must be measured independently, which may significantly increase the time until final
results are available, which goes to increase the costs of analysis. Finally, and most importantly, expression data (e.g. copy numbers of IL-2 relative to a house keeping gene), while important in the research setting, may not be fully representative of the bioactive protein levels, as not all mRNA will be fully translated and secreted.

The Luminex platform has several advantages compared to measuring cytokine/APP gene expression (e.g. mRNA) and traditional ELISA assays to measure cytokine concentrations. These include commercially available reagents and standards, rapid results (~3 hours), increase sensitivity compared to traditional ELISAs, quantification of each cytokine and APP, and the ability to measure multiple cytokines or APPs of interest in one small samples size (12.5 µl of serum or plasma). In addition, the collection of serum and plasma requires little effort and technical skills compared to the collection and processing of RNA.

In the present study, the human Th1/Th2 cytokine kits allowed the detection of cytokines in the supernatant of human PBMCs stimulated with the mitogens ConA and LPS, as expected. With the exception of TNFα in all three pinniped species, the human kits did not detect pinniped cytokines in a similar manner. For example ConA did not further increase the concentration of IL-10 in supernatant of harbor seal PBMCs compared to unstimulated cells (Figure 2). If, in fact, the antibodies were cross-reactive, it was possible that pinniped PBMCs needed additional incubation time (>48 hours) or higher mitogen concentrations to induce the secretion of cytokines at a concentration above the lowest standard curve value. However, as TNFα was detected in all three species, the previous scenario seems unlikely.

The human acute phase kit allowed the detection of APPs in the supernatant of human PBMCs, however, mitogen stimulation did not further increase their concentrations, suggesting that mitogens did not induce the secretion of acute phase proteins, at least with the conditions in our experiments. This was not surprising as APPs are predominately produced in the liver.
No pinniped APPs were detected using the human kits, and nearly all concentrations were near or below the lowest standard curve value.

As the human kits failed to cross-react with most pinniped cytokines, a newly available commercial canine cytokine kit was evaluated. It was reasonable to assume that canine antibodies were more likely to be cross-reactive with pinniped cytokines as these species are more evolutionarily related.

As the cytokine pattern was similar among the species, we concluded that the canine kit can be used to detect pinniped cytokines. Of clinical importance was the detection of three pro-inflammatory cytokines (IL-6, IL-8 and TNFα), the Th1 cytokine INFγ, and the Th2 cytokine IL-10. These cytokines are important to help define the direction of an immune response orchestrated by T helper (Th) cells. Th1 cells secrete INFγ and TNFα, which stimulate cell-mediated immunity to help combat intracellular pathogens (e.g. viruses), whereas Th2 cells produce IL-10 and IL-6, which inhibit cell mediated (Th1) immunity and promote humoral (i.e antibody mediated) immune responses to help combat extracellular pathogens (e.g. extracellular bacteria, parasites).

**Conclusion**

The Luminex platform and commercial canine kits were successful for measuring pinniped cytokines. In the future, this platform may have potential to help detect sub-clinical signs of inflammation, as well as evidence for the direction of the immune response in view of clinical infections, thus helping to refine health assessment and response to disease. For example, the cytokine profile can be measured when a stranded animal first comes into rehabilitation. If an animal has an increase in pro-inflammatory cytokines, these data may help veterinarians decide if treatment with anti-inflammatory and/or antibiotic drugs should be initiated. In addition, by monitoring an individual’s profile during its rehabilitation, it may help a
veterinarian decide, along with other health parameters (i.e. CBC, chemistry profile, physical exam), when an animal's health has improved (i.e. reduction of pro-inflammatory cytokines) enough to stop treatment and decisions about releasing an animal back into the wild. As another example, monitoring Th1 cytokines (IL-2 and INFγ) versus Th2 cytokines (IL-10, IL-4, IL-5) may help detect the direction of an immune response (Th1 cell-mediated vs Th2 humoral-mediated). This may help veterinarians decide when anti-parasitic and antibiotic treatments are necessary, as Th2 cytokines are higher during parasitic and extracellular bacterial infections.
References


Figure captions

Figure 1. Concentrations (mean + SD) of cytokines in the supernatant of human (Hu) PBMCs stimulated with no mitogen (NM), ConA, or LPS for 48 hours.

Figure 2. Concentrations (mean + SD) of cytokines in the supernatant of (top) harbor seals (Pv), (middle) grey seals (Hg), and (bottom) harp seals (Pg) PBMCs stimulated with no mitogen (NM), ConA, or LPS for 48 hours.

Figure 3. Concentrations (mean + SD) of acute phase proteins in the supernatant of human (Hu) PBMCs stimulated with no mitogen (NM), ConA, or LPS for 48 hours.

Figure 4. Concentrations (mean + SD) of cytokines in the supernatant of canine (Cf) PBMCs stimulated with no mitogen (NM), ConA, or LPS for 48 hours.

Figure 5. Concentrations (mean + SD) of cytokines in the supernatant of (top) harbor seals (Pv), (middle) grey seals (Hg), and (bottom) harp seals (Pg) PBMCs stimulated with no mitogen (NM), ConA, or LPS for 48 hours.

Figure 6. Concentrations (mean + SD) of cytokines in the supernatant of canine (Cf), harbor seal (Pv), grey seal (Hg), and harp seal (Pg) PBMCs stimulated with ConA (top) or LPS (bottom) after 48 hours.
**Table 1.** The lowest standard values calculated from the standard curve for each analyte in the three commercial kits. If a sample concentration was below the lowest standard curve value, it was given a value of half of the lowest standard value.

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<th><strong>Bio-Rad Human Th1/TH2</strong></th>
<th><strong>Lowest standard curve value (pg/ml)</strong></th>
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<td>IL-2</td>
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<tr>
<td>INFγ</td>
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<tr>
<td>GM-CSF</td>
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<td>TNFα</td>
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Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

**Graph A:**
- **Pv-NM**
- **Pv-ConA**
- **Pv-LPS**

**Graph B:**
- **Hg-NM**
- **Hg-ConA**
- **Hg-LPS**

**Graph C:**
- **Pg-NM**
- **Pg-ConA**
- **Pg-LPS**

Bars represent cytokine concentrations in pg/ml across different treatments.
Figure 6