

Effects of GM-CSF on Critically Ill Dog's Immune Cell Function

Michael Schacher, Amy E. DeClue, Sandra Bechtel, Juliana Amorim

Background

- Sepsis is the leading cause of death in people in intensive care units and a leading cause of death overall in the US.
- Recently, new insights into the pathophysiology of sepsis have led to the realization that immunosuppression along with the traditionally implicated cytokine storm contributes to morbidity and mortality.
- Moreover, we have preliminary data indicating that some dogs with critical illness develop an immunosuppressive phenotype similar to that of people with sepsis.
- GM-CSF reverses a similar immunosuppressive phenotype observed in dogs with cancer and thus might be a candidate intervention for reversing the immunosuppressive phenotype in critically ill dogs.

Hypothesis

We hypothesized that GM-CSF would improve phagocytic function, respiratory burst capacity, phagocytic expression of TLR4 and HLA-DR, and LPS-stimulated production of cytokines in cells from dogs with critical illness-induced immunodysfunction.

Materials and Methodology

Dogs presenting to the MU-Veterinary Health Center with critical illness were identified.

Glucose (mg/dl)	<84 7 points	84-102 8 points	103-164 9 points	165-273 10 points	>273 0 points
Albumin (g/dL)	<2.6 8 points	2.6-3.0 7 points	3.1-3.2 6 points	3.3-3.5 0 points	>3.5 2 points
Lactate (mmol/L)	<1.00 0 points	1.1-4.0 4 points	4.1-5.0 8 points	>5.0 12 points	
Platelet ct (x10 ³ /uL)	< 151 5 points	151-200 6 points	201-260 3 points	261-420 0 points	>420 1 point
Mentation Score – *assessed at admission	1 4 points	2 6 points	3 7 points	4 14 points	

Mentation: 0 = Normal; 1 = Able to stand unassisted, responsive but dull; 2 = Can stand only when assisted, responsive but dull; 3 = Unable to stand, responsive; 4 = Unable to stand, unresponsive

Figure 1: APPLE_{fast} scores were used to determine critically ill dogs. Scores greater than 15 without lactate, or greater than 20 with lactate resulted in a label of critically ill.

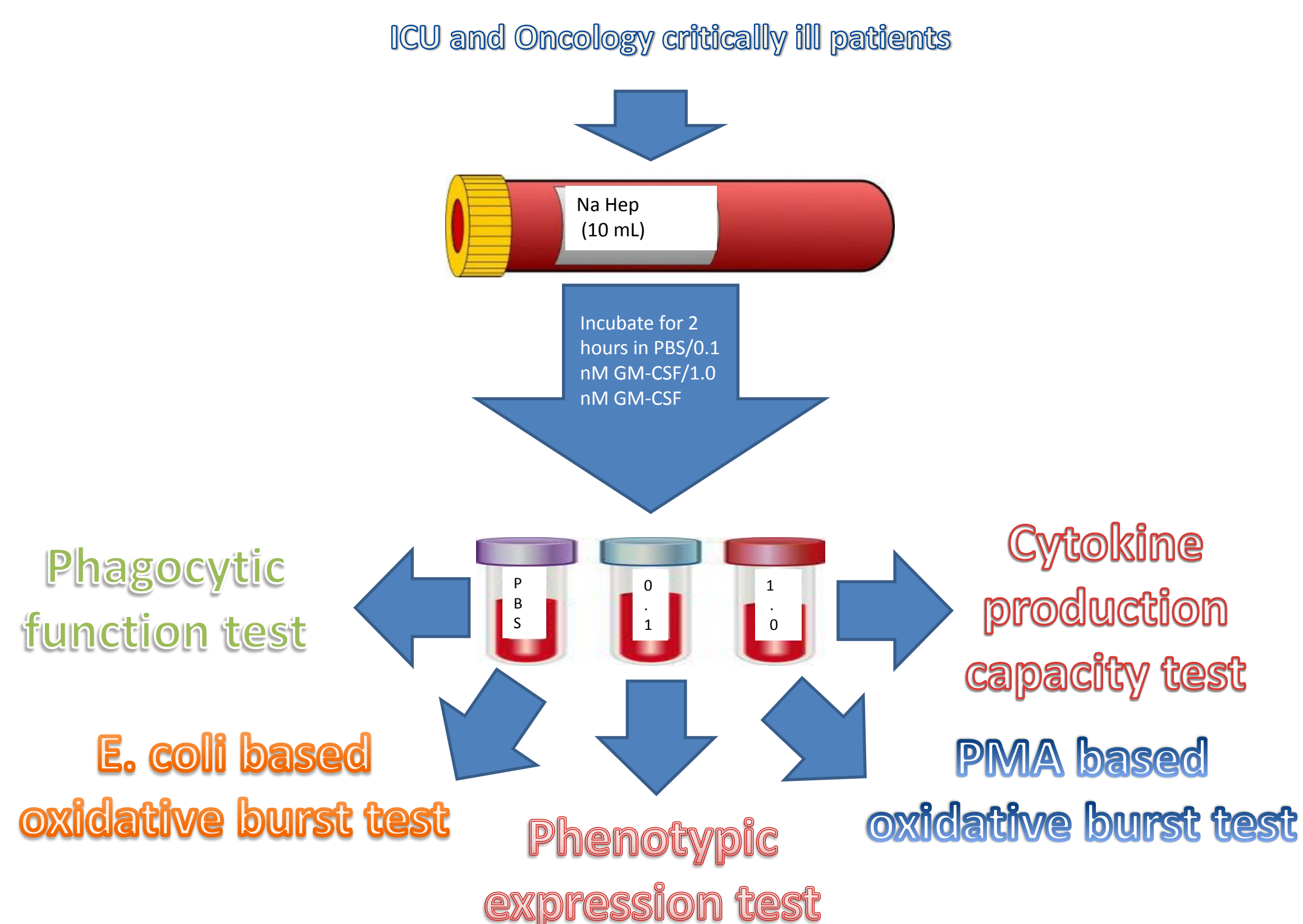


Figure 2: Assays following blood collection.

Phagocytic Function Assay:

100µL of heparinized blood was incubated in a 37°C water bath for 10 min with 20 µL FITC-labeled, opsonized *E. coli* bacteria or washing solution (negative control). Phagocytosis was arrested by placing the sample on ice and adding 100 µL of quenching solution in order to remove the FITC fluorescence of surface bound bacteria. The cells were washed, erythrocytes were lysed, the cells were washed again and 200 µL of DNA staining solution was added to exclude aggregation artifacts. Samples were analyzed by flow cytometry.

Respiratory Burst Function Assays:

100 µL of heparinized blood from healthy and CI dogs was incubated with 20 µL of either opsonized *E. coli* (biological stimulus) or phorbol 12-myristate 13-acetate (PMA; chemical stimulus) for 10 min at 37°C in a water bath. Samples were then incubated with 20 µL of dihydrorhodamine-123 as a fluorogenic substrate for oxygen intermediates for 10 min at 37°C in a water bath. This reaction was then extinguished, erythrocytes were lysed, the cells were washed and 200 µL of DNA staining solution was added to exclude aggregation. Samples were analyzed by flow cytometry.

Cytokine Production Capacity Assay:

Sodium heparinized whole blood was placed in 24 well plates, and stimulated with LPS from *E. coli* O127:B8 (final concentration, 100 ng/mL; Sigma-Aldrich), or phosphate buffered saline (PBS) control. Plates were gently mixed for 5 minutes, incubated for 24 hours at 37 °C in 5% CO₂, and then centrifuged (300g for 6 minutes); supernatant was collected and stored at -80 °C for batch analyses. TNF-α, IL-6 and IL-10 will be measured in duplicate from the supernatant with a canine specific multiplex bead-based assay at the culmination of the study (Millipore, MA, USA).

Phenotypic Expression Assay:

100 µL of heparinized blood from healthy and CI dogs was placed in 96 well plates with 4 µL of PE conjugated TLR4 antibodies, FITC conjugated HLA-DR antibodies, PE conjugated TLR4 antibodies and FITC conjugated HLA-DR antibodies, or FACS buffer. Antibodies were left to bind to their target for a period of 30 minutes in a dark room. Next, the wells were washed with 150 µL of FACS buffer, centrifuged, and the supernatant was discarded in order to wash away any antibody that had not bound. 200 µL of lysing solution was then added then centrifuged after 5 minutes; supernatant was discarded. This process was repeated, and then 100 µL of formalin PBS and 100 µL of FACS buffer were added to the wells and then transferred to FACS tubes. Samples were analyzed by flow cytometry.

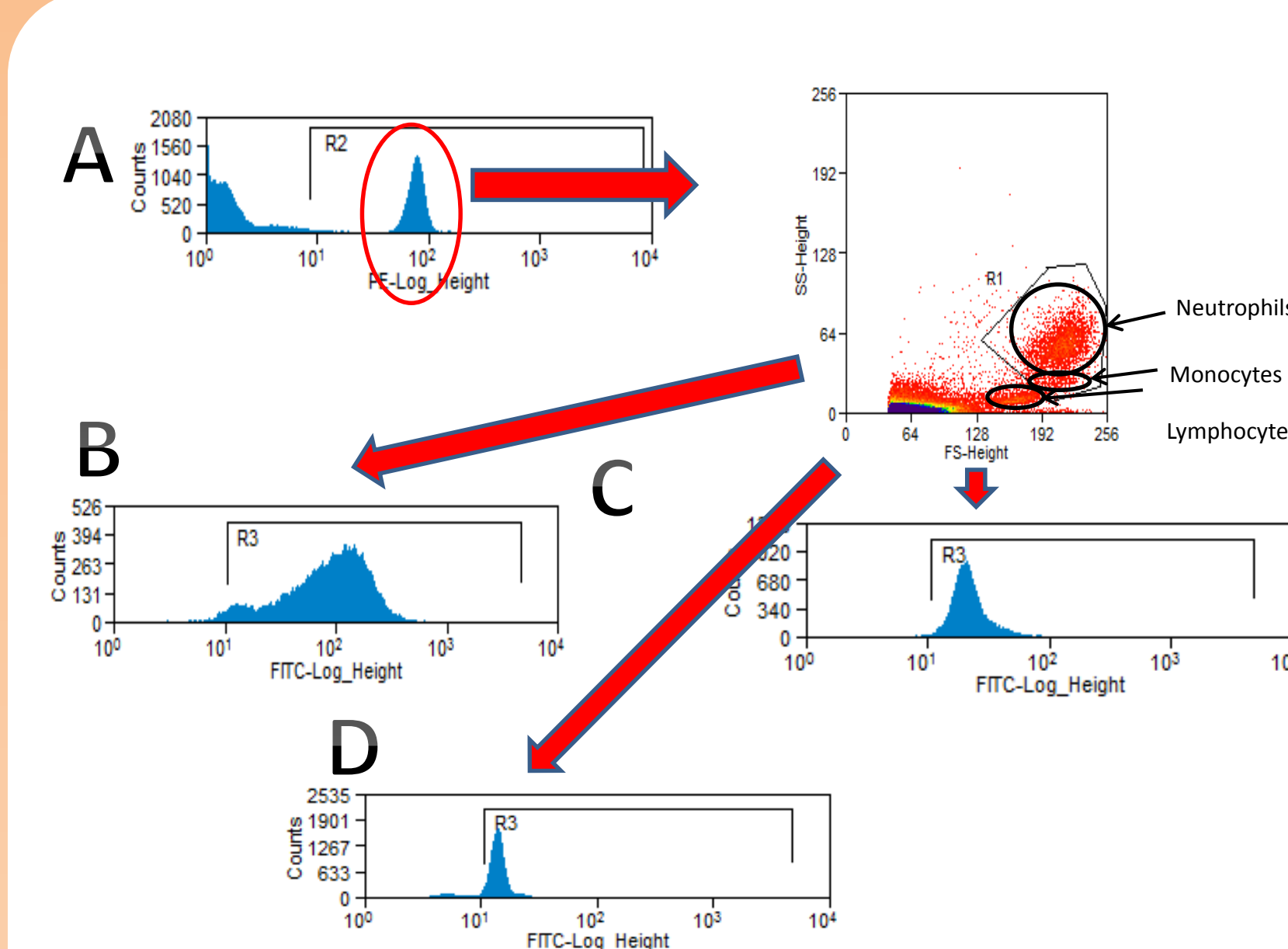


Figure 3: A) DNA positive cells were selected and then applied to a FS vs SS plot. Then phagocytes were gated and applied to a FITC histogram. Typical examples of cells positive for (B) phagocytosis of FITC-labeled *E. coli*, (C) *E. coli*-induced respiratory burst, and (D) PMA-induced respiratory burst.

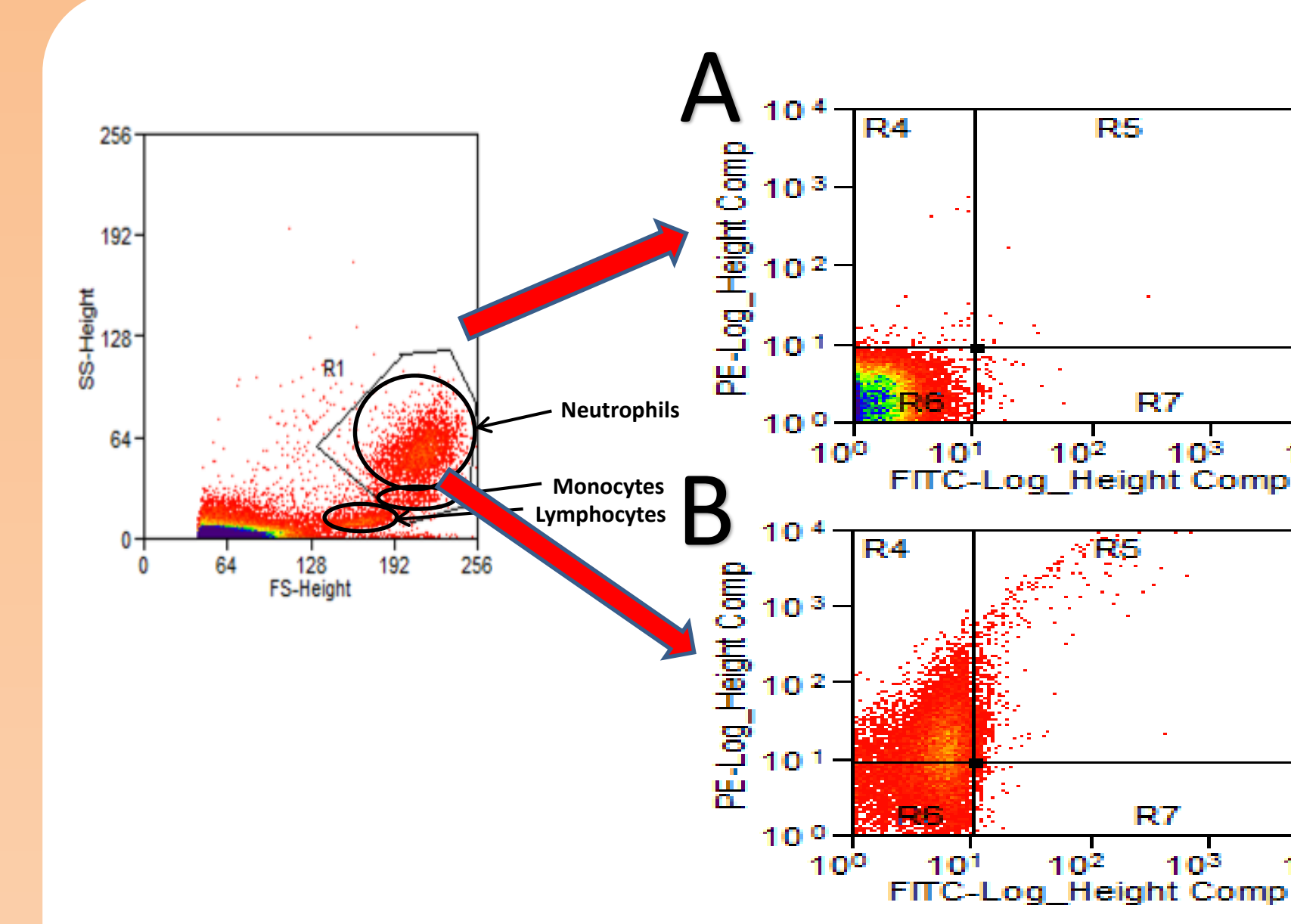


Figure 4: Flow cytometry results from the phenotypic assay were placed on a scatter plot based on the presence of FITC (HLA-DR) and PE (TLR4). A) demonstrates a typical control gate, from the FACS buffer added well, in which less than 1% of cells were located outside of the R6 gate. B) demonstrates cells positive for TLR4 in R4 and R5.

Results

Five dogs have been enrolled to date. Two of the dogs were castrated males, two of the dogs were spayed females and one of the dogs was an intact male. Breeds included a Shiba Inu, a Labrador Retriever, an American bulldog, an American Staffordshire terrier, and a mixed breed dog. Ages ranged from two to six years. Diagnoses for the pets included prostatitis, acute renal failure, mast cell tumor, lymphoma with neutropenia, and pneumonia with lung abscesses.

Results are preliminary at this time and data collection is ongoing.

Leukocyte cytokine production assay

Supernatant has been collected for the leukocyte cytokine production analysis. The supernatant is currently being stored at -80° C and will be analyzed at the conclusion of the study.

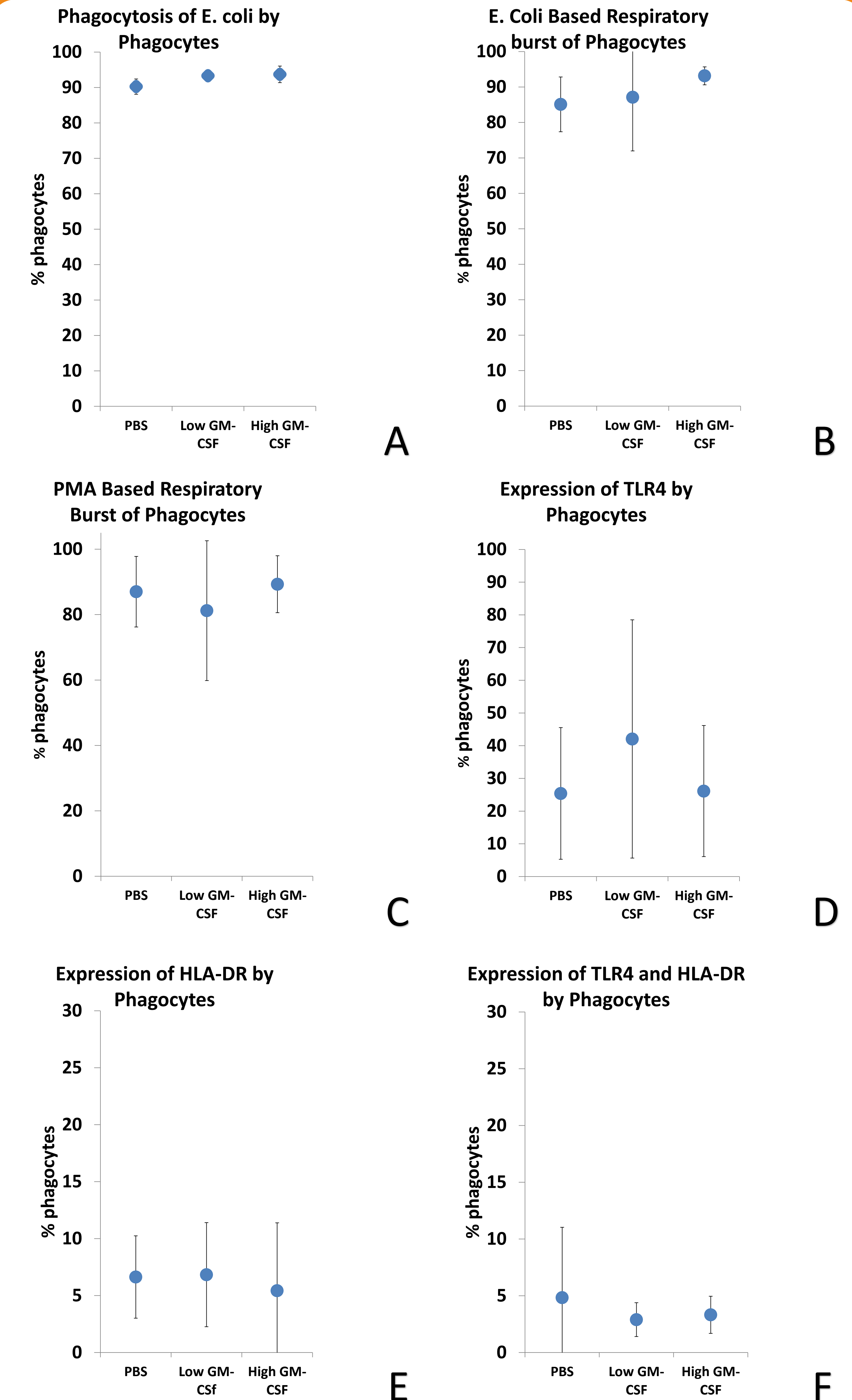


Figure 5: Analysis of percentage of phagocytes that A) underwent phagocytosis of FITC labeled *E. coli*, B) underwent respiratory burst following stimulation by *E. coli*, C) underwent respiratory burst following stimulation by PMA, D) expressed TLR, E) expressed HLA-DR, and F) expressed both TLR and HLA-DR following incubation with PBS, low and high concentrations of GM-CSF.

Conclusions

Although numbers are small, preliminary, it appears that GM-CSF may restore immune function from a phenotypic standpoint, with TLR expression, phagocytic function, and *E. Coli* based respiratory burst, however data collection is ongoing. Next steps include continuing the study until its conclusion and running the leukocyte cytokine production assay. Research into mechanisms that create the phenotype of critically ill immunosuppression are currently underway in our lab to gain further insight into the process. Our goal is to apply this research to a clinical setting.

Funding and Acknowledgments

A generous endowment established by IDEXX-BioResearch Comparative Internal Medicine Laboratory, University of Missouri