# Granulocyte-Macrophage Colony-Stimulating Factor Partially Restores Toll-Like Receptor-Mediated Functional Responses of Monocytes in Septic Shock

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## Abstract

Septic shock remains clinically challenging to manage. The dysregulated immune response seen in septic shock contributes to this complexity. However, biological response modifiers including the cytokine recombinant human granulocyte-macrophage colony-stimulating factor (rHuGM-CSF) might be important in managing both septicemia and septic shock. We hypothesized that *ex vivo* stimulation with rHuGM-CSF could restore functional and phenotypic responses of monocytes. The functional responses of complex whole blood and highly-enriched monocyte cultures were assessed from a panel of n=30 human study subjects (n=23, septic shock and n=7 healthy controls). Cultures were initially primed with rHuGM-CSF then stimulated with relevant toll-like receptor (TLR) ligands for short (4 h) or extended (48 h) time-periods. TLR-specific ligands included lipopolysaccharide (LPS, a surrogate of gram-negative bacterial infection) and double-stranded RNA (dsRNA, a surrogate of a viral infection). Endpoints included measurement of cell surface receptors by flow cytometry and cytokine secretion by multiplex bead-array technology. We found depressed expression of immune cell-surface markers on septic shock monocytes as compared to normal controls. However, a trend of partially restored cell surface expression of functionally important phenotypic markers on septic shock monocytes was associated with restored, or even augmented, cytokine secretion following rHuGM-CSF-priming and secondary stimulation with LPS or dsRNA. A dysregulated pattern of cell activation typically characterizes septic shock and may contribute to inadvertent immune suppression of monocytes. We have showed that rHuGM-CSF priming restored several important aspects of monocyte responsiveness to exogenous bacterial and viral stimuli. This approach could offer therapeutic utility that nonetheless requires empirical proof of concept.

**Keywords:** Monocyte; Anergy; Biomarkers; Septic shock; Granulocyte-macrophage colony-stimulating factor

## Introduction

Septic shock is a leading cause of mortality in critically ill patients and is a concern in post-surgical rehabilitation. Septic shock is complex and characterized by dysregulated immunity and an inability to effectively clear primary microbial infections [1]. The weakened immune state seen in septic shock increases host susceptibility to opportunistic secondary infections, super-infections like those associated with viruses, bacteriophages and bacterial or fungal infections [2]. Hospitalized trauma patients and immunosuppressed patients, such as those presenting with hematologic or solid malignancies, are often at high risk for developing sepsis and septic shock [3,4].

Monocytes serve important roles in the innate immune response to infection by linking innate and acquired immunity [5]. Circulating monocytes coordinate the response of the immune system to eliminate invading pathogens. Infection with gram-negative and gram-positive bacteria or fungal pathogens may lead to septic shock [6,7]. Septic shock involves a heterogeneous response of monocytes to bacterial components, such as lipopolysaccharide (LPS, or endotoxin) [8,9]. The

response of monocytes to bacterial cell wall components, such as LPS, forms the basis of the complex and heterogenic responses seen in septic shock, which display both pro-inflammatory and anti-inflammatory effects [10].

LPS is an immunogenic component of gram-negative bacterial cell walls and interacts with cell membrane-associated pattern-recognition receptor toll-like receptor 4 (TLR4) and the cell membrane-associated co-receptor CD14 [11]. Occupation of TLR4 transduces a signal via the MyD88 adaptor protein and the mitogen-activated protein kinases p38 MAPK and ERK I/II with subsequent activation of the transcription factors NF-κB p50/p65 and AP-1 [12,13]. This signaling pathway instructs *de novo* protein synthesis of immunologically important molecules. This includes expression of HLA-DR (MHC Class II), CD14, the co-stimulatory molecules CD40, CD80 and CD86, secretion of pro-inflammatory cytokines such as TNFα, IL-6 and IL-8, and augmented respiratory burst activity, seen as a rapid secretion of reactive oxygen species and hydrogen peroxide, which collectively display potent anti-microbial properties [14-16].

While the pro-inflammatory response to exogenous microbial "danger signals" like LPS and other antigens is essential for protective host immunity, excessive secretion of pro-inflammatory mediators by monocytes in septic shock is ultimately detrimental [17,18]. In septic

shock, the anti-inflammatory response drives monocyte dysfunction and tolerance of the monocyte to further LPS challenge, which may lead to near complete shutdown of the immune competence of monocytes; a state referred to as immune paralysis or immunological anergy of the monocyte [19,20]. In monocyte anergy, cell surface TLR4 expression is lost and expression of both HLA-DR and CD14 is markedly dampened [21,22]. Hyper-responsive monocytes in septic shock may also become immuno-suppressed and subsequently, apoptotic after exhausting metabolic or energetic resources following the initial antigenic challenge, or from sustained stimulation by LPS [23,24].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that is effective in the clinical management of a variety of conditions including complications from infectious diseases and in the management of infections in immuno-compromised patients presenting with hematological or solid tumors. GM-CSF stimulates the production and functional maturation of hematopoietic precursors and developing monocytes both *in vivo* and *in vitro* [25]. GM-CSF also reverses monocyte anergy seen in diseases like severe acute pancreatitis [26]. GM-CSF has also seen potential clinical utility in the management of minimal residual disease in human leukemia and other cancers [27-29]. Mouse models of septic shock have reinforced our appreciation that GM-CSF can recover functional responses of monocytes to LPS [30,31]. In addition, GM-CSF may restore monocyte activation in hematological malignancy following high-dose chemotherapy [32,33]. Thus, it seems reasonable that GM-CSF may reconstitute normal immune homeostasis from a position of dysregulated immunity that characterizes septic shock. However, there remains a paucity of mechanistic and functional studies that have explored the *ex vivo* response of septic shock monocytes to agents capable of restoring functional activity [34,35].

We explored the ability of rHuGM-CSF to partially restore the functional responsiveness of monocytes in septic shock on activation by TLR-dependent signals (i.e., the LPS-TLR4 axis), or viral ligands such as poly I:C, or double-stranded RNA (i.e., the dsRNA-TLR3 axis). We contrasted the responses of monocytes from septic shock and healthy control subjects. By examining a relevant, diverse population of septic shock subjects, our study explored the response of both highly enriched monocyte cultures, and whole blood cultures where the interactions of other cell types and secreted factors were permitted to influence the observed outcomes. The current study sets the foundation for future work detailing observations of restored monocyte responses under the influence of rHuGM-CSF priming *ex vivo*.

## Materials and Methods

### Subject recruitment

Thirty subjects were recruited to this study (n=7 healthy controls and n=23 critically ill subjects with septic shock). All septic patients had confirmed or suspected infection and fulfilled the accepted criteria for septic shock at the time [1]. Blood specimens were received within 48 h following diagnosis of septic shock. Investigators were blinded to patient status (septic shock case or healthy control) until completion of all *ex vivo* laboratory assays of cellular activation. Demographic and clinical parameters of research study subjects are shown in (Table 1). All subjects or their surrogates provided written informed consent and the study was approved by the University of Rochester Institutional Research Subjects Review Board (RSRB).

| Subject Age | Gender | Primary Infection | Apache Score | Saps II Score Day 1 |
|---|---|---|---|---|
| 71 | Male | Other | 16 | 49 |
| 77 | Male | Pulmonary | 36 | 89 |
| 51 | Male | Pulmonary | 8 | 49 |
| 71 | Male | Pulmonary | 35 | 74 |
| 83 | Male | Pulmonary | 21 | 38 |
| 72 | Male | Pulmonary | 38 | 76 |
| 80 | Male | Pulmonary | 36 | 51 |
| 48 | Male | Pulmonary | 15 | 19 |
| 30 | Male | Pulmonary | 12 | 3 |
| 28 | Male | Pulmonary | 18 | 30 |
| 64 | Male | Pulmonary | 27 | 47 |
| 57 | Male | Intra-abdominal | 9 | 31 |
| | | | 22.6 | 46.3 |
| | | | 19.5 | 48 |
| | | | 11.3 | 24.7 |

**Table 1a:** A summary of the demographics of the male subjects with septic shock that were a part of this study.

### *Ex vivo* culture of peripheral blood cells and experimental design

We designed three separate experiments to explore the immune restoring functions of rHuGM-CSF (PeproTech, Rocky Hill, NJ) on activation of monocytes in cultures of whole blood and in highly enriched mononuclear cell cultures ex vivo. In experimental platforms one and two, the objective was to test the response of mononuclear cells in whole blood cultures, which would permit other immune cells to interact and influence the outcome of the measured endpoints. Experimental endpoints included flow cytometric assays of cell surface-expressed function-associated markers and assays of cytokine secretion by multiplex cytokine array (Bio-Rad, Hercules, CA). In the third experimental platform, we wished to test for the ability of highly enriched monocyte cultures to respond to GM-CSF and contrast these outcomes to those obtained from unfractionated blood cultures obtained from platforms one and two. In the third experimental platform, the possible interactions of monocytes with other cellular components of whole blood were excluded, thereby addressing the issue of how an enriched population of mononuclear cells may respond to the immune-stimulating effects of LPS, or dsRNA, with, or without, prior rhGM-CSF-priming.

### Short-term culture (4 hrs)/whole blood platform (Experimental Platform 1)

In the first experimental platform, 1-2 ml of peripheral venous blood was anti-coagulated in preservative-free sodium heparin. For these assays, blood specimens were diluted 1:2 in complete culture medium (RPMI-1640 base media, Invitrogen, Carlsbad, CA), supplemented with 20 mM HEPES buffer (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 2.5 μg/ml gentamycin sulfate (Invitrogen, Carlsbad, CA), 8% v/v fetal bovine serum (FBS) (Tissue Culture Biologicals, Tulare, CA), $5.5 \times 10^{-2}$ mM beta-mercaptoethanol (Gibco, Carlsbad, CA), 1 mM sodium pyruvate (Gibco, Carlsbad, CA), $10^{-1}$ mM (each amino acid) MEM non-essential amino acids (Gibco, Carlsbad, CA), and seeded as 0.5 ml volumes into sterile, 12 x 75 mm, pyrogen-free, polypropylene, LP4 tubes (Corning Life Sciences, Lowell, MA). Cells were primed in the presence, or absence, of rHuGM-CSF (50 ng/ml) for 2 h, at 37°C. Cells were then immediately challenged for an additional 2 h in the presence of no other stimulus ("Resting"), LPS (1 ug/ml, E. coli-derived endotoxin, serotype 055:B5 in endotoxin-free water, Sigma-Aldrich, St. Louis, MO), or dsRNA (100 ng/ml, Poly(I:C), InvivoGen, San Diego, CA), which is a synthetic dsRNA analog reconstituted in endotoxin-free PBS. Four conditions were thus set up: (A) Resting cells without rHuGM-CSF, (B) rHuGM-CSF-primed cells, (C) rHuGM-CSF-primed cells followed by LPS stimulation, and (D) rHuGM-CSF-primed cells followed by dsRNA stimulation. At the conclusion of the assay, monocytes were harvested for quantitation of cell membrane expressed phenotypic markers by flow cytometry as described below.

### Long-term culture (48 hrs)/whole blood platform (Experimental Platform 2)

In this experiment, 2 ml of peripheral blood was anti-coagulated in sodium citrate to test the effect of long-term exposure (48 h in culture) of whole blood monocytes to rHuGM-CSF stimulated with/without TLR ligands (LPS-TLR4 axis and dsRNA-TLR3 axis). Each blood sample was diluted in complete RPMI1640 culture medium at a 1:3 ratio, and seeded into a 12-well culture dish (Corning Life Sciences, Lowell, MA) at 1 ml/well. Cultures were primed with/without rHuGM-CSF (50 ng/ml) for 4 h, at 37°C prior to secondary stimulation with/without, LPS (1 ug/ml), or dsRNA (100 ng/ml) for 48 h. In this way, six conditions were set up: (A) "Resting" cells without rHuGM-CSF, (B) Cells stimulated with LPS alone, (C) Cells stimulated with dsRNA alone, (D) rHuGM-CSF-primed cells, (E) rHuGM-CSF-primed cells and LPS stimulation, and (F) rHuGM-CSF-primed cells and dsRNA stimulation. At the conclusion of the assay, monocytes were analyzed for cell surface expression of various immune markers by flow cytometry as described below. Culture supernatants were aliquotted in duplicate and stored at -80°C until assayed for cytokine secretion by multiplex array analysis as described below.

### Long-term culture (48 hrs)/purified monocyte platform (Experimental Platform 3)

In this experiment, a monocyte-enriched fraction was prepared from 1-2 ml K3. EDTA anti-coagulated blood by centrifugal separation over a discontinuous solution of 13.5% w/v iodixanol (Metrizamide, Sigma-Aldrich, St. Louis, MO) cell separation medium. Briefly, whole blood was diluted 1:2 with sterile divalent cation-free phosphate buffered saline (PBS) that was supplemented with 2% v/v fetal bovine serum (F-PBS), and layered over an equal volume of 13.5% w/v iodixanol to enrich for peripheral blood mononuclear cells containing monocytes. Samples were centrifuged at 800 x g, 30 min, at 20°C, without brakes off and at the lowest acceleration. The low-density cells were harvested from the iodixanol/F-PBS interface, washed three times in warm complete RPMI1640 following which, cells were resuspended in complete culture medium, counted and seeded into a 6-well plate at 1 ml/well, or 106 cells/well.

### Flow cytometry of whole blood cultured cells

Flow cytometric analysis of whole blood cultured cells included assessment of cell surface function and activation-associated molecules, or receptors. These included the following anti-human monoclonal antibodies conjugated to either a fluorescein isothiocyanate (FITC), or phycoerythrin (PE) fluorochrome: MHC Class II (HLA-DR) (FITC; BD Biosciences, San Jose, CA), CD14 (FITC; BD Biosciences, San Jose, CA), CD71 (PE; Immunotech, Fullerton, CA), CD80 (PE; BD Biosciences, San Jose, CA), CD86 (PE; BD Biosciences, San Jose, CA), TLR2 (PE; Santa Cruz, Santa Cruz, CA), TLR4 (PE; Santa Cruz, Santa Cruz, CA), Tissue Factor (FITC; American Diagnostics, Hauppauge, NY), CD11b (PE; BD Biosciences, San Jose, CA), and CD54 (FITC; Immunotech, Fullerton, CA). Samples were stained at 4°C, for 30 min, in the dark. Samples were next lysed in BD FACS lysis solution (BD Biosciences, San Jose, CA) in the dark for 10 min, at room temperature, then centrifuged at 400 x g, 4°C, for 6 min, washed once in FACS wash buffer (divalent cation-free PBS supplemented with 2% v/v FBS and 0.2% w/v sodium azide), centrifuged at 400 x g, 4°C, for 6 min, and finally, each sample was resuspended and fixed in 250 μL of 2% w/v paraformaldehyde in FACS wash buffer prior to subsequent analysis using a FACScaliber flow cytometer (Becton Dickinson, Franklin, NJ) and CellQuest 3.1 software (Becton Dickinson, Franklin, NJ). In conjunction, the appropriate murine IgG isotypic controls were used for cell surface marker analysis (PE and FITC; BD Biosciences, San Jose, CA) to control against non-specific Fc-gamma receptor-mediated, non-specific binding of the antibodies. Data was acquired in real-time as geometric mean fluorescence intensity (MFI) and percent fluorescent positive cells (% positive). The instrument had a standard optical filter

configuration with band pass filters of 530/30-nm and 585/44-nm for FL1 (FITC-conjugated antibodies) and FL2 (PE-conjugated antibodies) data acquisition, respectively. For the analysis of forward-angle light scatter, side-angle light scatter, and cell surface receptor expression, data was acquired in real-time. The instrument was standardized prior to phenotypic analysis with calibration beads (FluoroSpheres 6-Peak; Dako Cytomation, Carpinteria, CA) and cleaned with sequential washes of distilled water, 10% v/v hypochlorite, and distilled water before data acquisition.

## Quantitation of inflammatory cytokines by multiplex cytokine array assay

The Bio-Plex 200 (Bio-Rad, Hercules, CA) was used to assess the amount of human cytokines in the supernatants of the long-term whole blood and whole blood-purified monocyte cultures; 50 µL of each undiluted supernatant was run. The cytokine bead standards were diluted in RPMI-base complete culture medium to correlate with the supernatant base matrix. A minimum of one blank (complete culture media alone) was run on each Bio-Plex 200 ninety-six well plate.

## Statistical analysis

Demographic data of all subjects are contained in Table 1. Phenotypic markers and cytokine measures were evaluated for distributional properties and transformed into the logarithm scale to meet model assumptions. Linear mixed models utilizing geometric mean data for phenotypic markers and observed cytokine values were used to assess each parameter as a function of condition (healthy *vs.* septic shock), treatment (± rHuGM-CSF and/or ± LPS, or dsRNA), and interaction of condition and treatment in short-term (4 hrs), or long-term (48 hrs) *in vitro* cell culture assays. Comparisons of the treatment groups with baseline within and over the conditions were carried out subsequent to the overall analyses. All analyses were carried out using SAS 9.2 (Copyright © 2009 SAS Institute Inc., Cary, North Carolina 27513, USA) on a Windows Vista Enterprise 32-bit platform. An alpha value of $p < 0.05$ was considered a statistically significant difference between pairs or groups of data, unless otherwise stated.

## Results

Phenotypic characterization of whole blood cultures, either 4 h (short-term) or 48 h (long-term) was performed as described above in Materials & Methods. Phenotypic characterization of whole blood purified monocyte cultures (third experimental platform) was not assessed due to an insufficient number of cells, which was accounted for in the initial experimental platform. However, cytokine secretion, a functional measurement, was assessed for the 48 h whole blood cultures and 48 h whole blood-purified monocyte cultures. The cumulative data was obtained from a blinded study of 30 individuals that when uncoded, revealed 23 septic shock subjects and 7 healthy subjects. Demographic data of all subjects are contained in Table 1.

| Subject Age | Gender | Primary Infection | Apache Score | Saps II Score Day 1 |
|---|---|---|---|---|
| 49 | Female | Blood | 47 | 10 |
| 66 | Female | Other | 39 | 10 |
| 39 | Female | Pulmonary | 60 | 11 |
| 44 | Female | Intra-abdominal | 22 | 7 |
| 74 | Female | Pulmonary | 97 | 14 |
| 65 | Female | Intra-abdominal | 72 | 9 |
| 88 | Female | Blood | 30 | 5 |
| 54 | Female | Pulmonary | 53 | 8 |
| 62 | Female | Intra-abdominal | 51 | 7 |
| 47 | Female | Pulmonary | 51 | 14 |
| 51 | Female | Pulmonary | 27 | 4 |
| | | | 23.4 | 49.9 |
| | | | 24 | 51 |
| | | | 8.7 | 21.6 |

**Table 1b:** A summary of the demographics of female subjects with septic shock that were a part of this study.

## Initial assessment of septic shock whole blood and whole-blood purified monocytes

Using flow cytometry, we found that cell surface expression of several markers was reduced on monocytes from subjects with septic shock compared with healthy controls, including TLR2 and TLR4, as well as CD11b, CD71, CD80 ($p < 0.05$), CD86, and HLA-DR (Figure 1). In addition, the supernatants from resting, septic shock, whole blood cultures, from which these monocytes were examined, had elevated average levels of IL-1 beta, IL-6 ($p < 0.05$), TNF-alpha, IL-8, and IL-10.
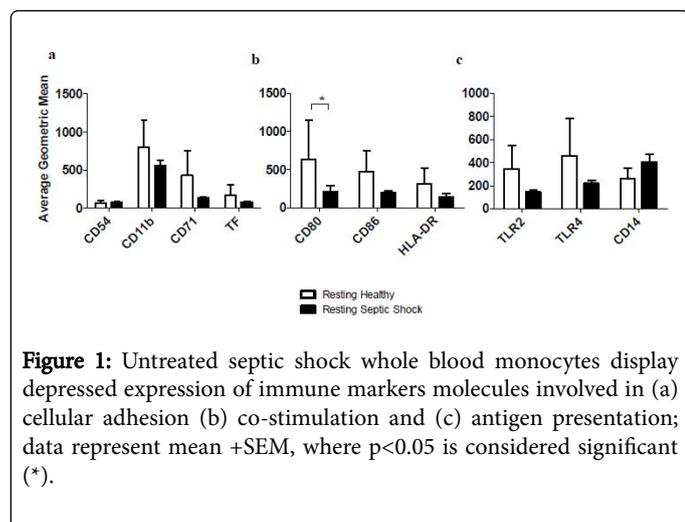
**Figure 1:** Untreated septic shock whole blood monocytes display depressed expression of immune markers molecules involved in (a) cellular adhesion (b) co-stimulation and (c) antigen presentation; data represent mean +SEM, where $p<0.05$ is considered significant (*).

Figure 2 compares the healthy and septic shock cytokine profiles of resting, whole blood cultures (Figure 2a, 2b) and resting, purified, whole blood monocyte cultures (Figure 2c, 2d) prior to any treatment. These cytokine profiles show a trend that fits with the data in Figure 1.
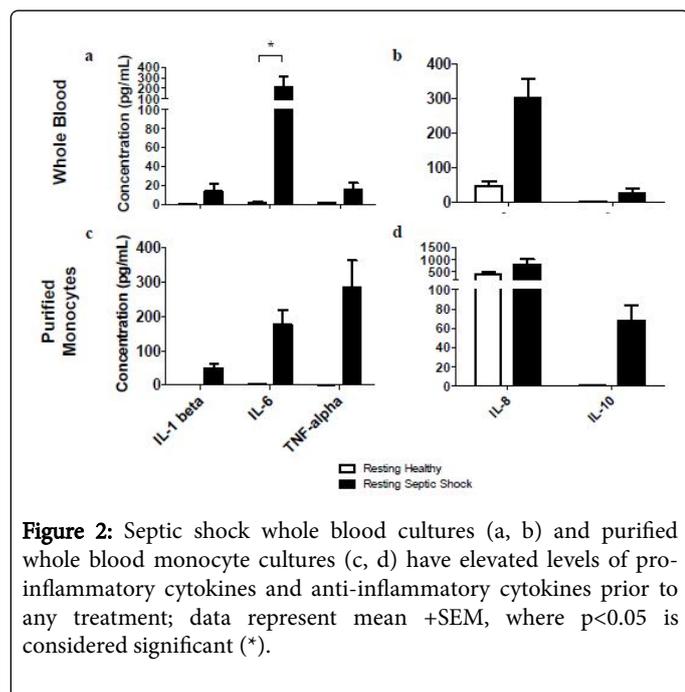


**Figure 2:** Septic shock whole blood cultures (a, b) and purified whole blood monocyte cultures (c, d) have elevated levels of pro-inflammatory cytokines and anti-inflammatory cytokines prior to any treatment; data represent mean +SEM, where $p<0.05$ is considered significant (*).

## Assessment of monocytes in short-term whole blood cultures (Experimental Platform 1)

rHuGM-CSF alone was unable to significantly enhance the expression of any of the monocyte markers examined (data not shown). Furthermore, in short-term 4 h cultures *ex vivo*, rHuGM-CSF had an almost negligable impact on the septic shock monocyte phenotypic response to TLR agonists, LPS, or dsRNA (data not shown). Healthy whole blood monocytes were used for comparison. Cell culture supernatants were not assessed for cytokine levels in this experimental platform, due to the short duration of the experiment, which is not applicable to cytokine detection.

## Assessment of monocytes in long-term whole blood cultures (Experimental Platform 2)

Priming septic shock whole blood cultures with rHuGM-CSF for 48 h had a stronger impact than in the short-term 4 hr cultures (Experimental Platform 1; Figures 3 and 4).
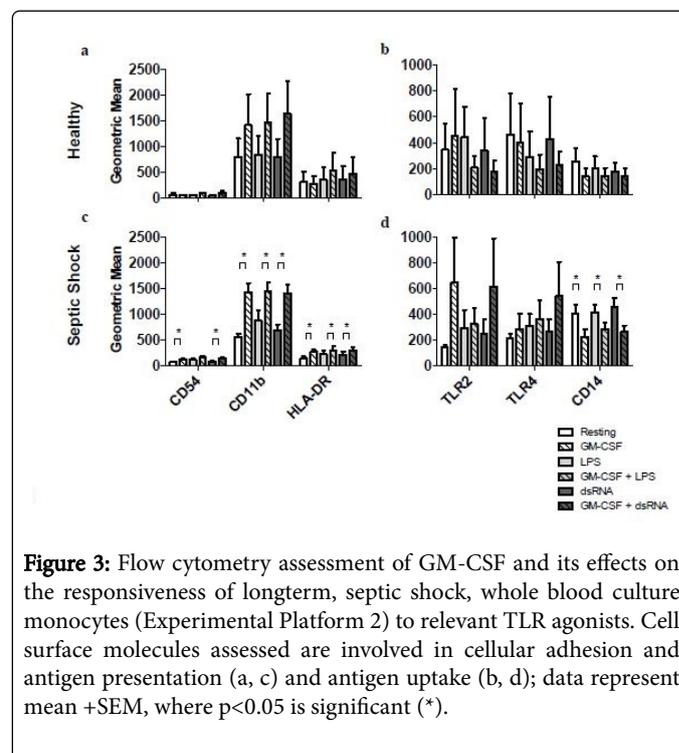


**Figure 3:** Flow cytometry assessment of GM-CSF and its effects on the responsiveness of longterm, septic shock, whole blood culture monocytes (Experimental Platform 2) to relevant TLR agonists. Cell surface molecules assessed are involved in cellular adhesion and antigen presentation (a, c) and antigen uptake (b, d); data represent mean +SEM, where $p<0.05$ is significant (*).

For example, long-term culture with rHuGM-CSF alone was able to significantly-enhance ($p<0.05$) septic shock monocyte levels of CD54, CD11b and HLA-DR, while also noticeably enhancing TLR2 and TLR4. Furthermore, prior to LPS activation, rHuGM-CSF-priming significantly-enhanced whole blood septic shock monocyte levels of CD11b and HLA-DR ($p<0.05$), and noticeably enhanced the expression levels of CD54, TLR2, and TLR. Moreover, in whole blood cultures that were primed with rHuGM-CSF alone or with LPS activation following rHuGM-CSF priming, CD14 expression levels were significantly decreased ($p<0.05$). rHuGM-CSF-priming also significantly-enhanced the levels of CD54, CD11b, and HLA-DR on whole blood septic shock monocytes, in response to dsRNA; TLR2 and TLR4 were also enhanced, while CD14 was significantly decreased ($p<0.05$). Whole blood healthy monocytes are provided for a phenotypic comparison. Monocyte phenotypic changes facilitated by rHuGM-CSF priming of septic shock whole blood, resulted in culture supernatant cytokine level changes (Figure 4). rHuGM-CSF alone was able to significantly-enhance septic shock whole blood levels of IL-8, while also enhancing IL-1 beta. Furthermore, rHuGM-CSF-priming significantly-enhanced the TNF-alpha septic shock whole blood culture responses to LPS; IL-1 beta, IL-6, and IL-8 were also enhanced. rHuGM-CSF-priming significantly-enhanced the septic shock whole blood culture IL-8 response to dsRNA, while also enhancing the IL-1 beta, IL-6, TNF-alpha, and IL-10 responses to this TLR3 agonist.
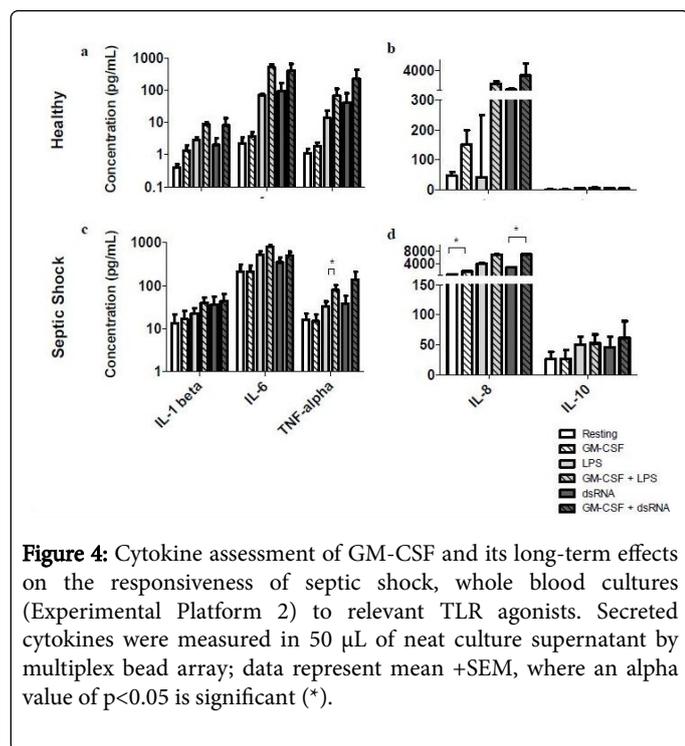
**Figure 4:** Cytokine assessment of GM-CSF and its long-term effects on the responsiveness of septic shock, whole blood cultures (Experimental Platform 2) to relevant TLR agonists. Secreted cytokines were measured in 50 µL of neat culture supernatant by multiplex bead array; data represent mean +SEM, where an alpha value of p<0.05 is significant (*).

### Assessment of monocytes in long-term cultures (Experimental Platform 3)

rHuGM-CSF-priming of purified septic shock monocyte cultures allowed for significant-enhancement of IL-6 and IL-8 secretion, while also enhancing secretion of IL-6 and TNF-alpha, in response to LPS, or dsRNA (Figure 5). Due to the amount of patient blood received, a phenotypic assessment could not be performed in this experimental platform.

### Discussion

Septic shock mortality remains at a consistently high level in hospital intensive care units [36]. As such, considerably more clinical and animal research must be done to understand the complex nature of this condition. Our clinical study adds valuable data to the field, not only in its examination of modulating the response of septic shock subjects' whole bloods to relevant toll-like receptor (TLR) ligands by the hematopoietic growth factor, GM-CSF, but in its focused effort to examine the rHuGM-CSF-modulation of a key cell in these responses, the monocyte. Known to possess immunomodulating properties, GM-CSF, was studied for its ability to enhance the monocyte response to relevant TLR ligands, such as LPS and dsRNA, in whole-blood and whole blood-purified monocyte *ex vivo* cell cultures.

Our initial examination of resting whole blood and whole blood-purified septic shock monocytes revealed depressed expression of CD80 (p<0.05), as well as, depressed expression of CD11b, CD71, CD86, HLA-DR, TLR2, and TLR4, when compared to healthy monocyte controls. The reduced expression of these important monocyte immune markers has been previously shown to be a characteristic of septic shock. Furthermore, the enhanced levels of pro-inflammatory cytokines, such as IL-6 (p<0.05), IL-8, TNF-alpha and of IL-10, an anti-inflammatory cytokine, support the septic shock

diagnosis of these blood samples. Monocytes examined in our whole-blood purified monocyte cultures displayed a similar cytokine secretion pattern.
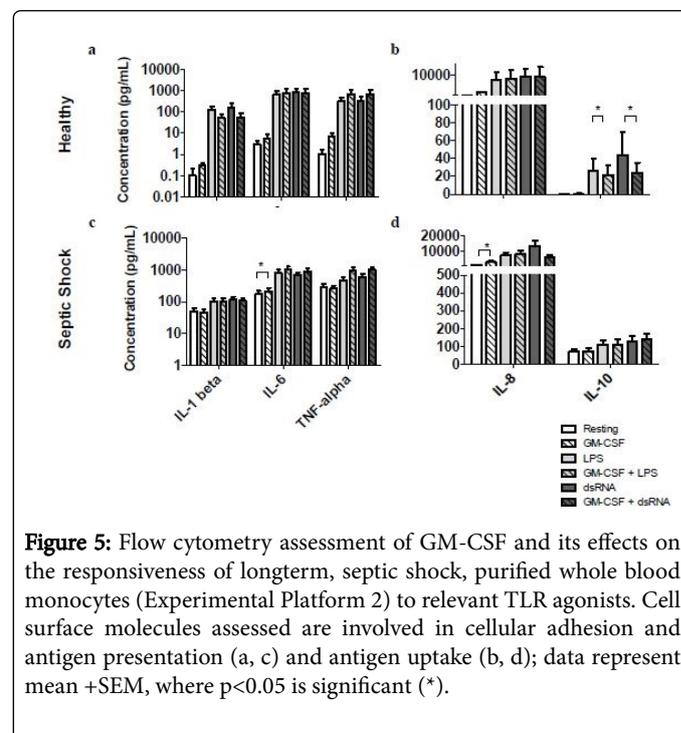


**Figure 5:** Flow cytometry assessment of GM-CSF and its effects on the responsiveness of longterm, septic shock, purified whole blood monocytes (Experimental Platform 2) to relevant TLR agonists. Cell surface molecules assessed are involved in cellular adhesion and antigen presentation (a, c) and antigen uptake (b, d); data represent mean +SEM, where p<0.05 is significant (*).

### IL-10/TNF-alpha ratio as a measure of responsiveness to rHuGM-CSF

The ratio of the anti-inflammatory cytokine IL-10, in relation to the pro-inflammatory cytokine TNF-alpha, decreased under conditions where rHuGM-CSF-priming occurred before stimulation with LPS or dsRNA (Figure 6). This trend was consistent when studying culture supernatants derived from both septic shock whole blood and their counterpart purified monocyte cultures and supports the potential immune-enhancing effect of rHuGM-CSF priming on septic shock monocytes prior to TLR activation [43].
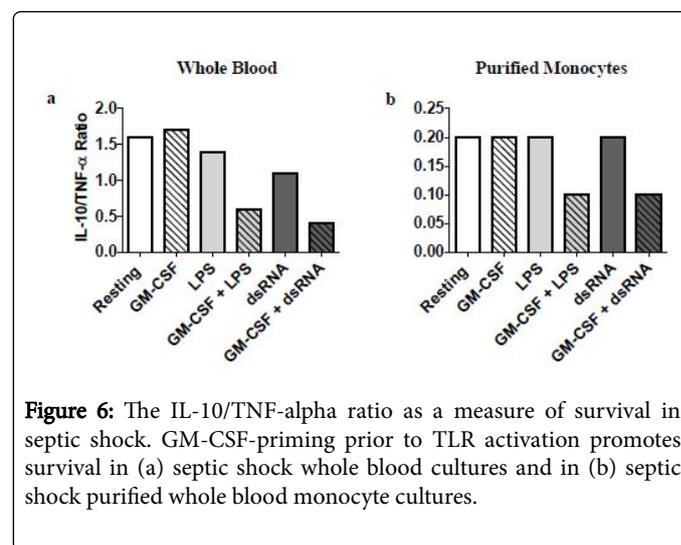


**Figure 6:** The IL-10/TNF-alpha ratio as a measure of survival in septic shock. GM-CSF-priming prior to TLR activation promotes survival in (a) septic shock whole blood cultures and in (b) septic shock purified whole blood monocyte cultures.

In the 4 h experimental platform, we observed no significant phenotypic changes in healthy or septic shock whole blood monocytes that were cultured with rHuGM-CSF alone, or together with LPS or dsRNA. There are two reasonable explanations for this. First, it is possible that although there were no effects of rHuGM-CSF on healthy or septic shock whole blood monocytes in the short-term culture platform, other cells in the blood may have been affected by the rHuGM-CSF. GM-CSF is a multi-functional biological response modifier that is produced by many cell types and has effects on the functional activities of various lymphocytes [37]. Second, a 2 h priming period may not be sufficient to see an average effect of rHuGM-CSF on monocytes. For instance, Brock, et al. found that enhanced leukotiene synthesis in alveolar macrophages required a minimum of 6 h GM-CSF pre-treatment before subsequent stimulation with a calcium ionophore [38].

GM-CSF, like another proposed septic shock therapy, IFN-gamma, targets the alteration of cellular activity, a broad-spectrum effect. Other septic shock therapies aimed at specific molecular targets, such as anti-TNF-alpha, have been ineffective in clinical trials [39]. Studies supporting the use of GM-CSF instead of IFN-gamma in septic shock treatment include those where IFN-gamma treatment of monocytes did not reconstitute defective pro-inflammatory cytokine production in sepsis after major visceral surgery [40]. Antibiotics, routinely used in treatment against bacterial-based septic shock, have the potential of exacerbating the inflammatory condition, as they provide "fuel for the fire" by destroying bacteria, thus releasing more LPS into an already dysregulated system [41].

In the second experimental platform, 4 h of rHuGM-CSF-priming enhanced the septic shock whole blood monocyte response to LPS, observed by an increase in monocyte expression of CD54, CD71, CD11b (p<0.05), TLR2, TLR4, and HLA-DR (p<0.05) after 48 h in culture. The enhanced monocyte immune phenotype supports the observed enhanced levels of IL-1 beta, IL-6, TNF-alpha (p<0.05), and IL-8 found with rHuGM-CSF-priming prior to LPS activation; IL-10 levels remained stable whether or not rHuGM-CSF was given prior to LPS activation. rHuGM-CSF-priming enhanced the septic shock whole blood monocyte response to dsRNA as well, with enhanced CD54 (p<0.05), CD11b (p<0.05), TLR2, TLR4, CD80, and HLA-DR (p<0.05). The enhanced monocyte immune phenotype supported the increased secretion of IL-1 beta, IL-6, TNF-alpha, and IL-8 (p<0.05) that were observed if rHuGM-CSF priming occurred prior to dsRNA activation; IL-10 levels did not significantly increase. Interestingly, septic shock monocyte CD14 levels significantly decreased when rHuGM-CSF was used to prime the cultures before activation with LPS or dsRNA. This may seem contradictory, as CD14 cooperates with TLR4 in the monocyte immune response to LPS and GM-CSF has been shown to activate mature macrophages and granulocytes. However, the findings of Kruger, et al. suggest that this CD14 downregulation, facilitated by GM-CSF, may represent an initial responsiveness to further monocyte differentiation to prepare for responsiveness to TLR ligands, such as LPS [42].

Experimental platform three sought to directly assess the effects of rHuGM-CSF, if any, on septic shock monocytes, where monocytes were isolated from the whole blood and put into culture. Whole blood contains a variety of cell types and serum components that could alter monocyte activity, making it important to confirm the effects of rHuGM-CSF on monocytes.

Due to a limiting amount of patient blood, we were only able to examine cell culture supernatants from whole blood purified monocyte cultures in this platform. Healthy whole blood culture control data is provided for comparison. rHuGM-CSF-priming resulted in a marginal enhancement of IL-8 alone (p<0.05), as well as in TNF-alpha in response to LPS, or dsRNA, with no change in IL-10 production. This further supports the importance of monocyte responsiveness to TLR-agonists, such as LPS and dsRNA.

Further support for beneficial effects of GM-CSF on septic shock monocytes is shown by Figure 6. It has been shown that the ratio of IL-10/TNF-alpha is a predictor of survival. The average IL-10/TNF-alpha ratio is higher in patients who die from septic shock [43]. In our studies, we found that the average IL-10/TNF-alpha ratio decreased when rhGM-CSF-priming was utilized prior to LPS or dsRNA activation of cell cultures of both septic shock whole blood and whole blood-purified monocytes. This would indicate a trend whereby GM-CSF is promoting survival in septic shock by promoting monocyte immune responsiveness.

In conclusion, our data trends with this clinical study group support our earlier studies and a thus further investigation of the biological response modifier, GM-CSF, in septic shock therapy. Our resting whole blood phenotypic data supports monocyte immunosuppression, while our phenotypic and cytokine data support rHuGM-CSF's ability to enhance septic shock monocyte responses to relevant toll-like receptor ligands, such as LPS and dsRNA.

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