OPEN

Effects of Mesenchymal Stem Cell Treatment on Systemic Cytokine Levels in a Phase 1 Dose Escalation Safety Trial of Septic Shock Patients*

Kenny Schlosser, PhD[1]; Jia-Pey Wang, MSc[1]; Claudia dos Santos, MD, MSc, FRCPC[2,3]; Keith R. Walley, MD[4]; John Marshall, MD, FRCSC, FACS[3]; Dean A. Fergusson, PhD[5]; Brent W. Winston, MD, FRCPC[6]; John Granton, MD[2]; Irene Watpool, RN, BScN[7]; Duncan J. Stewart, MD, FRCPC#[1,8]; Lauralyn A. McIntyre, MD, FRCPC, MHSc[7]; Shirley H. J. Mei, PhD, MSc[1]; on behalf of the Canadian Critical Care Trials Group and the Canadian Critical Care Translational Biology Group

***See also p. 1001.**

[1]Sinclair Centre for Regenerative Medicine, Ottawa Hospital Research Institute, Ottawa, ON, Canada.

[2]Department of Medicine, University of Toronto, Toronto, ON, Canada.

[3]Department of Surgery and Critical Care Medicine, Keenan Research Centre of the Li Ka Shing Knowledge Institute, St. Michael's Hospital, The University of Toronto, Toronto, ON, Canada.

[4]Centre for Heart Lung Innovation, University of British Columbia, Vancouver, BC, Canada.

[5]Department of Epidemiology and Community Medicine, University of Ottawa, Ottawa, ON, Canada.

[6]Department of Critical Care Medicine, Biochemistry and Molecular Biology, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada.

[7]Division of Critical Care, Department of Medicine, University of Ottawa, Ottawa, ON, Canada.

[8]Department of Cell and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada.

Drs. Stewart, McIntyre, and Mei are co-senior authors.

Drs. Schlosser, Stewart, McIntyre, and Mei, and Ms. Wang designed and did the study concept. Ms. Wang performed measurements for data acquisition. Drs. Schlosser, Stewart, McIntyre, and Mei, and Ms. Wang analyzed and interpreted the data. Dr. Schlosser drafted the initial article. All authors were involved in critical revisions of the article for important intellectual content.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (http://journals.lww.com/ccmjournal).

Drs. McIntyre's and Mei's institutions received funding from Ontario Institute of Regenerative Medicine (OIRM) Disease Team Grant (to Dr. Mei) and from the Canadian Institutes of Health Research (CIHR) Operating Grant (to Dr. McIntyre). Dr. Walley disclosed he was a founder and shareholder of Cyon Therapeutics. Dr. Fergusson's institution received funding from CIHR. Dr. Granton received other funding from Actelion, Bayer, and Bellepheron as a member of steering or adjudication committees for clinical trials in pulmonary hypertension and from Actelion and Bayer for support of pulmonary hypertension research. Dr. Granton disclosed off-label product use of stem cells. Dr. Stewart holds a patent for mesenchymal stem/stromal cell (MSC) therapy for the treatment of acute lung injury. Dr. Mei has received personal fees from Northern Therapeutics that are outside of this submitted work. The remaining authors have disclosed that they do not have any potential conflicts of interest.

For information regarding this article, E-mail: smei@ohri.ca

DOI: 10.1097/CCM.0000000000003657

Objectives: Cellular Immunotherapy for Septic Shock is the first-in-human clinical trial evaluating allogeneic mesenchymal stem/stromal cells in septic shock patients. Here, we sought to determine whether plasma cytokine profiles may provide further information into the safety and biological effects of mesenchymal stem/stromal cell treatment, as no previous study has conducted a comprehensive analysis of circulating cytokine levels in critically ill patients treated with mesenchymal stem/stromal cells.

Design: Phase 1 dose-escalation trial.

Patients: The interventional cohort ($n = 9$) of septic shock patients received a single dose of 0.3, 1.0, or 3.0 million mesenchymal stem/stromal cells/kg body weight ($n = 3$ per dose). The observational cohort received no mesenchymal stem/stromal cells ($n = 21$).

Interventions: Allogeneic bone marrow-derived mesenchymal stem/stromal cells.

Measurements and Main Results: Serial plasma samples were collected at study baseline prior to mesenchymal stem/stromal cell infusion (0 hr), 1 hour, 4 hours, 12 hours, 24 hours, and 72 hours after mesenchymal stem/stromal cell infusion/trial enrollment. Forty-nine analytes comprised mostly of cytokines along with several biomarkers were measured. We detected no significant elevations in a broad range of pro-inflammatory cytokines and biomarkers between the interventional and observational

cohorts. Stratification of the interventional cohort by mesenchymal stem/stromal cell dose further revealed patient-specific and dose-dependent perturbations in cytokines, including an early but transient dampening of pro-inflammatory cytokines (e.g., interleukin-1β, interleukin-2, interleukin-6, interleukin-8, and monocyte chemoattractant protein 1), suggesting that mesenchymal stem/stromal cell treatment may alter innate immune responses and underlying sepsis biology.

Conclusions: A single infusion of up to 3 million cells/kg of allogeneic mesenchymal stem/stromal cells did not exacerbate elevated cytokine levels in plasma of septic shock patients, consistent with a safe response. These data also offer insight into potential biological mechanisms of mesenchymal stem/stromal cell treatment and support further investigation in larger randomized controlled trials. (*Crit Care Med* 2019; 47:918–925)

Key Words: biomarkers; cell therapy; clinical trial; cytokines; mesenchymal stem cells; sepsis

Septic shock is a common and severe inflammatory response to infection, which leads to multiple organ dysfunction and death in 20–40% of patients (1). There is currently no effective pharmacotherapy that can improve outcomes; however, preclinical animal studies suggest mesenchymal stem/stromal cells (MSCs) can reduce inflammation and organ dysfunction, and enhance bacterial clearance and survival (2–6).

We previously completed a phase 1 dose-escalation trial (Cellular Immunotherapy for Septic Shock [CISS]) to investigate the safety and maximum tolerated dose of a single IV infusion of allogeneic bone marrow-derived MSCs in septic shock patients refractory to standard treatment (7). We found no adverse safety signals in prespecified and serious unexpected adverse events, or in the physiologic and clinical outcomes (e.g., mortality, ICU/hospital length of stay) of the interventional as compared with observational cohort. Although the sample size of the CISS phase 1 trial was not powered to detect clinical effects of MSC treatment, we hypothesized that changes in underlying biology may be detectable in the plasma of these patients. In particular, alterations in the levels of circulating cytokines may be especially relevant given that unbalanced cytokine production contributes to the development and severity of septic shock, and MSCs are known to exert immunomodulatory effects through secretion of paracrine/endocrine factors and/or direct interaction with host immune cells (8, 9), both of which may impact systemic cytokine levels. The plasma levels of a limited number of cytokines and markers (i.e., interleukin [IL]–6, IL-8, IL-1β, IL-10, angiopoietin-2 [ANGPT2], receptor for advanced glycation end products [RAGE], and surfactant protein D) in MSC-treated patients with septic shock or acute respiratory distress syndrome (ARDS) have previously been reported (7, 10, 11), but showed no clear MSC-related effects. However, more comprehensive profiling may reveal important insights into how groups of functionally related cytokines change in response to MSC treatment, given that additive or synergistic biologic effects could be mediated through small coordinated changes among many cytokines.

In the current study, we investigated whether plasma cytokine profiles in CISS patients provide further information related to the safety and/or potential biological effects of MSC treatment and inform the selection of an appropriate dose for future trials. Using a combination of single- and multianalyte profiling assays, plasma levels of 49 analytes were assessed serially over six different time points within 72 hours of MSC infusion and in relation to non-MSC treated septic shock patients (observational cohort) and healthy normal participants. Analytes consisted primarily of cytokine/chemokines (many with known pro- or anti-inflammatory activities), along with several other types of biologic markers or mediators related to cellular/organ dysfunction or the innate immune response.

MATERIALS AND METHODS

CISS Cohorts and Healthy Normal Subjects

The CISS trial was approved by Health Canada and the Ottawa Health Sciences Network Research Ethics Board (OHSN-REB No. : 20140809-01H) and registered on clinicaltrials.gov (NCT02421484). Details on trial design, participant recruitment, MSC source and preparation, and trial outcomes have been described previously (7). Briefly, the observational cohort consisted of 21 septic shock participants who met CISS eligibility criteria but did not receive MSCs, and the interventional cohort ($n = 9$) comprised three cohorts of three septic shock participants that received 0.3 (low dose), 1.0 (mid dose), or 3.0 (high dose) million freshly cultured cells per kg body weight, to a maximum of 300 million cells. Peripheral blood samples from healthy participants were obtained with informed written consent at a single center (OHSN-REB No. :2011470-01H).

Plasma Specimen Collection, Single- and Multianalyte Immunoassays, Data Analysis, and Statistics

Method details are described in the **online data supplement** (Supplemental Digital Content 1, http://links.lww.com/CCM/E387).

RESULTS

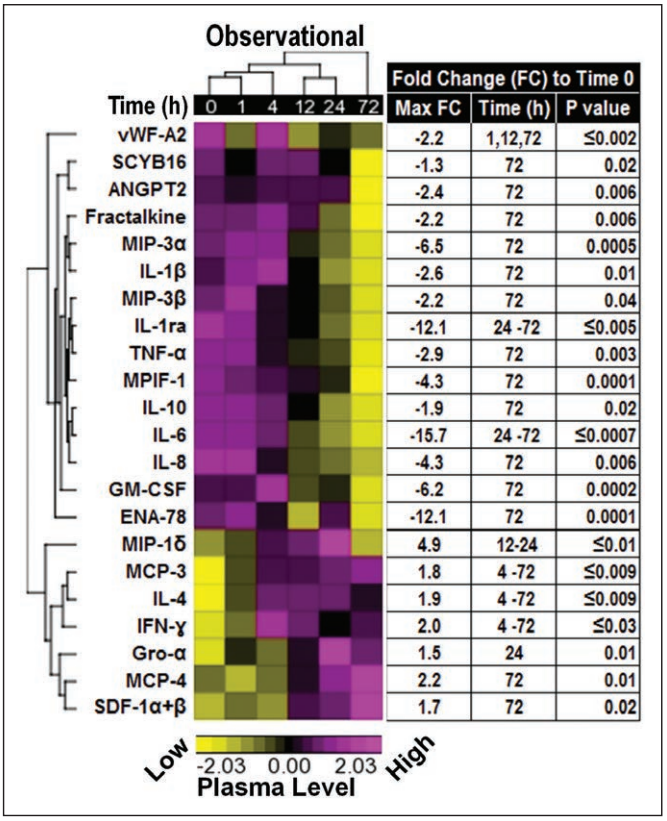
The Majority of Analytes Assessed Were Significantly Altered in Plasma of Septic Shock Patients Versus Healthy Subjects

Among 49 cytokines and biomarkers that were assessed in plasma, 47 were detectable under normal physiologic conditions in a separate cohort of healthy subjects ($n = 16$), and all were detectable in septic shock patients at trial enrollment (CISS 1 observational cohort, t0, $n = 21$). Basic characteristics of study participants are shown in **Supplementary Table 1** (Supplemental Digital Content 1, http://links.lww.com/CCM/E387). Descriptive statistics of plasma analyte levels are reported in **Supplementary Table 2** (Supplemental Digital Content 1, http://links.lww.com/CCM/E387). Thirty-nine analytes

were significantly elevated in septic shock patients (false discovery rate < 0.05; median 5.2-fold change; range 1.7- to 18,000-fold change) compared with healthy subjects (**Supplementary Fig. 1**, Supplemental Digital Content 1, http://links.lww.com/CCM/E387).

Septic Shock Patients Show Significant Perturbations in Plasma Cytokine Levels Within 72 Hours of Trial Enrollment

We next sought to define temporal changes in the systemic cytokine profile of the observational cohort (in the absence of MSC treatment) to inform changes associated with the natural course of septic shock in the ICU setting. A total of 22 analytes showed significant (adjusted $p < 0.05$) alterations in plasma levels at one or more time points within the first 72 hours of trial enrollment (**Fig. 1**). Among the cytokines that were altered, the majority (15/22 analytes) showed decreased levels, ranging in magnitude between –1.3- and –15.7-fold versus time 0 hours. By comparison, the changes exhibited by analytes that increased over time were generally more modest, ranging in magnitude from 1.5- to 4.9-fold. Among cytokines with potential pro-inflammatory activities, nine were significantly decreased (i.e., 1L-1b, IL-8, IL-6, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, ANGPT2, macrophage inflammatory protein [MIP]–α, MIP-3β, and myeloid progenitor inhibitory factor 1 [MPIF-1]), eight were unchanged (i.e., monocyte chemoattractant protein [MCP]–1, MCP-2, macrophage migration inhibitory factor, monokine induced by gamma, interferon gamma-induced protein 10, IL-2, IL-18, and MIP-1α), and only two were increased (i.e., interferon-γ and growth-regulated oncogene-α). Time course profiles for the 22 significantly altered analytes and 27 analytes that did not exhibit significant alterations are shown in **Supplementary Figures 2** and **3** (Supplemental Digital Content 1, http://links.lww.com/CCM/E387), respectively.



Fold Change (FC) to Time 0		
Max FC	Time (h)	P value
-2.2	1,12,72	≤0.002
-1.3	72	0.02
-2.4	72	0.006
-2.2	72	0.006
-6.5	72	0.0005
-2.6	72	0.01
-2.2	72	0.04
-12.1	24 -72	≤0.005
-2.9	72	0.003
-4.3	72	0.0001
-1.9	72	0.02
-15.7	24 -72	≤0.0007
-4.3	72	0.006
-6.2	72	0.0002
-12.1	72	0.0001
4.9	12-24	≤0.01
1.8	4 -72	≤0.009
1.9	4 -72	≤0.009
2.0	4 -72	≤0.03
1.5	24	0.01
2.2	72	0.01
1.7	72	0.02

Figure 1. Septic shock patients show significant alterations in plasma cytokine levels within 72 hr of trial enrollment in the absence of mesenchymal stem/stromal cell treatment. Hierarchical clustering and heatmap (*left*) of 22 cytokines that showed significant changes in plasma in the observational cohort over time ($n = 15–21$/time point). Columns denote median cytokine levels for each time point, and rows denote cytokines. Log2 transformed cytokine concentrations were shifted to mean 0 and scaled to SD of 1 for clustering and heatmap construction. *Right* summarizes the maximum observed fold change of each cytokine and time(s) at which significant changes were observed. *p* values were determined by nonparametric Kruskal-Wallis and Dunn's multiple comparison tests versus time 0. ANGPT2 = angiopoietin-2, ENA-78 = epithelial-derived neutrophil activating peptide 78, fractalkine = chemokine (C-X3-C motif) ligand 1, GM-CSF = granulocyte-macrophage colony-stimulating factor, Gro-α = growth-regulated oncogene α, IFN-γ = interferon gamma, IL = interleukin, IL-1ra = interleukin 1 receptor antagonist, MCP = monocyte chemoattractant protein, MIP = macrophage inflammatory protein, MPIF-1 = myeloid progenitor inhibitory factor 1, SCYB16 = small inducible cytokine B16, SDF-1α+β = stromal cell-derived factor-1 alpha and beta, TNF-α = tumor necrosis factor alpha, vWF-A2 = von Willebrand factor A2 domain.

No Significant Increase in Pro-Inflammatory Cytokines Was Detected After MSC Infusion

We next sought to investigate the effects of MSC treatment in the interventional versus observational cohort. At study enrollment, there were no significant differences in the baseline levels of any of the assessed analytes prior to MSC infusion between the observational and interventional cohorts (**Supplementary Table 3**, Supplemental Digital Content 1, http://links.lww.com/CCM/E387). In addition, plasma cytokine profiles revealed no discernible clustering of patients based on age, sex, Acute Physiology and Chronic Health Evaluation II score, or qualifying organ failure at study baseline (**Supplementary Fig. 4**, Supplemental Digital Content 1, http://links.lww.com/CCM/E387). Among the 49 assessed analytes, no significant differences were detected between the interventional and observational cohorts at any time point during the 72-hour study period (**Supplementary Fig. 5**, Supplemental Digital Content 1, http://links.lww.com/CCM/E387). Importantly, these included cytokines with pro-inflammatory activities and markers of cellular dysfunction/ injury, suggesting MSC infusion does not exacerbate the inflammatory phenotype of septic shock patients.

Principal Component Analysis Revealed Potential Dose- and Time-Dependent Effects of MSC Treatment

Small changes in multiple cytokines could potentially underlie additive or synergistic biologic effects; therefore, principal component analysis (PCA) was used to examine the aggregate response of all assessed analytes. PCA of 49 analytes assessed in all subjects across the entire time course of the study showed extensive overlap between the observational and interventional cohorts (**Fig. 2*A***), with no clear separation

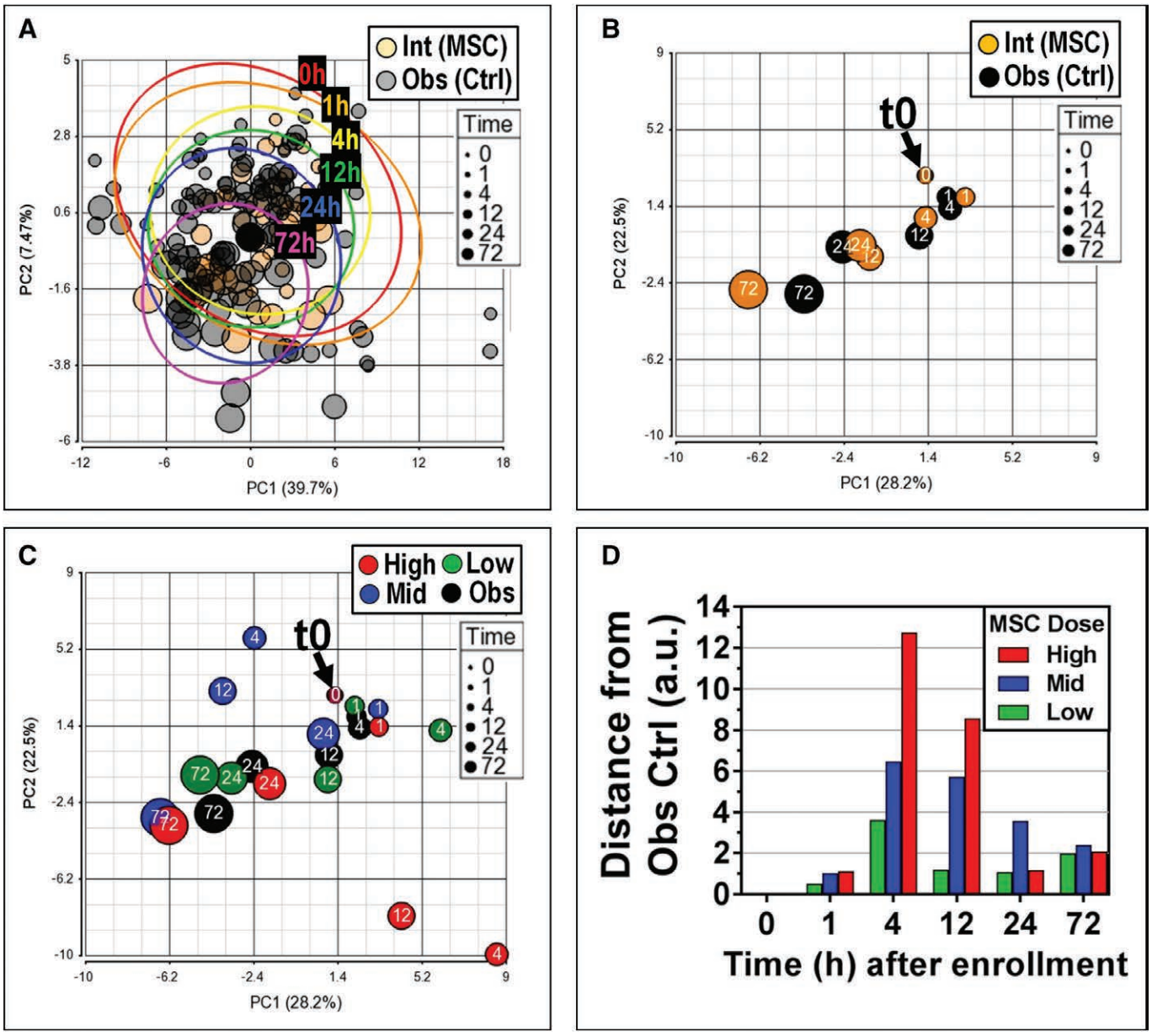


Figure 2. Principal component (PC) analysis (PCA) reveals potential dose- and time-dependent effects of mesenchymal stem/stromal cell (MSC) treatment. PCA of 49 analytes assessed in plasma of septic shock patients treated with (interventional [Int]) and without MSCs (observational [Obs]). **A**, PCA plot showing individual subjects (*circles*) across all time points (Obs, $n = 15–21$ subjects/time point; Int, $n = 8–9$ subjects/time point). PCA was performed on log2 transformed cytokine concentrations. Ellipses define region 2 SDs from centroid of each time group. **B**, PCA plot showing median value of each group of subjects (by treatment and time) after normalization of cytokine levels to study baseline time 0. **C**, PCA plot showing trajectory of the Int cohort after stratification by MSC dose. Data represent median value of each group of subjects (by treatment and time after normalization to study baseline). **D**, Distance between MSC dose groups and corresponding Obs group at each time point in the PCA plot of part C. Distances are in arbitrary units (a.u.) calculated from vector coordinates of the PCA plot.

between MSC-treated and non-treated patients. Of note, all MSC-treated patients were found within the broader range of values exhibited by the observational cohort, suggesting that MSC treatment does not cause any gross abnormalities in systemic cytokine profiles over the time course of the study. To better assess the relative trajectory of cytokine profiles between the observational and interventional cohorts over time, PCA was also conducted after normalizing analyte concentrations to study baseline (t0) and focusing on the median level for each treatment and time group (**Fig. 2*B***). The effects of time were more clearly delineated in this secondary analysis, as the aggregate cytokine profiles showed a progressive and linear movement away from study baseline (t = 0), with peak distance achieved at 72 hours. The observational and interventional cohorts showed very similar trajectories in the PCA plot; however, there was a divergence in trajectories after the interventional cohort was stratified by MSC dose (**Fig. 2*C***). In principal component space, maximum separation from the observational cohort occurred at 4 hours with the high MSC dose (**Fig. 2*D***), and decreased with time and lower MSC doses,

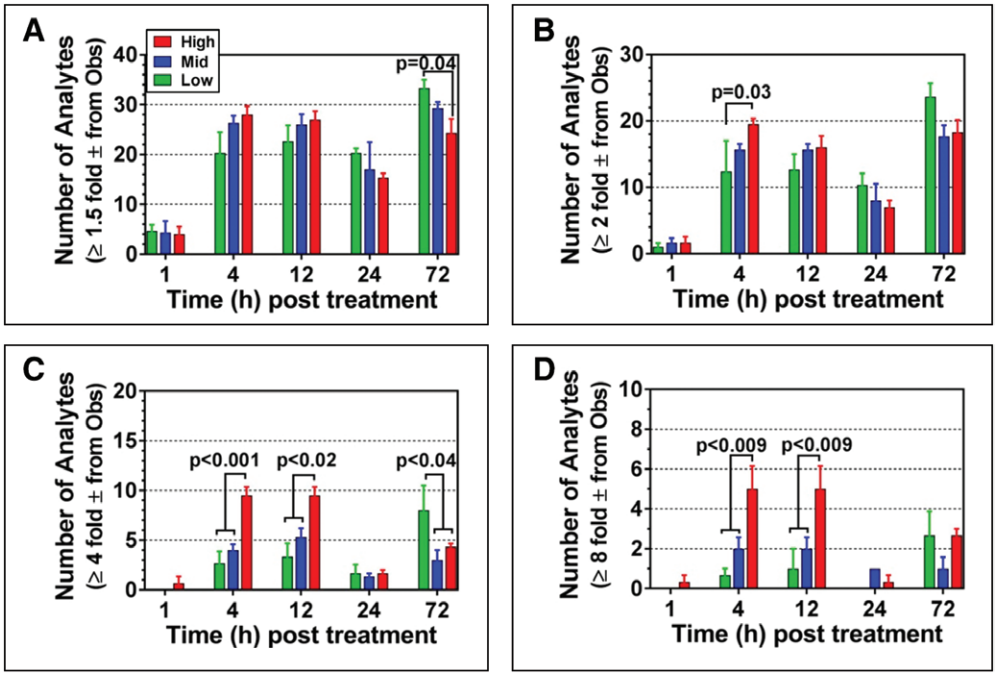


Figure 3. Higher mesenchymal stem/stromal cell (MSC) doses show greater perturbations in circulating analyte levels at early time points. Fold change to observational (Obs) group was determined after normalization of analyte levels to study baseline t0. For quantification purposes, altered analytes were defined by the magnitude of fold change (based on specified thresholds) as a surrogate for statistical tests that were limited by small sample sizes. **A**, Number of altered analytes greater than or equal to 1.5-fold up or down (±) from the Obs control. **B**, Number of altered analytes greater than or equal to two-fold up or down from the Obs control. **C**, Number of altered analytes greater than or equal to four-fold up or down from the Obs control. **D**, Number of altered analytes greater than or equal to eight-fold up or down from the Obs control. Differences between MSC dose groups were determined by Two-way analysis of variance and Tukey post test for all potential comparisons at each time point ($n = 3$ subjects/MSC dose/time point). Of note, one data point from the high MSC dose group at 4 hr and 12 hr was imputed using the mean value at each time point, respectively. Data are presented as mean ± SEM.

suggesting that perturbations in cytokine levels related to MSC treatment may be transient and dose-dependent.

Relationship Between MSC Dose and Perturbations in Systemic Cytokines

We next sought to leverage the comprehensive panel of assessed analytes to determine whether there was a relationship between MSC dose and the number of analytes that were altered in plasma. To mitigate statistical limitations of small sample sizes in the MSC dose groups, we defined alterations in plasma analyte levels strictly by the observed magnitude of fold change relative to the observational cohort. Furthermore, only changes greater than 1.5-fold from the observational cohort (up or down) were quantified to minimize the impact of potential spurious fluctuations in analyte levels. In this initial analysis, the number of altered analytes appeared to increase with increasing dose, specifically at 4 hours and 12 hours, although differences between doses did not reach statistical significance (**Fig. 3*A***). We speculated that MSC dose may also impact the magnitude of change in analyte levels, and therefore quantified the number of analytes that were altered under increasingly stringent thresholds ranging from greater than two-fold to eight-fold change from the observational control (**Fig. 3*B–D***). The positive linear relationship at 4 hours and 12 hours was preserved under these more stringent thresholds, and differences between doses reached statistical significance (Fig. 3*A–D*).

MSC-Treated Patients Show Transient Dose-Dependent Perturbations in Multiple Pro-Inflammatory Cytokines

We next investigated analyte-specific alterations in relation to MSC dose and known biological functions and sought to report all changes to mitigate the possibility of false negative errors in relation to potentially important safety signals. For this purpose, alterations in cytokine levels were again defined by the magnitude of fold change to the observational control, using a minimum quantification threshold of 1.5-fold. For clarity, **Figure 4** shows an abbreviated heatmap summarizing median alterations of each analyte and group of patients stratified by MSC dose and time. An expanded heatmap showing variability in analyte responses between individual patients is presented in **Supplementary Figure 6** (Supplemental Digital Content 1, http://links.lww.com/CCM/E387). The plasma cytokine profiles showed several differential patterns of change in relation to MSC dose. Although levels of some pro-inflammatory cytokines such as IL-1β, MIP-3α, and IL-2 appeared dampened at one or more time points across all three MSC doses (**Fig. 5*A***), other pro-inflammatory cytokines including IL-6, IL-8, MCP-1 showed discordant changes between doses (**Fig. 5*B***). These cytokines were selectively decreased by the mid MSC dose from 4 hours to 12 hours but showed elevated levels in the high MSC dose group during the same time interval. However, it is important to note that this transient spike in inflammatory cytokines in the high dose group was driven by a single patient, whereas the other two patients in the high dose group showed levels at or below the median level of the observational control (Fig. 5*B*). Furthermore, despite a relatively high magnitude of change, the spike in cytokine levels was generally within the broader range of variability exhibited by the observational cohort (Fig. 5*B*). Other cytokines such as MPIF-1, epithelial-derived neutrophil activating peptide 78, and macrophage-derived chemokine, which also may potentially contribute to the potentiation of

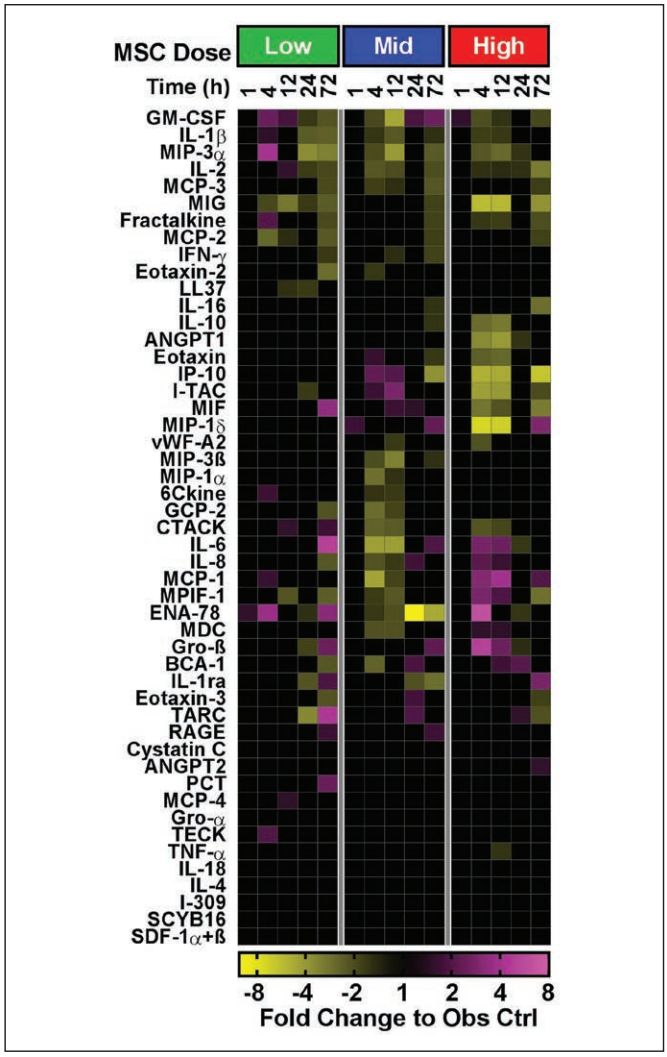


Figure 4. Plasma cytokine profiles show differential patterns in relation to mesenchymal stem/stromal cell (MSC) dose. Abbreviated heatmap showing median changes in levels of 49 analytes (rows) for each MSC dose and time group (columns; $n = 2$–3 subjects/MSC dose/time point). Analyte levels are presented as fold change (log2 scale) to the observational control (Obs Ctrl; $n = 15$–21 subjects/time point) after normalization to study baseline (time 0). Only changes of magnitude greater than or equal to 1.5-fold up or down from the Obs Ctrl group are shown. Note that changes are not statistically significant. 6Ckine = chemokine (C-C motif) ligand 21, ANGPT = angiopoietin, BCA-1 = B cell-attracting chemokine 1, CTACK = cutaneous T cell-attracting chemokine, ENA-78 = epithelial-derived neutrophil activating peptide 78, Eotaxin = eosinophil chemotactic protein, fractalkine = chemokine (C-X3-C motif) ligand 1, GCP-2 = granulocyte chemotactic protein 2, GM-CSF = granulocyte-macrophage colony-stimulating factor, Gro-α = growth-regulated oncogene α, Gro-β = growth-regulated protein beta, I-309 = chemokine (C-C motif) ligand 1, IFN-γ = interferon gamma, IL = interleukin, IL-1ra = interleukin 1 receptor antagonist, IP-10 = interferon gamma-induced protein 10, I-TAC = interferon-inducible T cell alpha chemoattractant, MCP = monocyte chemoattractant protein, MDC = macrophage-derived chemokine, MIF = macrophage migration inhibitory factor, MIG = monokine induced by gamma, MIP = macrophage inflammatory protein, MPIF-1 = myeloid progenitor inhibitory factor 1, PCT = procalcitonin, RAGE = receptor for advanced glycation end products, SCYB16 = small inducible cytokine B16, SDF-1α+β = stromal cell-derived factor-1 alpha and beta, TARC = thymus- and activation-regulated chemokine, TECK = thymus-expressed chemokine, TNF-α = tumor necrosis factor alpha, vWF-A2 = von Willebrand factor A2 domain.

inflammation through leukocyte recruitment and activation, showed a similar differential pattern of change between the mid and high MSC doses, which was again driven primarily by a single patient in the high MSC dose group (Fig. 5*B*). In addition, no dose-dependent safety signals were discernible among cellular markers of tissue injury/dysfunction including cystatin C, procalcitonin, and angiopoietin-2 (**Fig. 5*C***).

DISCUSSION

In this study, we investigated whether the plasma cytokine profiles of septic shock patients treated with MSCs provided information related to safety and/or other biological effects. No previous study has conducted as comprehensive an analysis of plasma cytokine and biomarker levels in critically ill patients treated with MSCs. Our results show that a single IV infusion of MSCs (dose from 0.3 to 3 million cells/kg) does not cause any gross abnormalities in systemic cytokine levels based on the assessment of a broad spectrum of 49 cytokines and biomarkers. Importantly, no significant increase in levels of known pro-inflammatory mediators or biomarkers of organ dysfunction was detected in relation to MSC treatment within the 72-hour time course of this study. Taken together with previously reported clinical data that showed no serious clinical or physiologic safety signals (7), this further evidence supports MSC treatment as well tolerated and safe for administration in critically ill patients with septic shock. In addition, stratification of the interventional cohort revealed dose-specific cytokine responses that suggest MSCs may alter sepsis biology.

Our finding that MSC treatment does not appear to exacerbate the systemic inflammatory phenotype of septic shock is supported by extensive cytokine profiling in plasma specimens sampled at six different time points within the first 72 hours of study enrollment. Thus, potential changes that may occur either acutely (within hours) or over a longer term (days) were subject to assessment. Furthermore, a relatively diverse range of 49 analytes were examined, including a large number of pro- and anti-inflammatory cytokines, as well as several other biomarkers of cell/organ dysfunction and injury (e.g., procalcitonin, cystatin C, ANGPT2, and RAGE). Alterations in these analyte levels were assessed before and after MSC treatment in the interventional cohort, and in relation to two separate control groups. Although no significant differences were detected between the interventional and observational groups, we sought to mitigate potential false negative errors by further stratifying the interventional group by MSC dose and reporting all changes in excess of 1.5-fold from the observational control. In the dose-stratified heatmap analyses, the high MSC dose appeared to elicit a temporary spike (from six-fold to 11-fold) in the levels of three potential pro-inflammatory cytokines IL-6, IL-8, and MCP-1; however, this effect was limited to a single patient, was transient in duration and generally fell within the broader range of variation exhibited by the observational cohort. Our second finding that MSC treatment might alter circulating cytokine levels was also supported by observations after stratification of the interventional group, in which the mid MSC dose

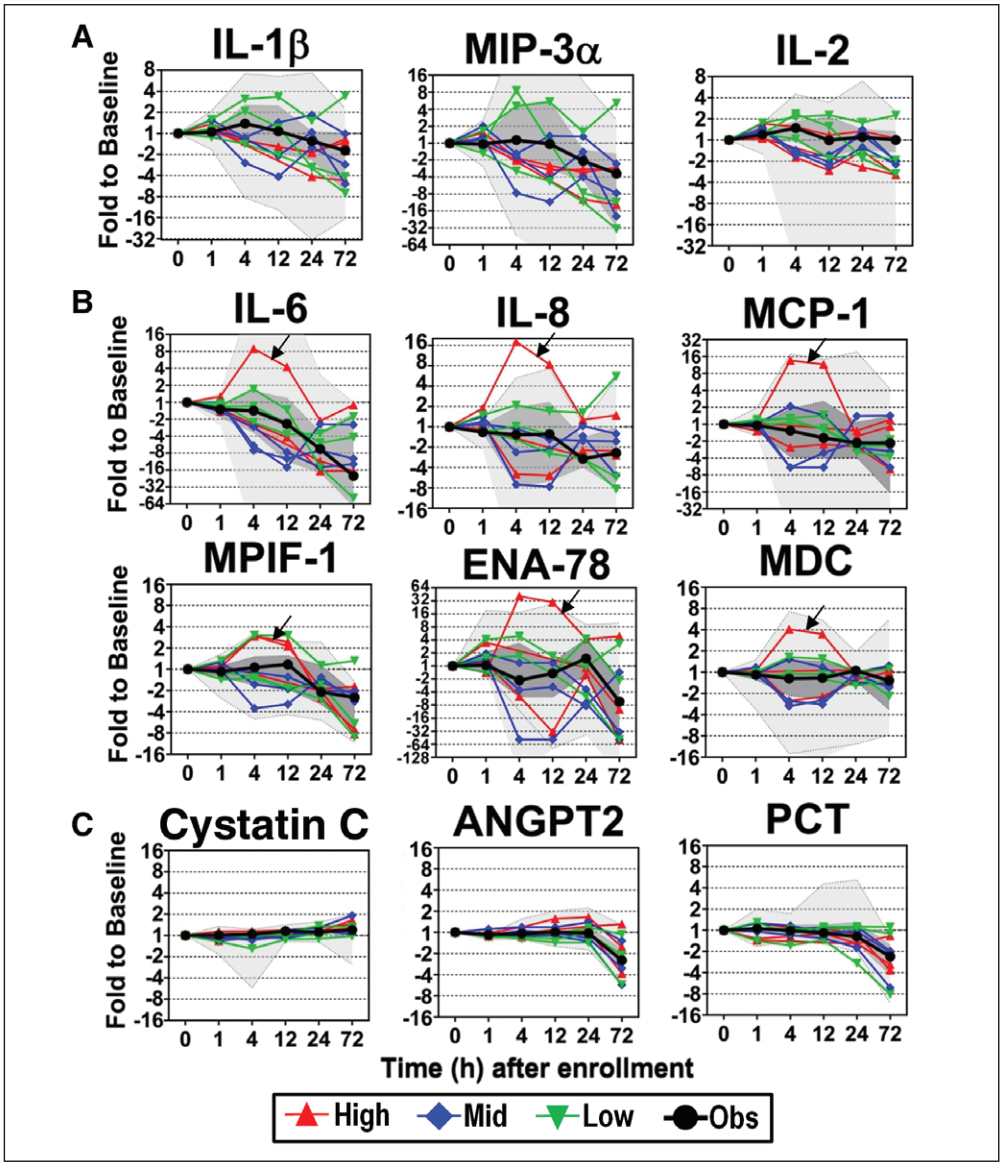


Figure 5. Changes in select analytes over time stratified by mesenchymal stem/stromal cell (MSC) dose. Data are presented as fold change to study baseline t0. Individual MSC-treated patients are shown color-coded by dose. Median levels of the observational group are shown with the interquartile range (*dark gray* shaded region) and minimum-maximum range (*light gray* shaded region). For clarity, the observational group range is clipped at the axis limit in some graphs. **A**, Mid and high MSC doses show similar dampening of some pro-inflammatory cytokines. **B**, Mid and high MSC doses show opposite changes in other cytokines with potential pro-inflammatory activities; however, discordance is driven by a single patient (denoted by *arrow*). **C**, No dose-dependent safety signals observed among known biomarkers of tissue injury/dysfunction. ENA-78 = epithelial-derived neutrophil activating peptide 78, IL-1β = interleukin 1 beta, MCP-1 = monocyte chemoattractant protein 1, MDC = macrophage-derived chemokine, MIP-3α = macrophage inflammatory protein 3 alpha, MPIF-1 = myeloid progenitor inhibitory factor 1.

(and one patient from the high MSC dose) appeared to selectively dampen the levels of several pro-inflammatory cytokines, albeit only transiently from 4 hours to 12 hours. These changes also fell within the range of variation exhibited by non-MSC treated patients, and therefore should be interpreted cautiously. Nevertheless, the observed alterations in systemic cytokine levels support the immunomodulatory mechanism of MSCs previously reported (8) and are consistent with previous reports that rapid MSC clearance or inactivation (by ~24 hr) may limit the duration of MSC effects (12, 13).

The broad scope of analytes, range of time points, and control groups examined in the current study distinguishes it from our previous report (7), and other early-phase studies that have investigated MSC treatment in patients with ARDS (10, 11). Our results are consistent with these previous ARDS studies, which have reported good safety profiles but limited evidence of clinical or physiologic efficacy signals of MSC therapy; however, it is important to note that these phase 1 trials were principally designed to assess MSC safety rather than therapeutic efficacy. In the current study, we observed marked perturbations in plasma cytokine levels that appeared to be both MSC dose- and time-dependent. From 4 hours to 12 hours, the number of altered cytokines progressively increased with higher MSC doses, and there were significant differences between the high versus mid and low doses.

One limitation of our study is the small sample size of the interventional cohort (7). Because statistical power is limited in this setting, we quantified and presented all changes to mitigate potential type II errors that could mask important safety signals. Similarly, we are cautious to interpret potential biological signals of MSC treatment because it is not yet clear how these biological effects may relate to clinical outcomes. Nevertheless, the coordinated patterns of change that were observed among groups of functionally related cytokines in response to specific MSC doses is suggestive and adds plausibility to their biological relevance.

Our finding that MSC infusion in septic shock patients did not exacerbate systemic cytokine levels or elevate other cellular markers of organ dysfunction and injury further supports the safety of MSC treatment. In addition, the demonstration that MSC treatment appeared to attenuate levels of several pro-inflammatory cytokines in a dose-specific manner (albeit

transiently), suggests that MSCs may potentially exert biologic effects. Whether these biological effects translate to beneficial effects in patients with septic shock remains a question (7). Collectively, these results support the need for larger phase II randomized controlled trials to rigorously examine clinical efficacy and safety, and to further explore the biology of MSCs in septic shock. These data also broaden our understanding of the circulating cytokine response to MSC infusion in humans, which may provide useful insight on the potential effects of MSC therapy in other acute inflammatory diseases.

ACKNOWLEDGMENTS

We gratefully acknowledge the support of the Cellular Immunotherapy for Septic Shock (CISS) research coordinators and assistants (Rebecca Porteous, Brigette Gomes, Heather Langlois, Shelley Acres, and Alana Aimable-Brathwaite) and the Canadian Critical Care Trials and Translational Biology Groups for their contribution to the study design of the CISS Phase I Trial and their critical review of this article.

REFERENCES

1. Angus DC, van der Poll T: Severe sepsis and septic shock. *N Engl J Med* 2013; 369:840–851
2. Mei SH, Haitsma JJ, Dos Santos CC, et al: Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 2010; 182:1047–1057
3. Gonzalez-Rey E, Anderson P, González MA, et al: Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009; 58:929–939
4. Krasnodembskaya A, Samarani G, Song Y, et al: Human mesenchymal stem cells reduce mortality and bacteremia in gram-negative sepsis in mice in part by enhancing the phagocytic activity of blood monocytes. *Am J Physiol Lung Cell Mol Physiol* 2012; 302:L1003–L1013
5. Németh K, Leelahavanichkul A, Yuen PS, et al: Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009; 15:42–49
6. Rabani R, Volchuk A, Jerkic M, et al: Mesenchymal stem cells enhance NOX2-dependent reactive oxygen species production and bacterial killing in macrophages during sepsis. *Eur Respir J* 2018; 51:1702021
7. McIntyre LA, Stewart DJ, Mei SHJ, et al; Canadian Critical Care Trials Group; Canadian Critical Care Translational Biology Group: Cellular Immunotherapy for Septic Shock. A phase I clinical trial. *Am J Respir Crit Care Med* 2018; 197:337–347
8. Le Blanc K, Davies LC: Mesenchymal stromal cells and the innate immune response. *Immunol Lett* 2015; 168:140–146
9. Spees JL, Lee RH, Gregory CA: Mechanisms of mesenchymal stem/stromal cell function. *Stem Cell Res Ther* 2016; 7:125
10. Wilson JG, Liu KD, Zhuo H, et al: Mesenchymal stem (stromal) cells for treatment of ARDS: A phase 1 clinical trial. *Lancet Respir Med* 2015; 3:24–32
11. Zheng G, Huang L, Tong H, et al: Treatment of acute respiratory distress syndrome with allogeneic adipose-derived mesenchymal stem cells: A randomized, placebo-controlled pilot study. *Respir Res* 2014; 15:39
12. Wang H, Cao F, De A, et al: Trafficking mesenchymal stem cell engraftment and differentiation in tumor-bearing mice by bioluminescence imaging. *Stem Cells* 2009; 27:1548–1558
13. Eggenhofer E, Benseler V, Kroemer A, et al: Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol* 2012; 3:297