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Intravenous Infusion of Human Adipose Mesenchymal Stem Cells Modifies the Host Response to Lipopolysaccharide in Humans: A Randomized, Single-Blind, Parallel Group, Placebo Controlled Trial

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ABSTRACT

In experimental models, mesenchymal stem cells (MSCs) can modulate various immune responses implicated in the pathogenesis of sepsis. Intravenous injection of lipopolysaccharide (LPS) into healthy subjects represents a model with relevance for the host response to sepsis. To explore the use of MSCs in sepsis, we determined their effect on the response to intravenous LPS in a randomized study in 32 healthy subjects with four treatment arms: placebo or allogeneic adipose MSCs (ASCs) intravenously at either 0.25×10^6, 1×10^6, or 4×10^6 cells/kg; all subjects received LPS intravenously (2 ng/kg) one hour after the end of ASC infusion (Trial Register number 2014-002537-63, clinicaltrials.gov identifier NCT02328612). Infusion of ASCs was well tolerated. The high ASC dose increased the febrile response, exerted mixed pro-inflammatory (enhanced interleukin-8 and nucleosome release) and anti-inflammatory effects (increased interleukin-10 and transforming growth factor-β release), and enhanced coagulation activation and reduced the fibrinolytic response. Blood leukocyte transcriptome analyses showed a biphasic effect of ASCs on the LPS response: at 2 hours post LPS, ASC-infused subjects displayed higher expression of genes involved in innate immune pathways, whereas at 4 hours post LPS these subjects had lower expression of innate immune pathway genes. Infusion of ASCs did not modify the "ex vivo" responsiveness of whole blood to various bacterial agonists. These results indicate that intravenous infusion of allogeneic ASCs (4×10^6 cells/kg) has a variety of proinflammatory, anti-inflammatory, and procoagulant effects during human endotoxemia. Further studies are needed to assess the safety and efficacy of ASCs in sepsis patients. STEM CELLS *2018;36:1778–1788*

SIGNIFICANCE STATEMENT

Mesenchymal stem cells have multiple immune modulatory properties that could benefit the treatment of sepsis. Infusion of mesenchymal stem cells improved outcome in preclinical sepsis models and is currently investigated in clinical trials in patients with sepsis or adult respiratory distress syndrome. This study used the human endotoxemia model to evaluate the biological activity of intravenously infused adipose mesenchymal stem cells in a controlled setting with relevance for sepsis pathophysiology. Infusion of adipose stem cells was well tolerated and at the highest dose tested associated with time-dependent proinflammatory and anti-inflammatory effects, as well as mild procoagulant features. These results may improve insight into the mechanism of action of mesenchymal stem cell therapy in sepsis.

INTRODUCTION

Sepsis is a clinical syndrome characterized by a dysregulated host response to an infection resulting in organ failure [1, 2]. Sepsis remains a major cause of morbidity and mortality worldwide despite the use of antibiotics and well-equipped intensive care facilities. In the last decade, hospital-treated sepsis had an estimated worldwide population incidence rate of

437 cases per 100,000 person years [3]. The current case fatality rate of sepsis is around 25% [3].

In sepsis, the balance between the pro-inflammatory and anti-inflammatory immune response is disturbed with a failure to return to homeostasis, resulting in sustained hyperinflammation with collateral tissue damage, combined with immune suppression [1, 2]. In spite of our increased knowledge of the pathogenesis of sepsis, immune modulatory therapies have thus far been unsuccessful in improving the outcome of this syndrome [1, 2, 4]. As such, there is a need for newer therapeutic approaches. Adult mesenchymal stem cells (MSCs) could potentially have clinical relevance in this context [5–9].

In recent years, MSCs have emerged as potent therapeutic tools based on their capacity to modulate immune responses and their low immunogenicity [5]. MSCs can exert many different immunomodulatory effects that theoretically could be beneficial in sepsis, including inhibition of microbial growth, reduction of pro-inflammatory cytokine release with concurrent enhancement of anti-inflammatory cytokine production, attenuation of inflammatory cell adhesion to endothelium, stimulation of endothelial regeneration, and inhibition of apoptosis [5–8]. In accordance, treatment with MSCs improved a variety of outcome parameters in animal models of sepsis [10–20].

Intravenous injection of purified lipopolysaccharide (LPS)—a component of the outer membrane of Gram-negative bacteria—into healthy subjects is a well-characterized model of human inflammation [21, 22]. While the model of human endotoxemia does not capture the full pathophysiology of clinical sepsis, it represents a controlled experimental setting in which the induction and regulation of systemic host responses with relevance for sepsis pathogenesis can be studied [21, 22]. Here, we sought to evaluate the effect of intravenous infusion of allogeneic adipose MSCs (ASCs) on the early inflammatory response to intravenous LPS.

Methods

Subjects and Study Design

This was a phase I, randomized, single-blind, parallel group, placebo-controlled study in 32 healthy male subjects (study acronym CELLULA; Trial Register number 2014-002537-63, clinicaltrials.gov identifier NCT02328612). The Dutch Central Committee on Research Involving Human Subjects (CCMO) and the Medical Ethics Committee of the Academic Medical Center (AMC), Amsterdam (the Netherlands) approved the study. Written informed consent was obtained from all subjects. Medical history, physical examination, hematological and biochemical screening, and electrocardiograms of all participants were normal. Block randomization was performed by the AMC pharmacist using nQuery; study subjects were blinded for treatment allocation.

ASCs were prepared at TiGenix SAU (Madrid, Spain) as described previously [23]. In short, allogeneic ASCs were obtained from adipose tissue from a healthy donor and expanded until population doublings 12–16, when cells were cryopreserved. Prior to administration, cells were thawed, recovered from cryopreservation by in vitro culture, formulated in Ringer's lactate (at a concentration of 2.5 million ASCs/ml) and infused intravenously (4 ml/minute) at 0.25×10^6, 1×10^6, or 4×10^6 cells/kg (n = 8 patients per dose). ASCs fulfilled the ISCT criteria for MSCs and were thoroughly checked for viability, population doublings, morphology, potency, identity, purity, sterility and genetic stability, among other quality controls. One hour after the end of intravenous ASC or placebo infusion, subjects were infused with LPS over one minute (2 ng/kg; from *Escherichia coli*, U.S. standard reference endotoxin, kindly provided by Anthony Suffredini, National Institute of Health, Bethesda, MD). This dose has been used extensively by us and others to induce reproducible inflammatory responses in healthy humans (for reviews, see [21, 22, 24, 25] and references herein).

Oral temperature, blood pressure, oxygen saturation (by pulse oximetry), respiratory rate, and heart rate were measured at half-hour intervals. Clinical symptoms characteristic of endotoxemia such as headache, chills, nausea, vomiting, photophobia and myalgia were recorded every 30 minutes using a graded scale (0, absent; 1, mild; 2, moderate; 3, severe).

EDTA-anticoagulated, citrate-anticoagulated, and heparin-anticoagulated venous blood was collected before treatment administration (baseline), directly before LPS injection (t = 0 hours) and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 24 hours thereafter. In addition, blood was collected in PAXgene tubes (Becton-Dickinson, Vianen, the Netherlands) at baseline 0, 2, 4, 6, and 24 hours for mRNA analysis. EDTA-anticoagulated and citrate-anticoagulated plasma was collected by centrifugation at 1,750g for 10 minutes at 4°C and stored at −80°C until analysis.

Assays

All measurements, except thrombin-antithrombin complexes (TATc; citrated plasma), were performed in EDTA-anticoagulated plasma. C-reactive protein (CRP) was measured by immunoturbidimetric assay (Roche Diagnostics, Almere, the Netherlands). Tumor necrosis factor (TNF), interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-12p40, interferon (IFN) γ, D-dimer, tissue-type plasminogen activator (tPA), plasminogen activator inhibitor type 1 (PAI-1), soluble intercellular adhesion molecule 1 (ICAM-1), soluble E-selectin, soluble vascular adhesion molecule 1 (VCAM-1), and myeloperoxidase (MPO) were measured with Luminex multiplex assay (Affymetrix eBioscience, Santa Clara, CA). Transforming growth factor beta (TGFβ) was measured by ELISA (eBioscience), as were LL-37 (Hycult Biotech, Uden, the Netherlands), elastase-α1-antitrypsin complexes and nucleosomes (both Sanquin, Amsterdam, the Netherlands) [26, 27], and TATc (Affinity Biologicals, Ancaster, Ontario, Canada). IL-2, IL-4, IL-5, IL-13, and IFNγ were only measured in supernatants of whole blood ex vivo stimulations.

Fibrin Generation Assay

Fibrin generation was assessed spectrophotometrically by the fibrin polymerization method as described previously [28]. In short, citrated platelet poor plasma was diluted in 25 mM Hepes, 137 mM NaCl, 0.1% ovalalbumine with or without 25.000 ASCs (from two different donors) in phosphate buffered saline, and with or without monoclonal antihuman tissue factor antibody (Sekisui, Stamford, CT; 10 μg/ml) in round bottom 96 well plates with Immunolon 2-high-binding coating (Fisher Scientific, Landsmeer, the Netherlands). Plasma was incubated at 37°C for 5 minutes and fibrin generation was initiated by prewarmed 25 mM Hepes, 137 mM NaCl, 0.1% ovalbumin, and 17 mM CaCl2. Absorption at 405 nm was measured for 60 minutes with 15 seconds intervals in the

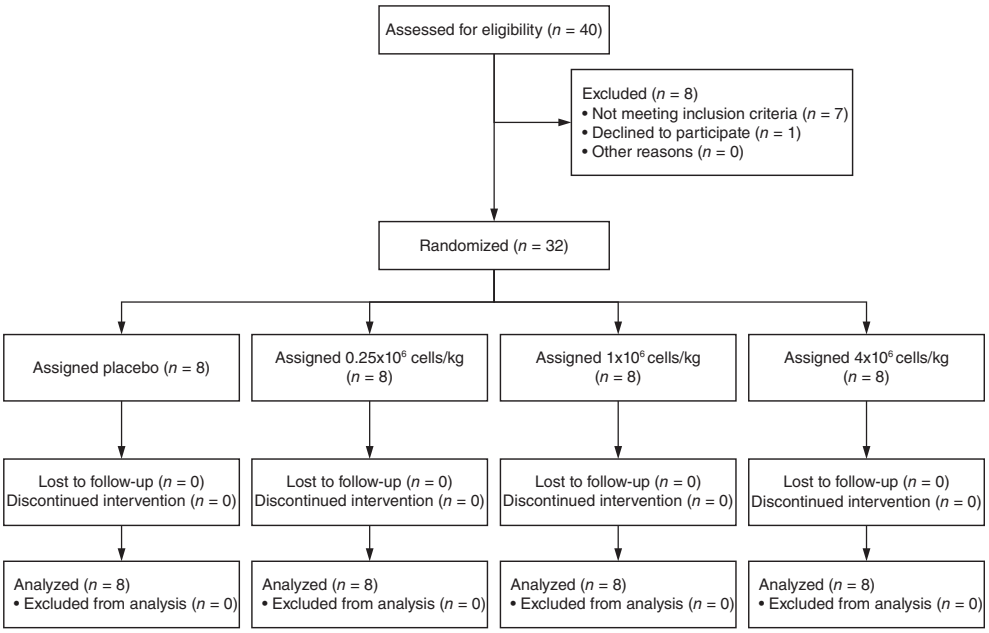

Figure 1. Flowchart of the study.

SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as percentage tissue factor clot activity.

RNA Preparation and Microarrays

RNA was isolated from PAXgene tubes using the PAXgene Blood miRNA isolation kit (PreAnalytiX, Qiagen, Venlo, the Netherlands) as described by the manufacturer. Total RNA yield and purity (260 nm:280 nm) were determined spectrophotometrically (Nanodrop). RNA integrity was assessed by bioanalysis (Agilent). RNA with integrity number (RIN) > 6 was included for microarrays, which applied to all samples obtained. RNA was hybridized to the Affymetrix Human Genome U219 96-array chip and scanned by means of the GeneTitan instrument (Affymetrix) at the Cologne Center for Genomics, Cologne, Germany. Preprocessing and quality control of the scans were performed by using the affy method (version 1.36.1) [29] in the R statistical computing environment (version 3.3.2). Array data were background corrected by Robust Multi-array Average (RMA), quantiles-normalized, and summarized by median-polish. The resultant 49,386 probe intensities were log-transformed and filtered by means of a 0.5 variance cutoff using the genefilter method [30]. In this way, 24,646 expressed probes in at least one sample were recovered. The occurrence of nonexperimental chip effects was evaluated by means of the surrogate variable analysis method (version 3.4.0) [31] and corrected by the empirical Bayes method combat [32]. Comparison between groups was performed by moderated *t* statistics implemented in the empirical Bayesian linear models method limma (version 3.14.4) [33–36]. Throughout Benjamini–Hochberg multiple comparison adjusted probabilities [37] (adjusted $p < .05$) defined significance. Pathway analysis was performed by means of Ingenuity pathway analysis (IPA, www.ingenuity.com) specifying the Ingenuity knowledgebase as reference and human species. All other parameters were default. Significance was evaluated by Fisher's exact test Benjamini–Hochberg adjusted *p* values (adjusted $p < .05$). Normalized and non-normalized array data are accessible through the Gene Expression Omnibus with accession GSE108685.

Whole Blood Stimulations

Heparin-anticoagulated whole blood was obtained at baseline 0, 4, and 24 hours, and incubated ex vivo for 4 or 24 hours without stimulus (control) or stimulated with LPS (200 ng/ml; from *E. coli* 0111:B4, ultrapure; Bio-Connect, Huissen, the Netherlands), Lipoteichoic acid (LTA 10 μg/ml; from *Staphylococcus aureus*, purified; Bio-Connect, Huissen, the Netherlands), heat-killed *S. aureus* or *E. coli* (both equivalent to 0.5×10^8 CFU/ml), or anti-CD3/CD8 (respectively 1 μg/ml and 5 μg/ml, both Sanquin, Amsterdam, the Netherlands). Cytokines were measured in supernatants as readouts for monocyte activation (TNF-α, IL-1β, IL-10) or lymphocyte activation (IL-2, IL-4, IL-5, IL-13, IFN-γ) using assays described above.

Statistical Analysis

Between-group comparisons of all variables, except cytokine data of whole blood stimulations, were done by two-way repeated measures ANOVA, if significant followed by Bonferroni post hoc analysis. Cytokine data of whole blood stimulations was analyzed by Kruskal–Wallis test followed by a Mann–Whitney *U* test where appropriate. Data are expressed as mean and SD. Analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). A two-sided *p*-value < .05 was considered significant.

Results

Vital Signs, Clinical Symptoms, and Acute Phase Response

In all, 32 male subjects were enrolled and completed the study between November 2014 and March 2015 (Fig. 1). Demographics

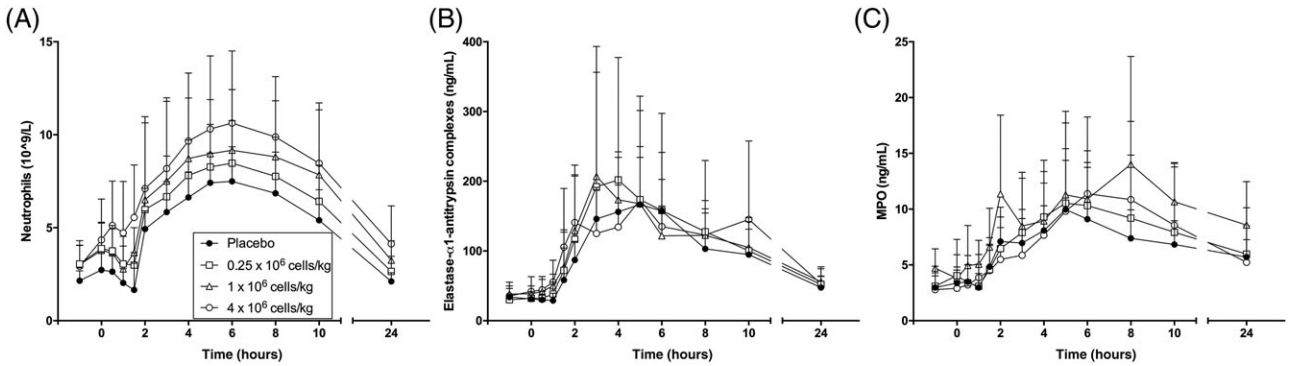

Figure 2. Adipose mesenchymal stem cells (ASC) infusion does not influence lipopolysaccharide (LPS)-induced neutrophil responses. Mean (with SD) neutrophil counts **(A)**, plasma elastase-α1-antitrypsin **(B)**, and MPO **(C)** levels after intravenous LPS administration (2 ng/kg, t = 0) 1 hour after the completion of intravenous infusion of Ringer's lactate (placebo) or ASCs (0.25×10^6, 1×10^6, or 4×10^6 cells/kg). Differences between groups were not significant. Abbreviation: MPO, myeloperoxidase.

did not differ between treatment groups (Supporting Information Table S1). Intravenous infusion of ASCs, at any dose, was well tolerated; arterial oxygen saturation was >96% in all subjects throughout the study and respiratory rates were not different between groups (data not shown). Intravenous injection of LPS induced a transient febrile response, peaking after 2–3 hours (38.0 $\pm$ 0.1°C), which was not influenced by infusion of ASCs at 0.25×10^6 or 1×10^6 cells/kg (Supporting Information Fig. S1). Subjects treated with ASCs at 4×10^6 cells/kg displayed an enhanced febrile response (peak temperature 39.1 $\pm$ 0.1°C; $p < .01$ vs. placebo). LPS administration was associated with tachycardia and transient flu-like symptoms, which were not modified by ASC infusion (data not shown). A total of six nonserious adverse events were reported during the study in five subjects, two of which were considered related to ASC infusion (throat irritation and pruritus; Supporting Information Table S2). The acute phase response induced by LPS, as measured by plasma CRP levels at 24 hours, was not influenced by ASC infusion (Supporting Information Table S3). Together, these data indicate that ASC infusion in healthy subjects challenged with intravenous LPS has a good safety profile and that high dose ASCs (4×10^6 cells/kg) potentiated the rise in body temperature during human endotoxemia.

ASC Infusion Does Not Influence LPS-Induced Leukocyte Responses

As expected [22], infusion of LPS induced a transient monocytopenia and lymphocytopenia; these responses were not influenced by ASC infusion (Supporting Information Fig. S2). LPS injection also elicited a neutrophilic leukocytosis (Fig. 2A), accompanied by neutrophil degranulation as indicated by transient rises in the plasma concentrations of elastase-α1-antitrypsin complexes (Fig. 2B) and MPO (Fig. 2C), which were not significantly modified by ASC infusion. The plasma levels of LL-37, a peptide present in lysosomes of neutrophils (and macrophages) and implicated in the antimicrobial properties of ASCs [38], were not altered in any of the treatment groups (data not shown). These results suggest that ASC infusion has no major effects on neutrophil responses during human endotoxemia.

ASC Infusion Has Both Proinflammatory and Anti-Inflammatory Effects on LPS-Induced Activation of the Cytokine Network

To obtain insight into the effect of ASCs on the LPS-induced activation of the cytokine network, we measured a set of proinflammatory cytokines (TNF, IL-1β, IL-6, IL-8, and IL-12p40) and anti-inflammatory cytokines (IL-10 and TGF-β) (Fig. 3). Intravenous LPS elicited transient rises in the plasma concentrations of TNF, IL-6, IL-8, IL-12p40, and IL-10 (Fig. 3). ASC infusion did not modify LPS-induced TNF, IL-6, or IL-12p40 release, albeit in the high dose group there was a tendency toward reduced IL-12p40 release. Administration of all three ASC doses was associated with increased LPS-induced IL-8 release ($p < .01$, <.05, and <.05 vs. placebo, respectively). Infusion of ASCs at 4×10^6 cells/kg was associated with higher plasma IL-10 and TGF-β levels after LPS injection (Fig. 3, $p < .01$ vs. placebo). IL-1β remained undetectable in virtually all samples and was not different between groups (data not shown). Together, these results suggest that ASC infusion has mixed proinflammatory (enhanced IL-8 release) and anti-inflammatory effects (trend to reduce IL-12p40 and increased IL-10 and TFG-β release) on the cytokine network during human endotoxemia.

Infusion of ASCs Exerts Procoagulant Effects Without Influencing LPS-Induced Endothelial Cell Activation

Activation of coagulation, fibrinolysis and endothelial cells are hallmark features of sepsis [39, 40], and the human endotoxemia model is suitable to study the mechanisms influencing these responses [41]. LPS injection elicited transient increases in the plasma levels of TATc (Fig. 4A) and D-dimer (Fig. 4B), reflecting activation of the coagulation system. Infusion of ASCs at 4×10^6 cells/kg was associated with increased plasma TATc and D-dimer concentrations relative to the control group ($p < .01$ and <.001, respectively); these increases occurred early, that is, already before LPS injection for TATc and directly after LPS administration (before a rise was detected in the control group) for D-dimer. ASCs at 1×10^6 cells/kg also enhanced LPS-induced D-dimer release ($p < .05$ vs. placebo). LPS provoked transient increases in the plasma concentrations of tPA (indicating activation of fibrinolysis; Fig. 4C) and PAI-1 (a main inhibitor of fibrinolysis; Fig. 4D). High dose ASCs (4×10^6 cells/kg) inhibited the LPS-induced increase in plasma

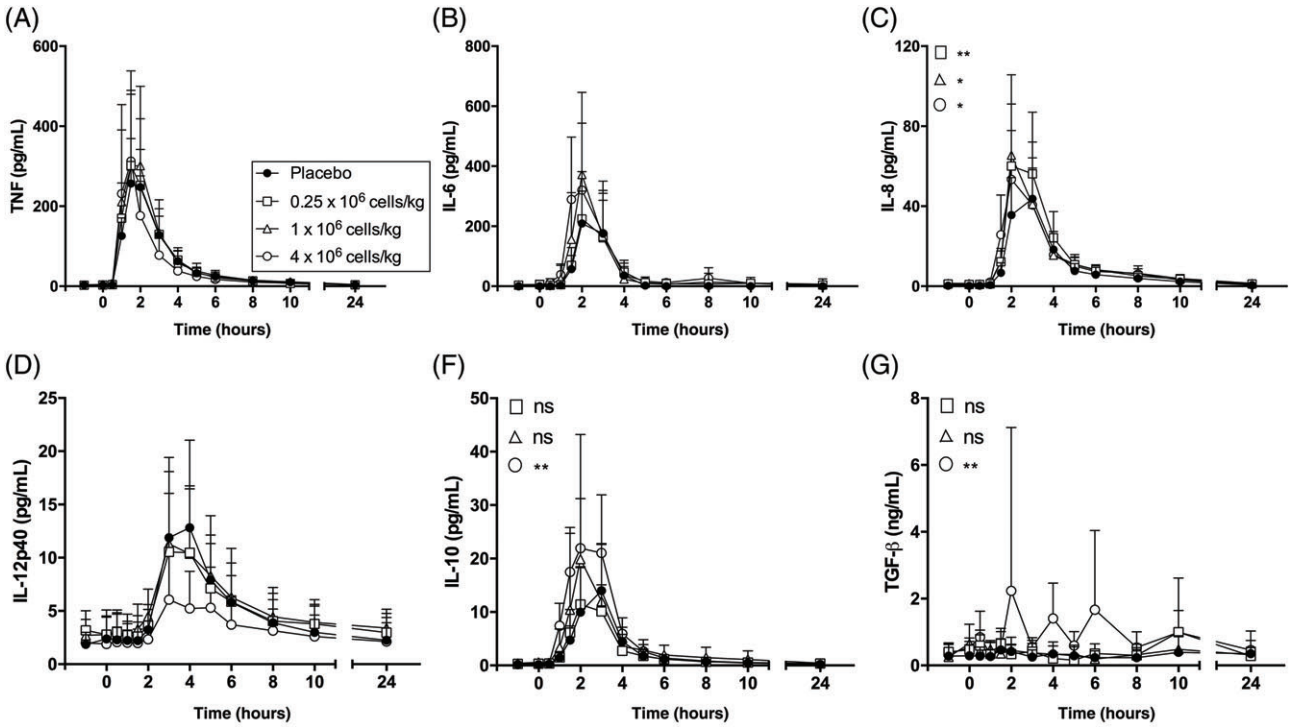

Figure 3. Adipose mesenchymal stem cells (ASC) infusion modifies lipopolysaccharide (LPS)-induced cytokine release. Mean (with SD) plasma concentrations of TNF **(A)**, IL-6 **(B)**, IL-8 **(C)**, IL-12p40 **(D)**, IL-10 **(E)**, and TGF-β **(F)** after intravenous LPS administration (2 ng/kg, $t = 0$) 1 hour after the completion of intravenous infusion of Ringer's lactate (placebo) or ASCs (0.25×10^6, 1×10^6, or 4×10^6 cells/kg). IL-8 levels were significantly changed at baseline (**) and T0-1 (*) after treatment with 0.25×10^6 cells/kg, at baseline (**) and T0 (*) after treatment with 1×10^6 cells/kg and at T1 (**) and T1.5 (*) after treatment with 4×10^6 cells/kg. IL-10 levels were significantly changed at T1 (***) and T1.5 (**) after treatment with 4×10^6 cells/kg. *, $p < .05$; **, $p < .01$; ***, $p < .001$ versus the control group (two-way repeated measures ANOVA). Abbreviations: TGFβ, transforming growth factor beta; TNF, tumor necrosis factor.

tPA levels ($p < .01$ vs. placebo). ASC infusion did not modify PAI-1 concentrations. Endothelial cell activation after LPS injection was reflected by rises in the plasma concentrations of soluble E-selectin (Fig. 4E) and soluble VCAM-1 (Fig. 4F); this response was not altered by ASC infusion. Likewise, the LPS-induced increase in plasma soluble ICAM-1 concentrations, which can be derived from endothelial cells or leukocytes, was not affected by ASC infusion (Fig. 4G). Previous investigations have documented procoagulant activity associated with MSCs from different sources [42–47], which has been linked to expression of tissue factor [42–47], a protein capable of activating the coagulation cascade via Factor VII [48]. In accordance, ASCs used in our in vivo study enhanced fibrin generation in vitro, which could be completely inhibited by an anti-tissue factor antibody (Fig. 4H). Together, these results indicate that ASC infusion at a high dose is associated with a transient procoagulant effect, detectable already before LPS injection and possibly tissue factor dependent, and a blunted fibrinolytic response.

ASC Infusion Increases Plasma Nucleosome Levels

Sepsis is associated with elevated plasma levels of nucleosomes, which is considered to reflect enhanced cell death [49, 50]. Considering that ASCs exert antiapoptotic effects [5, 6, 8, 10], we were interested to determine the impact of ASC infusion on nucleosome release during endotoxemia. Intravenous LPS elicited a transient rise in plasma nucleosome levels peaking after 3 hours (Fig. 5). Infusion of ASCs at 4×10^6 cells/kg was accompanied by an early increase in plasma nucleosome levels peaking after 1.5 hours that was significantly higher than in control subjects ($p < .01$).

ASC Infusion Modifies the Blood Leukocyte Transcriptome in a Time-Dependent Biphasic Manner

In order to better understand the capacity of ASCs to influence the host response during endotoxemia, we performed a genome-wide scan of gene expression in blood leukocytes. At 2, 4, and 6 hours after LPS administration, the leukocyte transcriptome in the placebo group was characterized by a dramatic alteration in gene expression with >8,000 significantly different genes relative to baseline (Supporting Information Fig. S3A). At 24 hours, only three genes were significantly different in expression. Ingenuity pathway analysis revealed significant overrepresentation of various canonical signaling pathways (Supporting Information Fig. S3B). Overexpressed genes were associated to prototypical innate immune pathways, including nuclear factor (NF)-kB signaling, Toll-like receptor (TLR) signaling, triggering receptor expressed on myeloid cells (TREM)-1 signaling, and IL-6 signaling; underexpressed genes were predominantly associated to metabolic and adaptive immune pathways. Comparing the LPS-induced leukocyte transcriptome, ASC groups at low and medium dose revealed no statistically significant differences to the placebo group (Supporting Information Fig. S4). However, we uncovered statistically significant alterations in subjects treated with high dose (4×10^6 cells/kg). Two hours after LPS administration,

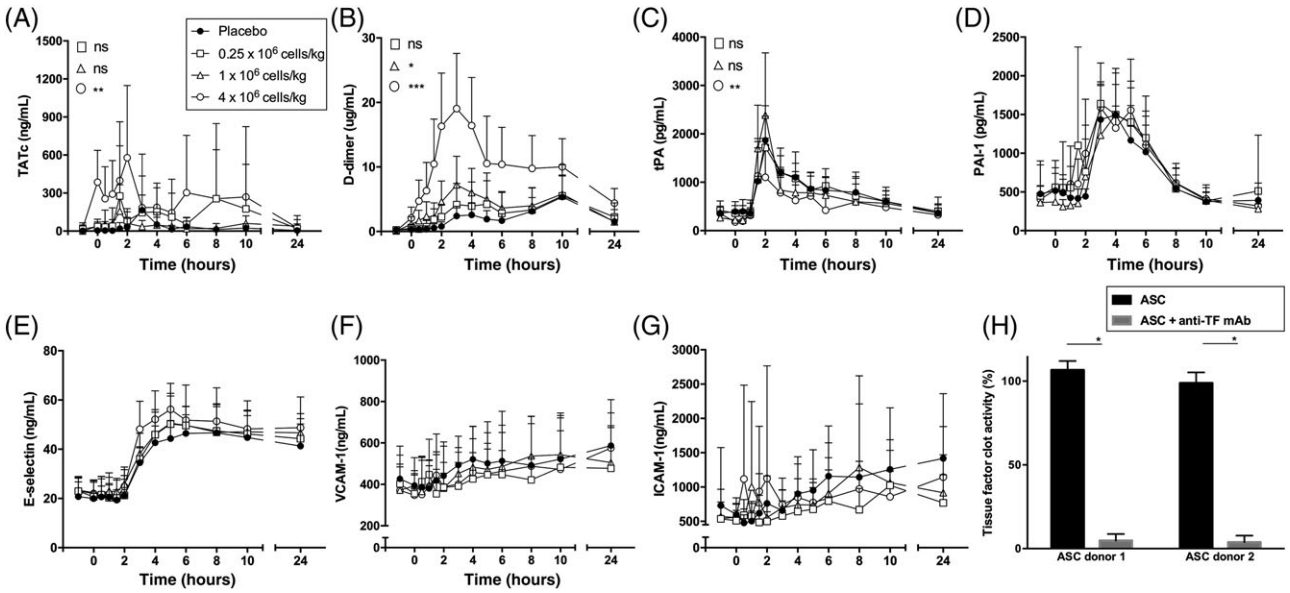

Figure 4. ASC infusion has a procoagulant effect without influencing lipopolysaccharide (LPS)-induced activation of endothelial cells. Mean (with SD) plasma concentrations of TATc **(A)**, D-dimer **(B)**, tPA **(C)**, PAI-1 **(D)**, soluble E-selectin **(E)**, soluble VCAM-1 **(F)**, and soluble ICAM-1 **(G)** after intravenous LPS administration (2 ng/kg, t = 0) 1 hour after the completion of intravenous infusion of Ringer's lactate (placebo) or ASCs (0.25×10^6, 1×10^6, or 4×10^6 cells/kg). TATc levels were significantly changed at T0 (***), T0.5 (**), T1-1.5 (***), and T2 (**) after treatment with 4×10^6 cells/kg. D-dimer levels were significantly changed at T2 (**), T3 (***), and T4 (*) after treatment with 1×10^6 cells/kg and at T1.5-6 (***) and T8 (*) after treatment with 4×10^6 cells/kg. tPA levels were significantly changed at T0 (**) and T6 (*) after treatment with 4×10^6 cells/kg. *, $p < .05$; **, $p < .01$; ***, $p < .001$ versus the control group (two-way repeated measures ANOVA). **(H):** in vitro fibrin generation in normal pool plasma by ASCs. Tissue factor (TF) dependence is shown by a blocking TF antibody in ASCs of two different donors. Mean (with SD) tissue factor clot activity (%) of ASCs derived from two different donors (N = 4). *, $p < .05$. Abbreviations: ASC, adipose mesenchymal stem cells; ICAM-1, intercellular adhesion molecule-1; PAI-1, plasminogen-activator inhibitor type I; TATc, thrombin-antithrombin complexes; tPA, tissue-type plasminogen activator; VCAM-1, vascular cell adhesion molecule-1.

138 genes were significantly overexpressed and 236 genes were underexpressed in high-dose ASC-treated subjects relative to the control group. Supporting Information Table S4 depicts the topmost significant genes (adjusted $p < .05$) considering fold changes >2 or <−2. At 4 hours, we found 56 overexpressed genes and 92 underexpressed genes, and at 6 hours we detected 4 overexpressed and 7 underexpressed genes in high-dose ASC-treated subjects relative to controls; no differences were observed at 24 hours. Pathway analysis of the altered genes at 2 hours revealed significant overrepresentations of overexpressed genes linking with predominantly innate immune mechanisms including NF-κB signaling, TLR signaling, and p38 mitogen activated protein kinase (MAPK) signaling (Fig. 6A). No significant associations were uncovered for underexpressed genes. At 4 hours, analysis of overexpressed genes in the high-dose ASC group revealed overrepresentation for various metabolic, protein degradation, and cell growth pathways. Interestingly, underexpressed genes in the high-dose ASC group at 4 hours significantly overrepresented a number of innate immune pathways that included NF-κB signaling, TNF receptor signaling, and production of nitric oxide and reactive oxygen species in macrophages. Supporting Information Figure S5 illustrates the biphasic effect of high-dose ASC infusion on LPS-induced NF-κB signaling in blood leukocytes. Overall, these findings suggest that high-dose ASC treatment may influence the blood leukocyte host response in a time-dependent biphasic manner with innate immune mechanisms enhanced early after LPS administration (2 hours) and blunted shortly thereafter (4 hours).

ASC Infusion Does Not Modify Leukocyte Responsiveness upon "Ex Vivo" Stimulation

Sepsis is associated with a reduced responsiveness of monocytes and lymphocytes upon stimulation [2, 51]. This immune suppressive feature can be reproduced by intravenous injection of LPS, that is, blood leukocytes obtained several hours after LPS administration are less capable of releasing cytokines upon restimulation ex vivo [52, 53]. Considering the broad immune modulatory actions of ASCs, we wished to establish whether ASC infusion is able to modify cellular hyporesponsiveness during endotoxemia. For this, we stimulated the whole blood obtained at baseline, directly before LPS injection and at 4 and 24 hours thereafter, ex vivo with bacterial agonists (LPS, LTA, *S. aureus*, or *E. coli*) or with a T cell stimulus (anti-CD3/CD28) for 4 or 24 hours. Blood leukocytes harvested 4 hours after LPS injection showed a reduced capacity to release cytokines upon stimulation, which was not influenced by ASC infusion at any dose. Data are shown for TNF and IL-10 after 24-hour LPS stimulation (Fig. 7A) and for IFN-γ and IL-13 after 24-hour stimulation with anti-CD3/CD28 (Fig. 7B).

Discussion

Ample preclinical evidence indicates that MSCs can be an effective adjunctive therapy in patients with sepsis or noninfectious critical illness [5–9]. A recent meta-analysis including 20 controlled experiments in preclinical sepsis models reported that treatment with MSCs reduced the odds of mortality of

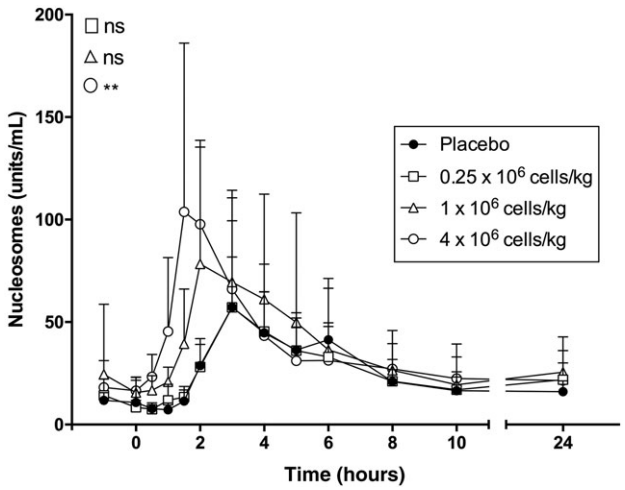

Figure 5. Infusion of adipose mesenchymal stem cells (ASCs) enhance lipopolysaccharide (LPS)-induced nucleosome release. Mean (with SD) plasma concentrations of nucleosomes after intravenous LPS administration (2 ng/kg, $t = 0$) 1 hour after the completion of intravenous infusion of Ringer's lactate (placebo) or ASCs (0.25×10^6, 1×10^6, or 4×10^6 cells/kg). Nucleosome levels were significantly changed at T0.5 (**) and T1-2 (***) after treatment with 4×10^6 cells/kg. **, $p < .01$; ***, $p < .001$ versus the control group (two-way repeated measures ANOVA).

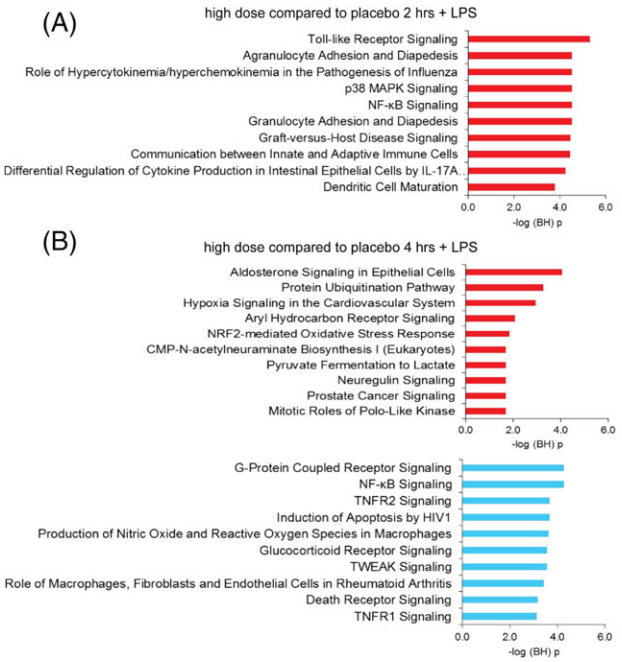

Figure 6. Time dependent effects of adipose mesenchymal stem cells (ASC) infusion on the lipopolysaccharide (LPS)-induced leukocyte transcriptome. **(A):** Ingenuity pathway analysis of the high ASC dose group at 2 hours after LPS administration revealed statistically significant enrichment for only overexpressed genes (red). Typical pro-inflammatory pathways, notably NF-kB signaling, were potentiated by the high cell dose.**(B):** Ingenuity pathway analysis of the high ASC dose at 4 hours after LPS administration revealed statistically significant enrichment for both overexpressed (red) and underexpressed (turquoise) genes. Most particular, NF-kB signaling was underexpressed in high cell dose samples compared to placebo after LPS administration. No differences were uncovered at baseline. Only 1 gene (*NFKBIA*) was differentially expressed at 0 hours in high cell dose compared to placebo. Array data are accessible through the Gene Expression Omnibus with accession GSE108685 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108685).

experimental sepsis by approximately 73% over a range of investigational conditions [54]. To further explore the safety and potential use of intravenous infusion of ASCs in the treatment of sepsis patients, we used the well-described model of human systemic inflammation induced by intravenous injection of LPS [21, 22] to obtain insight into the effect of ASCs on the induction of host immune responses with relevance for sepsis pathogenesis. We show that intravenous infusion of ASCs is well tolerated, at any dose tested. At a high dose (4×10^6 cells/kg), ASCs had clear signs of biological activity, exerting a variety of proinflammatory, anti-inflammatory, and procoagulant effects during human endotoxemia. Notably, while many previous studies examined the effect of MSCs in inflammation and sepsis models in animals [5–9, 54], this investigation is the first to study the effect of MSCs on inflammatory responses in humans.

Excessive neutrophil activation may cause tissue injury in sepsis [55]. MSCs have been reported to inhibit neutrophil oxidative burst and degranulation in vitro, as measured by extracellular release of MPO and elastase [56, 57], and to strongly attenuate neutrophil-mediated damage in a model of immune complex-mediated vasculitis in vivo [56]. However, we did not detect a significant effect of ASCs on the induction of neutrophilic leukocytosis or neutrophil degranulation during human endotoxemia.

MSCs can promote the repolarization of monocytes and/or macrophages from a type 1 (proinflammatory) to a type 2 (anti-inflammatory) phenotype characterized by increased secretion of the anti-inflammatory cytokine IL-10, augmented phagocytosis, and reduced TNF and IFN-γ production [58–60]. In animal models of sepsis and endotoxemia, MSCs from a variety of sources inhibited proinflammatory cytokine release and concurrently amplified IL-10 secretion [10, 20, 61–63]. The capacity of MSCs to reprogram monocytes/macrophages and to enhance their IL-10 release was essential for their protective role during polymicrobial sepsis in mice [10]. Here, we showed that infusion of high-dose ASCs enhanced not only the release of IL-10 upon LPS injection, but also elicited the release of TGF-β, which was not induced in subjects receiving placebo or lower doses of ASCs. TGF-β has pleiotropic functions and is involved in both suppressive and inflammatory immune responses [64]. TGF-β can render macrophages and monocytes hyporesponsive to LPS [65] and therefore may play an anti-inflammatory role during endotoxemia and sepsis [66]. Hence, elevated IL-10 and TGF-β levels in subjects infused with high-dose ASCs point to an anti-inflammatory effect. This is further supported by a tendency to also reduce IL-12p40 release and by the alteration of the blood leukocyte gene expression at 4 hours after LPS showing reduced activation of NF-κB signaling, TNF receptor signaling, and production of nitric oxide and reactive oxygen species. On the other hand, infusion of high-dose ASCs was also associated with proinflammatory effects, most notably enhanced IL-8 release, and increased TLR, MAPK, and NF-kB signaling in blood leukocyte gene expression at 2 hours after LPS injection. In accordance, short-term exposure of MSCs to LPS in vitro can induce a proinflammatory response [67, 68] and a comprehensive

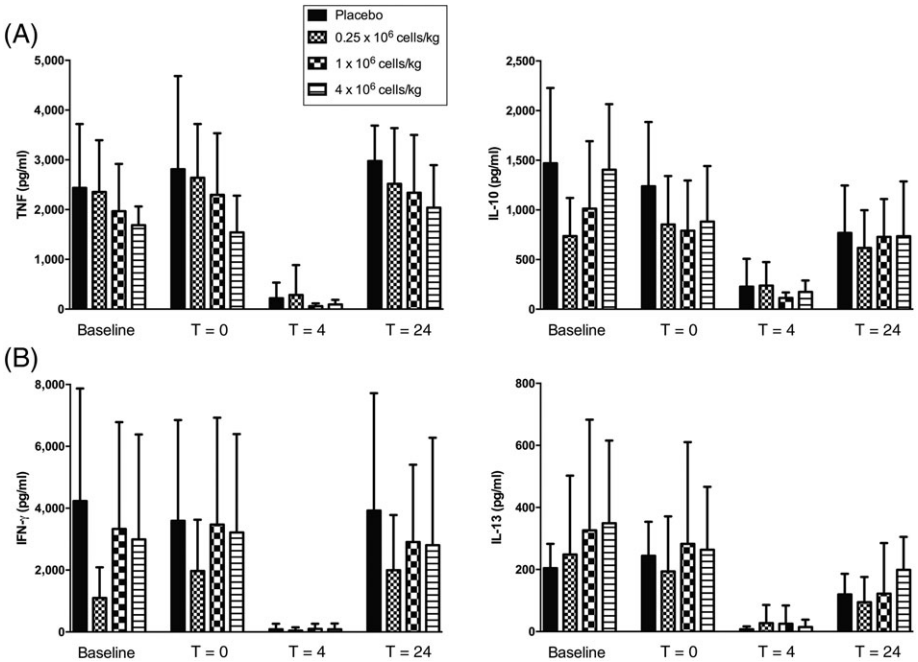

Figure 7. Adipose mesenchymal stem cells (ASC) infusion does not modify the "ex vivo" responsiveness of blood leukocytes harvested after intravenous administration of lipopolysaccharide (LPS). Whole blood was obtained before ASC infusion (baseline), before LPS injection (t = 0) and at 4 or 24 hours thereafter, and "ex vivo" stimulated with LPS **(A)** or anti-CD3/CD28 **(B)** for 24 hours. Data are means with SD. While at t = 4 hours blood leukocytes released lower concentrations of all cytokines shown when compared with baseline or t = 0, no differences between treatment groups were observed. Abbreviation: TNF, tumor necrosis factor.

analysis of gene expression in LPS-stimulated MSCs revealed induction of multiple proinflammatory genes with NF-κB as the central node [69]. Together, these results suggest that ASC infusion has mixed proinflammatory and anti-inflammatory effects during human endotoxemia, which may vary in a time-dependent manner.

In our study, infusion of high ASC dose was associated with a transient procoagulant effect as reflected by elevated plasma levels of TATc and D-dimer, which already became apparent before or shortly after LPS injection, that was not related to any clinical adverse event. In additional in vitro studies, we showed that the ASCs used here, like MSCs from other sources [42–47], express tissue factor-dependent procoagulant activity. Tissue factor is the main driver of coagulation activation after intravenous LPS injection in humans [70, 71], and we consider it likely that MSC-associated tissue factor is responsible for the enhanced coagulation activation in this model. Akin to our results, patients infused with bone-marrow derived MSCs for treatment of complications of hematopoietic stem cell transplantation showed a rise in plasma TATc without clinically evident thromboembolic events [42]. Likewise, infusion of placenta-derived decidual stromal cells resulted in a transient rise in plasma D-dimer levels in the absence of any thrombotic event in these patients [46]. High-dose ASC infusion inhibited the release of tPA, which is expected to reduce fibrinolysis, which may further contribute to a procoagulant effect. Procoagulant effects of MSCs in vivo have also been documented in experimental animals; for example, infusion of high doses of MSCs into mice was associated with the formation of fibrin clots in lungs [44] and in a porcine model of acute myocardial infarction, intracoronary delivery of bone-marrow-derived MSCs was associated with in situ microvascular thrombosis [45]. Although caution is warranted and close monitoring of patients is needed, the clinical relevance of the procoagulant effects of MSCs in humans is not certain considering that MSCs of a variety of sources have been proven safe in patients and thromboembolic events have not been reported [72, 73].

Comprehensive analyses of the blood leukocyte transcriptome suggested a biphasic effect of ASCs on LPS-induced gene expression. Most strikingly, ASC infusion was associated with early enhancement (2 hours post-LPS) and later suppression (4 hours post-LPS) of NF-κB signaling in blood leukocytes. Importantly, the inflammatory microenvironment is required for MSCs to activate gene expression pathways and exert their immunoregulatory capability [74, 75], which therefore may require some hours from the time of infusion. Thus, proinflammatory cytokines like IFN-γ, TNF, and IL-β can activate MSCs to exert immunosuppressive functions [76, 77]. Our data suggest that the initial inflammatory response elicited by intravenous LPS may have contributed to activate the ASCs, resulting in the immune suppressive effects of ASCs detected beyond 2 hours post LPS. This also points to a limitation of the human endotoxemia model, which might not be the best option to investigate MSC immunomodulatory properties, due to differential kinetics between the transient and short LPS effects and the time needed by MSC for activation and activity. This model is associated with activation of innate immune pathways that—although resembling responses seen in patients with sepsis in a qualitative way—occurs in a very rapid, short-lived, and transient way, unlike sepsis-associated alterations, which are more severe and sustained. Because of this limitation, subjects received ASCs prior to LPS, whereas posttreatment would better resemble the clinical scenario.

Release of nucleosomes is considered to reflect cell death [78, 79]. Elevated circulating levels of nucleosomes have been detected in several conditions, including trauma, ischemia–reperfusion injury, and sepsis [49, 50, 78, 79]. Here, we report the transient release of nucleosomes after intravenous injection of LPS into healthy subjects. Preliminary investigations done in our laboratory suggest that the main cellular source of circulating nucleosomes in this model is neutrophils. Infusion of ASCs was associated with an earlier and stronger rise in plasma nucleosome levels upon intravenous LPS injection, suggestive of enhanced neutrophil turnover. Possibly, the increased early proinflammatory response as detected by the blood transcriptome played a role herein. Although these data contrast with the effect of MSCs in animal models of sepsis and endotoxemia, where MSCs diminished apoptosis of cells in various organs [11, 14, 80], they are not necessarily contradictive. Indeed, intravenous injection of low-dose LPS into healthy subjects induces only a mild and transient response, whereas experimental sepsis in animals or clinical sepsis on the intensive care unit are associated with sustained hyperinflammation with injury to multiple cell types in multiple organs [2, 51, 78]. Additionally, it cannot be ruled out that the rise in plasma nucleosomes might originate from the clearance and elimination of the infused ASCs, as it is known that MSCs are short lived after administration [81, 82].

Sepsis is associated with immune suppression characterized by (among other) a reduced capacity of blood monocytes and lymphocytes to release proinflammatory cytokines upon stimulation ex vivo [2, 51]. The model of human endotoxemia reproduces this state of "reprogramming" in blood leukocytes and has been used to study the mechanisms involved and to evaluate potential strategies to prevent this [52, 53, 83, 84]. MSCs exert broad immune modulatory effects on many different cell types including monocytes and lymphocytes, characterized by inhibition of (proinflammatory) type 1 cytokines and enhancement of (anti-inflammatory) type 2 cytokines [58–60, 85]. As such, in theory MSCs could potentiate this feature of immune suppression after in vivo exposure to LPS. However, using a panel of monocyte and lymphocyte stimulants, we were not able to detect such an effect in this rapid and transient model. It remains to be established whether MSCs influence cellular reprogramming at tissue levels and/or during conditions of more sustained immune suppression.

MSCs are now tested as a potential adjunctive therapy in inflammatory diseases including sepsis and acute respiratory distress syndrome (clinicaltrials.gov identifiers NCT02883803) [86, 87]. While the immune modulatory effects of MSCs have been studied in a variety of animal models of inflammation and infection [5–9, 54], the current investigation is the first to report on the effects of MSCs in humans, using a well-established model of systemic inflammation induced by intravenous injection of LPS. Our results indicate that in this transient condition of mild inflammation intravenous infusion of allogeneic ASCs was safe and exerted a variety of time-dependent proinflammatory and anti-inflammatory effects, as well as procoagulant features. Despite the limitations of this model, some of our findings confirm earlier immunomodulatory effects in animal models of sepsis. Further testing of ASCs in sepsis patients is warranted to assess their safety and efficacy, to determine their effects on the host response during sepsis, and to enhance insight in their mechanism of action.

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Author Contributions

D.P.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; L.A.v.V.: conception and design, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; B.P.S.: collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; A.M. and R.L.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; E.M.K., C.v.V., M.A.P., J.G., M.P.R., W.D., E.L., and A.F.d.V.: conception and design, data analysis and interpretation, final approval of manuscript; T.v.d.P.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

M.A.P., J.G., M.P.R., W.D., and E.L. are full time employees of TiGenix. T.V.D.P. received research funding for mouse studies with stem cells used in the study described in this article. The other authors indicated no disclosure to declare.

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