

# Comparison of Single and Repeated Dosing of Anti-Inflammatory Human Umbilical Cord Mesenchymal Stromal Cells in a Mouse Model of Polymicrobial Sepsis

**Barbara Fazekas**

National University of Ireland Galway <https://orcid.org/0000-0001-9771-6133>

**Senthilkumar Alagesan**

National University of Ireland Galway

**Luke Watson**

Orbsen Therapeutics Ltd.

**Olivia Ng**

National University of Ireland Galway

**Callum M. Conroy**

National University of Ireland Galway

**Cristina Català**

Institut d'Investigacions Biomediques August Pi i Sunyer

**Maria Velascode Andres**

Institut d'Investigacions Biomediques August Pi i Sunyer

**Neema Negi**

National University of Ireland Galway

**Jared Q. Gerlach**

National University of Ireland Galway

**Francisco Lozano**

Institut d'Investigacions Biomediques August Pi i Sunyer

**Stephen J. Elliman**

Orbsen Therapeutics Ltd.

**Matthew D. Griffin** (✉ [matthew.griffin@nuigalway.ie](mailto:matthew.griffin@nuigalway.ie))

National University of Ireland Galway <https://orcid.org/0000-0002-8701-8056>

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## Research Article

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# Abstract

Mesenchymal stromal cells (MSCs) ameliorate pre-clinical sepsis and sepsis-associated acute kidney injury (SA-AKI) but clinical trials of single-dose MSCs have not indicated robust efficacy. This study investigated immunomodulatory effects of a novel MSC product (CD362-selected human umbilical cord-derived MSCs [hUC-MSCs]) in mouse endotoxemia and polymicrobial sepsis models.

Initially, mice received intra-peritoneal (i.p.) lipopolysaccharide (LPS) followed by single i.p. doses of hUC-MSCs or vehicle. Next, mice underwent cecal ligation and puncture (CLP) followed by intravenous (i.v.) doses of hUC-MSCs at 4 hours or 4 and 28 hours. Analyses included serum/plasma assays of biochemical indices, inflammatory mediators and the AKI biomarker NGAL; multi-color flow cytometry of peritoneal macrophages (LPS) and intra-renal immune cell subpopulations (CLP) and histology/immunohistochemistry of kidney (CLP).

At 72 hours post-LPS injections, hUC-MSCs reduced serum inflammatory mediators and peritoneal macrophage M1/M2 ratio. Repeated, but not single, hUC-MSC doses administered at 48 hours post-CLP resulted in increased survival, lower serum concentrations of inflammatory mediators, lower plasma NGAL and reversal of sepsis-associated depletion of intra-renal T-cell and myeloid cell subpopulations. Hierarchical clustering analysis of all 48-hour serum/plasma analytes demonstrated partial co-clustering of repeated-dose hUC-MSC CLP animals with a Sham group but did not reveal a distinct signature of response to therapy. It was concluded that repeated doses of CD362-selected hUC-MSCs are required to modulate systemic and local immune/inflammatory events in polymicrobial sepsis and SA-AKI. Inter-individual variability and lack of effect of single dose MSC administration in the CLP model are consistent with observations to date from early-phase clinical trials.

## Introduction

Sepsis is characterised by life-threatening organ dysfunction due to a dysregulated host response to infection caused by bacterial, fungal, viral, and parasitic pathogens (1). Each year, sepsis affects nearly 50 million people worldwide, frequently leading to severe systemic consequences and organ injuries, including acute kidney injury (AKI), resulting in approximately 11 million deaths (2, 3). Mesenchymal stromal cells (MSC) are multipotent cells with extensive immune-modulatory properties that can be isolated from various tissues (4). It is now well recognised that MSC offer a potential disease-modulating therapy for sepsis and sepsis-associated AKI (SA-AKI) (5, 6). Several recent studies using mouse and rat cecal ligation and puncture (CLP), lipopolysaccharide (LPS) or faecal peritonitis models have shown that systemic administration of MSC has the potential to reduce inflammation, counteract bacterial infection and improve the repair of injured tissue in sepsis (7–11), including SA-AKI, (10, 12–15) by modulating the balance between pro-inflammatory and anti-inflammatory states.

A recent trial in healthy adults confirmed that preventative intravenous (i.v.) treatment with  $4 \times 10^6$  MSC/kg produces early immunomodulatory effects on the host response to LPS (16). Furthermore, administration

of allogeneic MSC to patients with septic shock (NCT01849237, NCT02421484, and NCT02328612) and sepsis-related acute respiratory distress syndrome (NCT01775774) demonstrated good safety and tolerability in Phase 1 clinical trials (16–19). Despite these encouraging results, Galstyan et al. also reported that a single dose of i.v. MSC did not prevent death from sepsis-related organ dysfunction, raising the possibility that additional doses may be necessary to derive meaningful clinical benefit (17). A recent non-sepsis, phase 2 trial (NCT01602328) in which single doses of allogeneic MSC were delivered intra-aortically to patients with sterile AKI following cardiopulmonary by-pass surgery also failed to demonstrate a beneficial effect on organ dysfunction and patient survival despite promising pre-clinical and phase 1 trial results (20). Whether repeated dosing during the early course of sepsis and other acute inflammatory syndromes could augment or extend the disease-modulating effects of MSC remains relatively under-investigated at pre-clinical and translational levels. Furthermore, incomplete knowledge of the mechanism of action, dose response and optimal clinical indices for MSC administration in sepsis limits the potential for designing successful trials (5, 21, 22).

In the current study, we performed pre-clinical investigation of the anti-inflammatory effects of CD362-selected human umbilical cord-derived MSC (hUC-MSC) in mouse models of sepsis. This surface marker-selected hUC-MSC is a novel therapeutic product that has demonstrated evidence of efficacy in rat models of bacterial pneumonia and sepsis when administered early after disease onset (23, 24). As a clinical-grade investigational medicinal product (IMP), hUC-MSC is currently undergoing phase I/II trial in patients with moderate to severe acute respiratory distress syndrome (ARDS) due to COVID-19 (NCT03042143). We aimed to demonstrate the anti-inflammatory potential of single doses of hUC-MSC in mouse models of LPS- and CLP-induced endotoxemia/polymicrobial sepsis (25, 26) and, in the latter, to determine whether a second administration of hUC-MSC during the early disease course resulted in greater or more frequent beneficial effects on systemic inflammation and organ-specific injury, exemplified by SA-AKI.

## Materials & Methods

*Cells:* Anti-CD362<sup>+</sup>-selected hUC-MSC were cultured from ethically-sourced human umbilical cord tissue obtained from Tissue solutions Ltd. (Glasgow, U.K). Primary isolation and expansion cultures of CD362<sup>+</sup> hUC-MSC was carried out as previously described (19, 23, 27). Cryopreserved vials ( $1 \times 10^7$  in 1mL) of anti-CD362<sup>+</sup>-selected hUC-MSC were thawed, transferred into 9mL of phosphate buffered saline (PBS). After live/dead analysis either via trypan blue dye or automated cell counter (NucleoCounter® NC-200™, Chemometec A/S, Denmark), the required numbers of cells were pelleted via centrifugation at 400xg for 5 minutes and the cells were re-suspended in 100ml of sterile saline for intra-peritoneal (i.p.) or i.v. injection.

*Animal Procedures:* All animal procedures were carried out under the license (no. 255/17) from the Animal Experimentation Ethical Committee, University of Barcelona and under authorisation (AE19125/P082 and AE19125/P066) from the Health Products Regulatory Authority, Ireland, and

approved by the NUI Galway Animal Care Research Ethics Committee. All procedures were performed in licensed animal facilities at NUI Galway and University of Barcelona.

For the LPS model of endotoxemia, 10-12 week-old male C57BL/6 mice from Charles River Ltd., Kent, UK were used. Mice were injected i.p. with 5µg/g LPS (LPS 0111:B4, catalogue no. L2630, Sigma Aldrich, UK) in 100ml of sterile saline. The mice were housed in groups of 3-5 mice/cage during the study in individually ventilated cages. Single i.p. injections of  $2.5 \times 10^5$  hUC-MSC or equal volumes of vehicle (sterile saline) were administered 4 hours after LPS injections. The animals were monitored every 4 hours until the end of the study using a distress score sheet and support measures according to a pre-determined protocol. Humane euthanasia was performed at the defined experimental end-point or earlier if animals exceeded the pre-defined severity score threshold. At the time of euthanasia, peritoneal exudates were collected for flow cytometry analysis by carefully flushing 5mL of sterile PBS into and out of the peritoneal cavity.

Cecal ligation and puncture (CLP) was performed on 8-12 week-old, male C57BL/6 mice (Charles River Ltd., UK). The mice received buprenorphine 0.1 mg/kg (Richter Pharma AG, Austria) subcutaneously 25-30 minutes before the procedure and were anesthetized with 1.8-2% isoflurane (with O<sub>2</sub> flow of 0.5L/min) at NUI Galway or with Anesketin (100mg/mL; Dechra Veterinary Products SLU, Spain) and Rampun (20 mg/mL; Bayer, Germany) at University of Barcelona. The lower half of the abdomen was shaved and cleaned with 4% chlorhexidine or povidone-iodine and incised 1cm vertically along the midline. The cecum was externalized and the distal 50% was ligated using 4.0-6.0M sutures. Cecal material was released by 'through and through' puncture with a 21-gauge needle and a drop of fecal matter was exuded before reinstating the cecum into the peritoneal cavity and suturing the muscle and skin closed. Sham-operated mice underwent an identical procedure, including opening the peritoneum and exposing the bowel, but without ligation and perforation of the cecum. Mice received 0.5mL of Gelofusine (Braun Melsungen AG, Germany) by i.p. instillation prior to wound closure. Post-operative support consisted of buprenorphine diluted given subcutaneously (s.c.) every 8-12 hours until the pre-determined end-points (48 or 72 hours for individual experiments). Administration of  $1 \times 10^6$  hUC-MSC or equivalent volumes of vehicle (sterile saline) was carried out i.v. via the tail vein at 4 hours or at 4 and 28 hours following CLP. Frequent monitoring and support was carried out according to an ethically-approved protocol. Humane euthanasia was performed at the defined experimental end-point or earlier if animals exceeded a pre-defined severity score threshold.

*Blood Sampling and Tissue Procurement:* Venous blood samples to a maximum volume of 20mL were drawn intermittently from tail and facial veins by aseptic technique using 25-21 gauge needles and were collected into heparin (VWR International, Dublin, Ireland)-containing tubes. A terminal blood sample was drawn by cardiac puncture at the time of euthanasia. Serum was collected in micro-tubes with serum gel and clotting activator (Sarstedt, Wexford, Ireland). Plasma and serum samples were prepared by centrifugation at 10,000xg for 10 minutes. Serum samples were stored at -80°C and subsequently analysed for biochemical parameters by NationWide Laboratories (Lancashire, UK). Spleen, lungs, kidneys and liver were dissected immediately after euthanasia.

*Multicolour flow cytometry:* For flow cytometry of mouse peritoneal macrophages (LPS model), 100mL of freshly-prepared peritoneal exudate cells re-suspended in FACS buffer (PBS, 2% fetal calf serum and 0.05% sodium azide) were labelled with the following combinations of fluorochrome-labelled antibodies at 4°C for 30 minutes: anti-Ly6C -PerCP-Cy<sup>™</sup>5.5 (clone-AL-21), anti-CD11b -APC-Cy7 (clone-M1/70), anti-CD68-PE (F1/11), anti-CD206-APC (MR5D3) from BD Pharmingen<sup>™</sup> (BD Bioscience, Berkshire, UK). Cell viability was analysed by SYTOX<sup>™</sup> blue dead cell stain (Thermo-Fisher Scientific, Dublin, Ireland) according to manufacturer's instructions. For flow cytometry of kidney cells, single-cell suspensions were prepared from freshly-dissected kidneys by collagenase/DNase digestion and were enriched for CD45<sup>+</sup> bone marrow-derived cells by magnetic column separation. Kidney cell suspensions were stained with panels of fluorochrome-coupled monoclonal antibodies. A detailed protocol for preparation and flow cytometry analysis of kidney cell suspensions is available in **Supplementary Methods**. For all flow cytometry analyses, labelled cells were washed and re-suspended in FACS buffer and immediately analysed on a FACS Canto II cytometer (BD Biosciences). Data files were subsequently analyzed using FlowJo v6 software (Ashland, OR, USA). Details and examples of the gating strategies used to define and enumerate specific immune cell subpopulations are provided in **Supplementary Table 1** and **Supplementary Figure 1**.

*Immunoassays:* Plasma neutrophil gelatinase-associated lipocalin (NGAL) concentration was quantified with the mouse Lipocalin-2/NGAL Duo-Set ELISA Development kit (R&D Systems, Minneapolis, MI, USA) according to the manufacturer's suggested protocol (details in **Supplementary Methods**). For multiplex quantification of cytokines and chemokines in serum, the Bio-Plex Pro mouse cytokine standard 23-plex assay (Bio-Rad, Accuscience) was used according the manufacturer's instructions. Samples were analysed on a Bioplex 200 multiplex ELISA system (Bio-Rad, Accuscience).

*Histology and Immunohistochemistry:* Kidneys were dissected and placed in 10% neutral buffered formalin for 24 hours at room temperature before being processed in a Leica Tissue Processor ASP300. Tissues were wax-embedded in a Leica EG1150 wax embedder fitted with EG1130 Cold Plate, and 5-µm sections were cut using a Leica RM12235 microtome. Sections were transferred to Superfrost Plus microscope slides (Fisher Scientific Ireland) and dried overnight at room temperature. Histologic staining of sections for hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) were performed using standard protocols (details provided in **Supplementary Methods**).

For immunohistochemical staining, kidney tissue sections were dewaxed in xylene then hydrated in graded ethanol solutions. Heat-mediated antigen retrieval was performed with Tris/EDTA buffer pH 9.0 at 90°C for 30 minutes. Sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub>, and incubated with avidin-biotin blocking solution (SP-2001, Vector Laboratories, Burlingame, CA, USA) to reduce nonspecific staining. Next, the slides were incubated for one hour at 4°C with rabbit anti-mouse NGAL monoclonal antibody (1:2000; ab216462, Abcam, Cambridge, UK), followed by incubation with biotinylated goat anti-rabbit IgG secondary antibody (BA-1000). For colorization, an avidin-biotin horseradish peroxidase complex (Vector Laboratories) and 3,3-diaminobenzidine substrate solution (Sigma-Aldrich) were applied to the slides at room temperature for 30 and 5 minutes respectively and the slides were counterstained with Gill no. 3

hematoxylin (Sigma-Aldrich) for 30 seconds. Negative control slides were prepared by staining under identical conditions but without adding the primary antibody.

*Semi-quantitative Scoring of Kidney Tissue Sections:* Stained sections of kidney were analyzed in blinded fashion by light microscopy at 40X magnification using an Olympus BX43 bright-field microscope (Olympus, Center Valley, PA) and with IS TCapture software (Tucsen Photonics Co., Fujian, China). For each kidney, twenty non-overlapping fields of a stained section were captured, and the positively stained area was scored by a blinded observer for (A) tubular dilatation, cast and necrosis (PAS) and (B) NGAL expression (28) Scoring was carried out on a 0-4 semi-quantitative scoring scale (details in **Supplementary Methods**). Mean scores were calculated for each individual kidney, and final results were expressed as group means  $\pm$  SD.

*Statistical Analysis:* Results were expressed as means  $\pm$  SD, and differences between conditions were tested statistically by ANOVA and post-hoc tests where indicated using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Significance was assigned at  $p < 0.05$ . Unsupervised hierarchical clustering and the generation of the corresponding heat map were performed using the Morpheus online visualization and analysis suite (Broad Institute, Cambridge, MA, <https://software.broadinstitute.org/morpheus>). All data, except gamma glutamyl-transpeptidase (Gamma GT) and albumin-globulin ratio (Albumin:Globulin), were subjected to log<sub>2</sub> transformation in Excel (version 2013, Microsoft, Redmond, WA), prior to clustering. The hierarchical relationship of data patterns for all animals was established by average linkage, Euclidean distance method.

## Results

*A single dose of hUC-MSC modulated inflammatory response to LPS in mice:* To initially establish a modulatory effect of hUC-MSC on acute systemic inflammatory response in mice, the LPS endotoxemia model was used. As shown in **Figure 1A**, i.p. administration of  $2.5 \times 10^5$  hUC-MSC 4 hours after LPS injection was associated with higher 72-hour survival compared to a control group that received LPS followed by i.p. saline (70% vs. 40% survival). However, the difference did not reach statistical significance and the average body weight losses and total distress scores over 72 hours were closely comparable for the two groups (**Figure 1B&C**). Despite the limited effects on physical determinants of acute illness, multiplexed analysis of soluble inflammatory mediators in serum of animals that survived to 72 hours revealed significant reductions in hUC-MSC recipients in comparison to the saline group (**Figure 1D**). Notably, circulating concentrations of key mediators of innate (e.g. IL-1b, IL-6, GM-CSF, TNFa, IL-10, MCP-1, MIP-1b) and Th1- and Th2-type adaptive immune responses (e.g. IL-2, IFNg, IL-12, IL-3, IL-4, IL-5, IL-13, IL-9) were lower in hUC-MSC-treated compared to saline-treated animals. Concentrations of the Th17-type mediator IL-17, the eosinophil chemoattractant, eotaxin and the chemokine CCL5 were unaffected by hUC-MSC administration (data not shown). To assess the immunomodulatory effects of hUC-MSC at the site of delivery, CD11b<sup>+</sup>/Ly6C<sup>-</sup> peritoneal macrophages from surviving animals were analysed by flow cytometry for surface expression levels of the M1 and M2 markers CD68 and CD206. As shown in **Figure 1E**, peritoneal macrophages from hUC-MSC-treated

animals had lower CD68 surface expression and lower CD68/CD206 ratio compared to those from saline-treated animals. Overall, these results demonstrated that a single dose of hUC-MSC had distinct anti-inflammatory and immune-modulatory effects in a simplified sepsis-like model with limited influence on overall disease severity.

*Repeated doses of hUC-MSC modified survival and systemic inflammation following CLP-induced polymicrobial sepsis in mice:* To assess (i) whether the anti-inflammatory effects of hUC-MSC could be reproduced in a more clinically-relevant model and route of administration and (ii) whether a repeat dose of cells early in the disease course could more potently modulate the course of sepsis, experiments were subsequently performed in the mouse CLP model. Groups of animals underwent CLP or sham procedures and received i.v. injections of  $1 \times 10^6$  hUC-MSC or equivalent volumes of saline at 4 hours only or at 4 and 28 hours after CLP. In the first such experiment, survival over a prolonged period (7 days) was determined (**Figure 2A**). As shown, CLP animals administered saline alone at both time points had 40% 7-day survival compared to 100% for sham controls. Seven-day survival of groups that received hUC-MSC at 4 hours only (single dose) or at 4 and 28 hours (double dose) did not differ significantly from that of the untreated group. It was noted, however, that mortality was delayed in both hUC-MSC-treated groups, being lower than that of untreated CLP animals at 48 and 96 hours. To explore this further, a second study with a duration of 48 hours was performed using the same groupings and group sizes. As shown in **Table 1**, when the 48-hour survivals of the combined total animals for each of the 4 groups from the 2 experiments were compared, those that received two doses of hUC-MSC had 95% survival compared to 75% and 74% respectively for untreated and single dose-treated groups. Overall, the survival analysis following CLP was consistent with a modest beneficial effect of repeated early dosing of anti-inflammatory hUC-MSC.

To investigate further, serum samples collected from surviving animals at the end-point (48 hours post-CLP) of the second experiment were analyzed by multiplex assay for soluble mediators of innate and adaptive immune responses (**Figure 2B**). As shown, serum concentrations of the majority of these cytokines and chemokines were significantly elevated in untreated CLP compared to sham animals. As with the LPS model, serum concentrations of IL-17, eotaxin and CCL5 were unaffected by CLP (data not shown). With the exception of IL-12p70 and IFN $\gamma$ , all CLP-induced mediators were also significantly elevated in the single-dose hUC-MSC-treated group. For the double-dose hUC-MSC group, however, multiple innate immune mediators (IL-1a, IL-1b, IL-6, IL-10, G-CSF, KC, MCP-1, MIP-1a, MIP-1b) and some Th1- and Th2-type adaptive immune mediators (IFN $\gamma$ , IL-2, IL-3, IL-9) did not differ significantly from the sham group. The findings supported a conclusion that repeated dosing of hUC-MSC is necessary for detectable modulation of systemic inflammation 48 hours after onset of polymicrobial sepsis.

*The CLP model was not associated with multi-organ failure:* To determine whether the CLP model was associated with multi-organ failure that may have been modulated by single- and/or double-dose hUC-MSC administration, biochemical analyses reflecting liver, kidney and pancreas function were performed on 48-hour serum samples collected at the termination of the second experiment (**Figure 3**). As shown, there were trends for individual group results consistent with adverse changes in liver and pancreas

function tests (AST, albumin, Amylase, ALT, GGT) in CLP compared to sham groups. In contrast, apart from a mild increase in urea in one group, serum markers of kidney function (creatinine, urea, potassium) were not different among the groups. These results indicated that, while the CLP model we established resulted in systemic inflammation and reduced survival, multi-organ failure was not present at the time and anti-inflammatory effects of repeated hUC-MSC administration were observed.

*Repeated dosing of hUC-MSC modulated the severity of acute kidney injury following CLP-induced polymicrobial sepsis in mice:* To more clearly determine whether the anti-inflammatory effect of repeated hUC-MSC administration was associated with end-organ protection, we focussed on the kidneys. Additional analyses of AKI were performed on 48-hour samples from each group of the second CLP experiment. Neutrophil gelatinase-associated lipocalin-2 is a biomarker that increases rapidly in blood and urine following the onset of sterile AKI as well as SA-AKI (29, 30). As shown in **Figure 4A**, plasma NGAL concentrations were markedly increased in CLP compared to sham control animals at 48 hours. In the double-, but not the single-dose, hUC-MSC group, however, the average plasma NGAL of animals surviving to 48 hours was lower than that of the untreated CLP group. At the same time-point, the intensity of renal tubular staining for NGAL, determined by immunohistochemical staining of kidney tissue sections, was also markedly increased in CLP compared to sham controls (**Figure 4B&E**). Although not reaching statistical significance, there was a trend towards lower renal tubular NGAL staining among the double-dose hUC-MSC group compared to untreated and single-dose hUC-MSC groups. To support the conclusion that plasma NGAL reflected severity of AKI rather than being a non-specific marker of sepsis-associated inflammation, there was a significant correlation between plasma NGAL and renal tubular NGAL staining intensity at 48 hours among all CLP animals (**Figure 4C**). A less sensitive analysis of acute renal inflammatory injury (semi-quantitative scoring of PAS-stained tissue sections for tubular damage and interstitial cell infiltrates) failed to demonstrate clear differences among the sham and CLP groups (**Figure 4D&E**).

In kidney cell suspensions from same experimental groups, magnetic enrichment and multi-colour flow cytometry were used to quantify total (CD45<sup>+</sup>) intra-renal immune cells as well as several myeloid and lymphoid immune cell subpopulations (**Figure 5**). As shown, kidneys of the surviving animals from the untreated and single-dose hUC-MSC CLP groups demonstrated reduced numbers of total immune cells and of all myeloid and lymphoid subpopulations compared to the sham controls. For the double-dose hUC-MSC-group, however, intra-renal numbers of total immune cells as well as of CD4<sup>+</sup> T-cells, CD4<sup>-</sup>/CD8<sup>-</sup> (double negative) T-cells, neutrophils and Ly6C<sup>-</sup>/F4/80<sup>+</sup> macrophages did not differ significantly from those of sham controls. These observations were consistent with sepsis-associated depletion of intra-renal myeloid and lymphoid immune cell populations at the time-point studied that was partially reversed by double- but not single-dose i.v. hUC-MSC. Overall, the results for analyses focussed on the kidneys at 48 hours after onset of polymicrobial sepsis, suggested that, despite the inconsistent rise in urea/creatinine and overt acute tubular necrosis, there was a substantial acute injury response and intra-renal immune cell depletion. Furthermore, repeated i.v. dosing of hUC-MSC was associated with evidence of amelioration of this SA-AKI.

*Cluster analysis of multiple blood analytes reveals response trends and intra-group heterogeneity following repeated dosing of hUC-MSc in CLP.* In keeping with the complex nature of polymicrobial sepsis, many of the analyses shown in **Figures 2-5** for untreated, single- and double-dose hUC-MSc-treated CLP groups were associated with high intra-group variability and with trends toward beneficial effects of repeated dosing or non-significant differences to the sham control group. To better understand whether these results represented evidence of a specific hUC-MSc-associated “responder profile” among that experimental group, an unsupervised hierarchical clustering analysis was performed of all factors quantified in blood of surviving animals at the 48-h time-point after initiation of CLP (**Figure 6**). As shown, the cluster containing all 5 of the sham group animals was divided from the rest of the animals by the greatest Euclidean distance and this same cluster also included 2/10 double-dose i.v. hUC-MSc-treated CLP animals. Other clusters contained mixed populations from each of the 3 CLP groups. Overall, while suggesting that animals treated with two sequential i.v. doses of hUC-MSc were more likely than those from other groups to resemble non-septic animals, this analysis highlighted the wide variability in systemic markers of sepsis for the CLP model and did not indicate a distinct signature of disease modulation following hUC-MSc administration.

## Discussion

Sepsis and its frequent complication SA-AKI are major public health challenges due to the continued lack of effective treatments and disappointing results from late-stage clinical trials (2, 3). Several recent pre-clinical studies have reported results indicating that MSC of various sources and their products have positive effects on disease severity and survival in models of sepsis and AKI (7, 8, 11–15). Nonetheless, the clinical benefits of MSC in sepsis and SA-AKI remain unproven with only limited data available from human patients (5). To date, clinical trials have documented that single-dose i.v. MSC infusion in the setting of LPS administration, sepsis and ARDS is safe and feasible, (16–19) but does not overtly reduce death from sepsis-related organ failure (17). In this study, we compared the effects of single- and double-dose i.v. administration of a distinctive human MSC therapeutic product (CD362-enriched hUC-MSc) in the mouse CLP model of polymicrobial sepsis. Initially, in keeping with recently-reported results for this same MSC product in rat models of bacterial pneumonia and sepsis (23, 24), we confirmed the potential for early administration of hUC-MSc to modulate LPS-induced systemic inflammation in mice. In a mouse CLP model of polymicrobial sepsis, however, our results indicated that single doses of hUC-MSc administered i.v. 4 hours following induction of fecal peritonitis exerted no clear beneficial effect on systemic inflammation or organ-specific (renal) tissue injury. In contrast, administration of a second i.v. dose of hUC-MSc 24 hours later resulted in multiple signals of ameliorated inflammation and renal injury by 48 hours following onset of sepsis. Despite this, a survival benefit for the double-dose regimen was not definitively demonstrated. Our results highlight the importance of performing *in vivo* studies of MSC therapeutic products in multiple models and of reporting both positive and negative pre-clinical outcomes in order to better inform clinical translation and trial design. Taken together, our results also provide evidence that the limited or absent benefit of early, single-dose regimens of anti-inflammatory MSC products in sepsis trials may be at least partially overcome by repeated dosing.

In the mouse LPS model, a single, early dose of hUC-MSC modulated systemic levels of both innate and adaptive inflammatory mediators for at least 72 hours, albeit without an overt effect on severity scores and survival. Phenotypic analysis of peritoneal macrophages provided evidence that hUC-MSC administration was associated with a predicted local immune modulatory effect – skewing of macrophages toward M2 polarization, which has been linked to resolution of inflammatory injury and promotion of tissue repair (31, 32) and is likely to be mediated by MSC cross-talk with resident myeloid cells (33). Indeed, recent studies have reported that therapeutic immunomodulation by MSC in the setting of sepsis may be dependent on their phagocytosis by myeloid cells (mononuclear phagocytes) which then undergo alternative activation resulting in the production of IL-10 and other paracrine anti-inflammatory mediators (7, 27, 34). Following confirmation of hUC-MSC biological activity in mice, the mouse CLP model allowed for characterization of anti-inflammatory effects related to repeated dosing and quantification of kidney-specific effects of hUC-MSC in the setting of polymicrobial sepsis and SA-AKI. In our hands, this model was associated with moderately severe sepsis (approximately 70%, 50% and 40% survival at 48, 96 and 168 hours respectively in untreated animals) without overt liver and kidney failure. Nonetheless, analysis of serum/plasma and kidney tissue at 48 hours indicated significant systemic inflammation and renal injury response. These latter analyses provided the clearest evidence for a modulatory effect of the double-dose hUC-MSC regimen on the severity of polymicrobial sepsis when compared to single-dose administration which, in contrast to results recently reported in a rat CLP model (24), was indistinguishable from the saline-treated CLP group across all indices examined. In particular, the clinically-relevant AKI biomarker, NGAL, proved to be a valuable discriminator of sepsis severity and treatment effect in this model and may be an important biomarker for future clinical trials of cell therapies in sepsis and SA-AKI (29, 35).

Our results for the survival benefit of i.v. MSC in small animal models of sepsis are modest compared to some other reports (36, 37) but, notably, are in keeping with observed effects of allogeneic MSCs in human clinical trials (5, 16–18, 38). In this regard, we would highlight the potential role of publication bias – specifically, selective publication of results reflecting positive effects – as an important driver of unrealistic expectations for the efficacy of single-dose MSC regimens and other advanced therapies in the earliest stages of clinical translation (39). Indeed, Sun et al., in a recent meta-analysis of 29 animal studies of the efficacy of MSC therapies in sepsis, detected significant publication bias and lack of clarity in regard to optimal cell dose among these pre-clinical reports (40). Nevertheless, we also report distinctive positive findings that may help to advance translational goals for UC-MSCs in sepsis or other systemic and organ-specific inflammatory diseases, such as AKI, liver or respiratory diseases. In both models, these molecular changes indicate complex interaction of hUC-MSC with the Th1 and Th2 immune response. Such effects on T-effector cell activation T helper phenotype balance may play a key role in modulation of the acute phase of sepsis as indiscriminate, dysregulated activation of immune effectors resulting in high levels of circulating cytokines contribute to multiple-organ failure (41) and, in the case of T-cells, may be followed by widespread apoptosis and subsequent immune deficiencies (42). It has also been shown that MSC administration may decrease localized tissue inflammation by regulating cytokine homeostasis and decreasing the traffic of immune cells into organs (41). In keeping

with this, our quantitative analysis of a range of intra-renal immune cell populations 48 hours following CLP-induced sepsis revealed sepsis-induced deficiencies affecting both innate and adaptive effectors, including loss of double-negative T cells which have been recently reported to be early responders to AKI (43). Notably, for double- but not single-dose hUC-MSC-treated animals, there was evidence of reversion of intra-renal immune cell depletion.

Given the inherent variability we and others observe with individual outcomes for the mouse CLP model, we hypothesized that hierarchical clustering analysis of quantitative readouts for a range of circulating inflammation-related mediators at 48 hours would help to better define a distinctive “responder signature” among MSC-treated animals. Although 60% of the double-dose group clustered in a pattern that separated them from the majority of the single dose- and untreated groups and was closer to the sham group, no very clear multi-analyte profile of disease modulation could be identified. A principal component analysis (PCA) approach yielded a similar conclusion (data not shown). It should be acknowledged that data from animals that failed to survive to 48 hours could not be acquired and it is possible that analyses at one or more earlier time-points could provide a more distinctive responder/non-responder separation. Nonetheless, this analysis highlights the complexity of inter-individual variation that is inherent to animal models of sepsis and cell therapies even with close attention to principles of good experimental design (44, 45) and that reflects similar challenges faced in the clinical application of novel therapies to sepsis (18, 20, 38, 46, 47).

## Abbreviations

MSC: Mesenchymal stromal cells

SA-AKI: sepsis-associated acute kidney injury

hUC-MSC: human umbilical cord-derived MSC

LPS: lipopolysaccharide

CLP: caecal ligation and puncture

Intravenous: i.v.

Intraperitoneal: i.p.

NGAL: neutrophil gelatinase-associated lipocalin

## Declarations

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**Competing interests:** BF, NN, JQG CC, MVdA and MDG have no potential conflicts of interest to declare. SA, LW, ON, CMC and SJE are paid employees of Orbsen Therapeutics Ltd., a cell therapy company that is developing CD362-selected human umbilical cord-derived MSCs for clinical applications. FL is founder and ad-honorem scientific advisor of Sepsia Therapeutics.

**Ethics approval:** All animal procedures were carried out under the license (no. 255/17) from the Animal Experimentation Ethical Committee, University of Barcelona and under authorisation (AE19125/P082 and AE19125/P066) from the Health Products Regulatory Authority, Ireland, and approved by the NUI Galway Animal Care Research Ethics Committee. All procedures were performed in licensed animal facilities at NUI Galway and University of Barcelona.

**Consent to participate:** Not applicable.

**Consent for publication:** “The pre-print version of this article is present on <https://www.researchsquare.com/article/rs-547210/v1>. This article is not published nor is under publication elsewhere.”

**Availability of data and material:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Authors' contributions:** BF took part of conception and design, performing experiments, data analysis and interpretation, provided financial support and drafted the manuscript. SA took part of conception and design, performing experiments, data analysis and interpretation. LW, ON and CM performed experiments. YG took part of conception and design. NN gave technical assistance. JQG performed data analysis and interpretation. CC and MVA performed experiments and data analysis. FL provided study material. SJE

and MG took part of conception and design, data analysis and interpretation and provided financial support. All authors read and approved the final manuscript.

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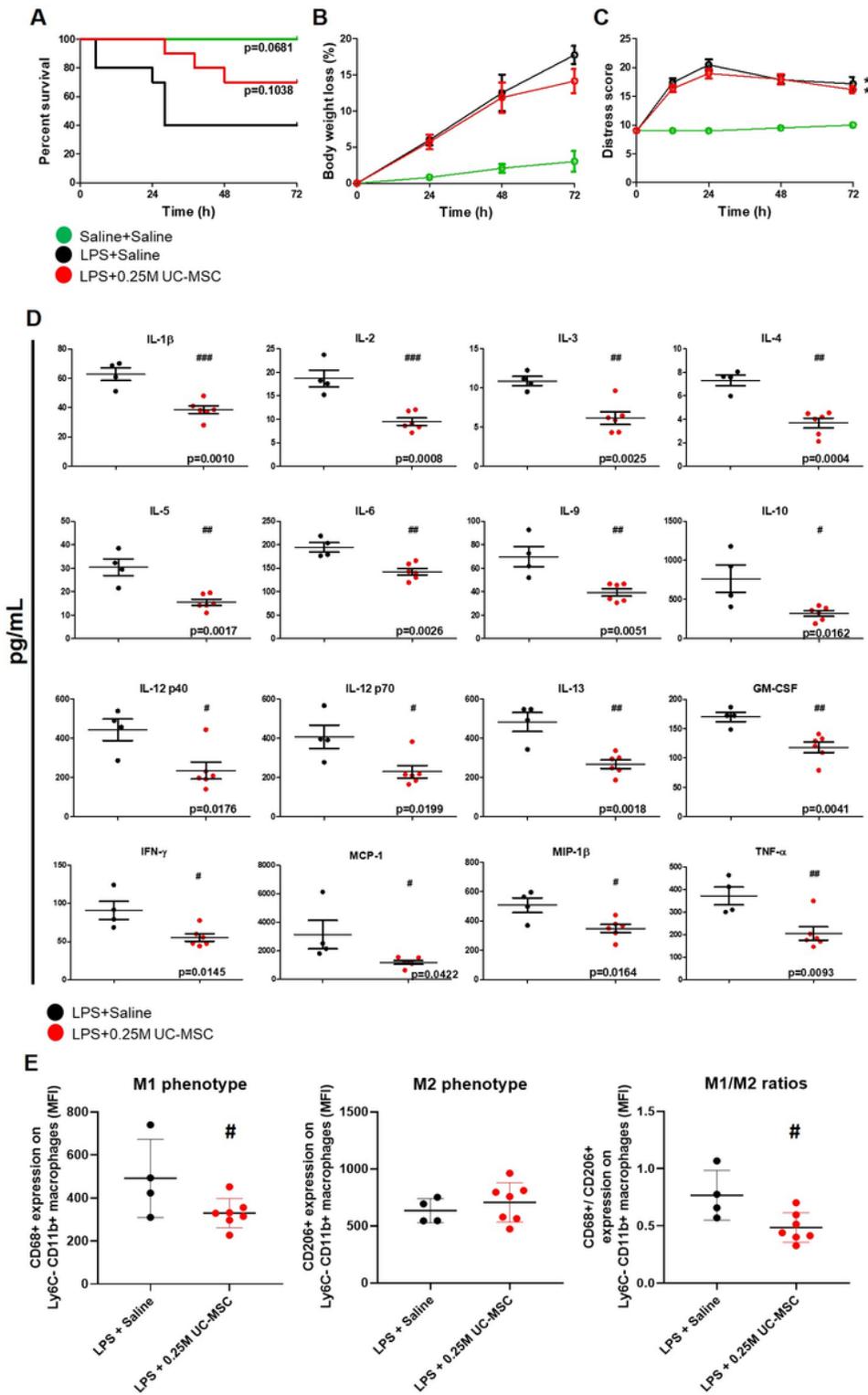
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## Tables

**Table 1:** Survival to 48 hours following CLP in combined groups from two experiments comparing effects of saline, single-dose hUC-MSC and double-dose hUC-MSC

Experimental Group (Procedure/IV inject. at 4 + 28 hrs)	Time-point (No. of animals surviving)					
	0 hrs	12 hrs	24 hrs	36 hrs	48 hrs	% Survival to 48 hrs
SHAM / Saline + Saline	14	14	14	14	14	<b>100</b>
CLP / Saline + Saline	20	20	20	20	15	<b>75</b>
CLP / hUC-MSC + Saline (single-dose)	19	19	19	19	14	<b>74</b>
CLP / hUC-MSC + hUC-MSC (Double-dose)	20	20	20	20	19	<b>95</b>

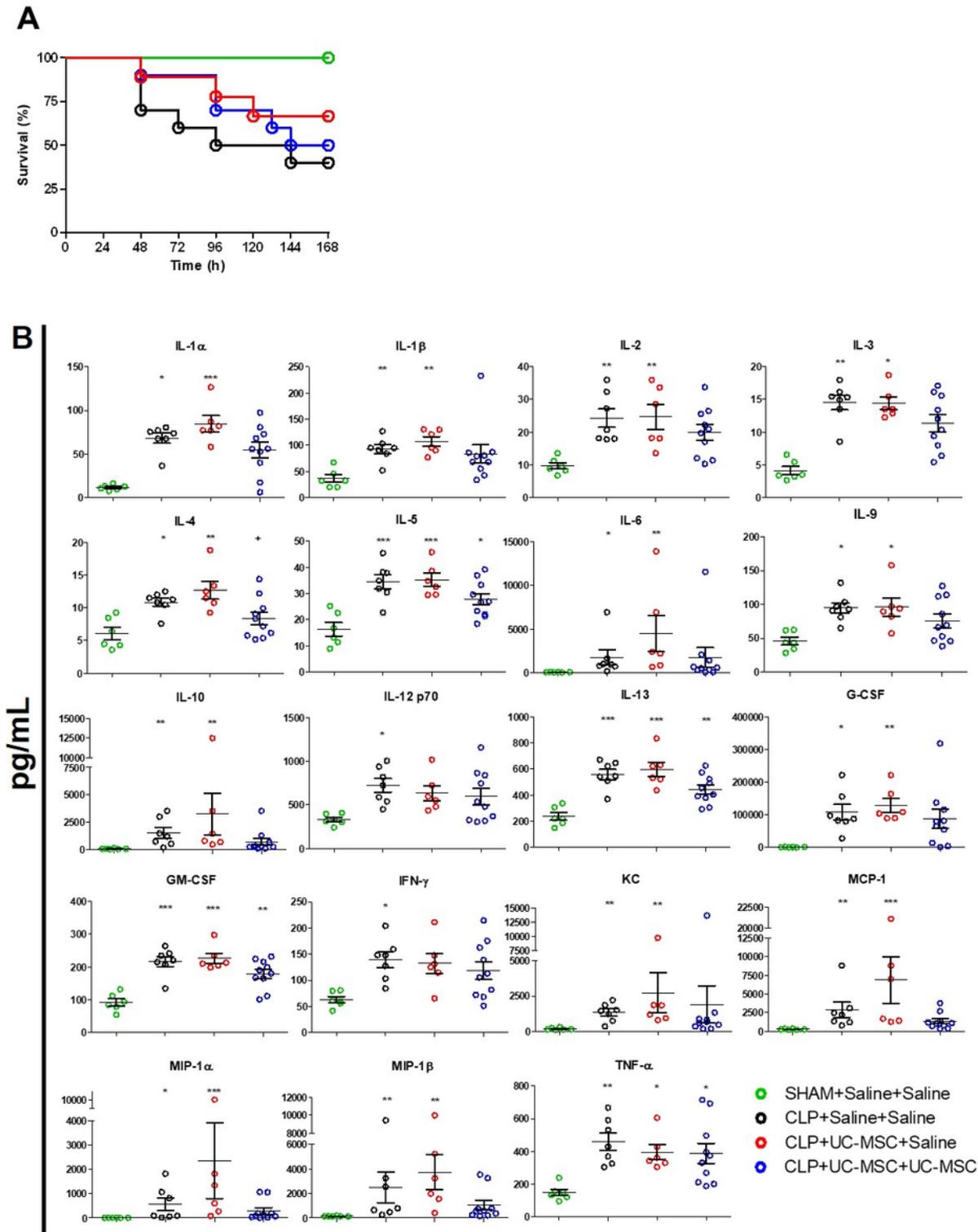
## Figures



**Figure 1**

Effects of single-dose intra-peritoneal hUC-MSC administration on survival, systemic inflammatory response and peritoneal macrophage polarization following LPS: A-C) Survival curve, percentage of body weight loss data and distress score of groups of mice over 72 hours following intra-peritoneal injection of saline alone (SHAM+Saline [n=4]), LPS and saline (LPS+Saline [n=10]) or LPS and 0.25x10<sup>6</sup> hUC-MSC (LPS+0.25M UC-MSC [n=10]). D) Serum concentrations of inflammatory cytokines and chemokines in

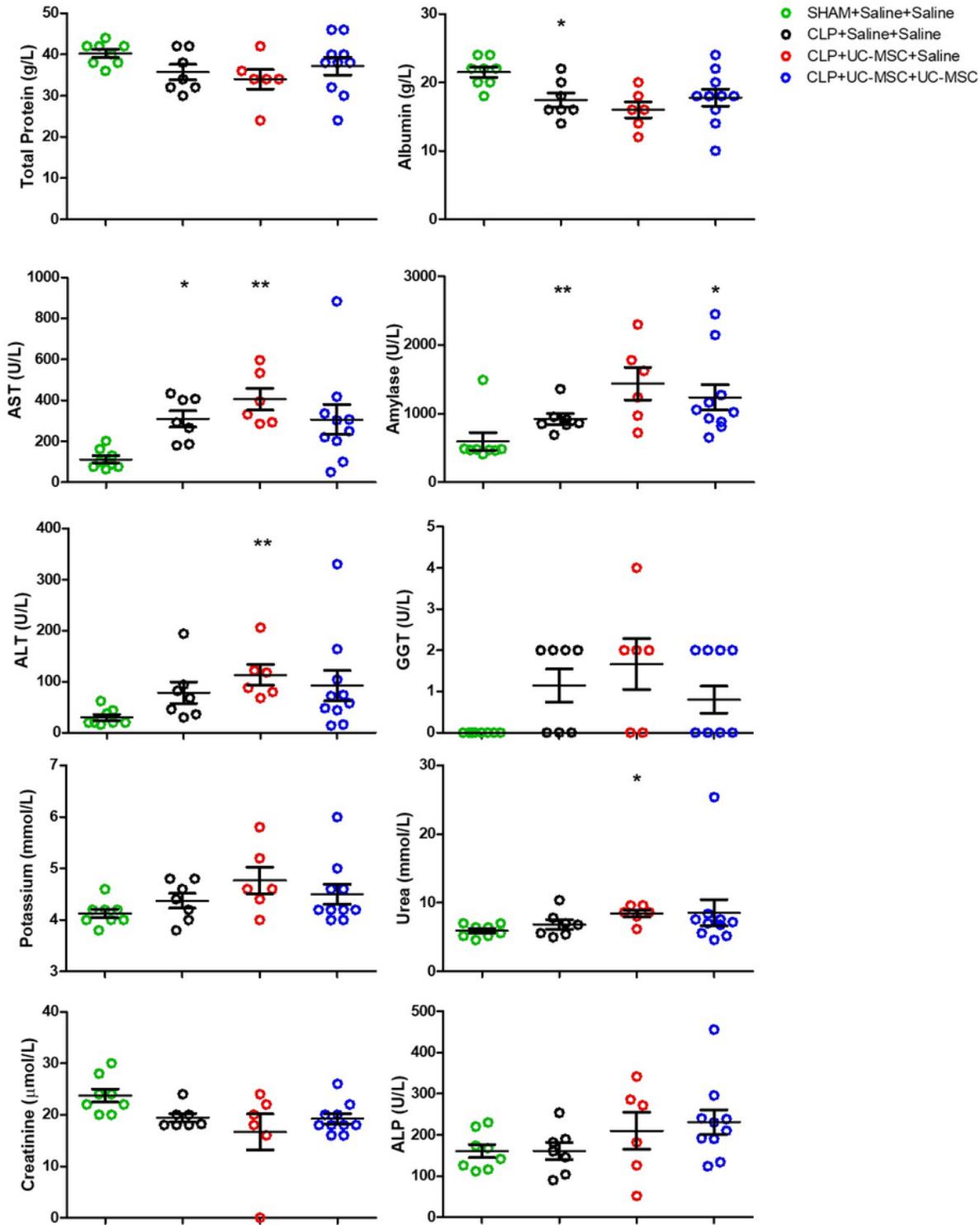
mice surviving to 72 hours following LPS and saline (LPS+Saline [n=5]) or LPS and 0.25x10<sup>6</sup> hUC-MSC (LPS+0.25M UC-MSC [n=5]). E) Mean fluorescence intensities (MFI) of Ly6C-/CD11b+ peritoneal macrophages for M1 marker CD68 (left) and M2 marker CD206 (middle) as well as CD68/CD206 ratios for mice surviving to 72 hours following LPS and saline (LPS+Saline [n=4]) or LPS and 0.25x10<sup>6</sup> hUC-MSC (LPS+0.25M UC-MSC [n=5]). Statistical analysis: A) Long-rank test of Kaplan-Meier survival curve, p values indicate statistical comparison with LPS+Saline group. B) Repeated measures ANOVA with Bonferroni post-test. C) Friedman test with Dunn's post-test, 95% confidence interval. D) Non-Gaussian distribution: Unpaired T test, p<0.05. Gaussian distribution: Non-parametric Mann-Whitney test p<0.05. E) Unpaired t-test. \*Significantly different from SHAM groups (p<0.05). #Significantly different from LPS+Saline group (#p<0.05, ##p<0.01, ###p<0.001).



**Figure 2**

Effects of single- and double-dose intravenous hUC-MSC on survival and systemic inflammatory responses following CLP-induced polymicrobial sepsis: A) Survival curves for groups of mice following sham procedure with saline injections at 4 and 28 hours (SHAM+Saline+Saline, [n=5]), CLP procedure with saline injections at 4 and 28 hours (CLP+Saline+Saline, [n=10]), CLP procedure with 1x10<sup>6</sup> hUC-MSC at 4 hours and saline injection at 28 hours (CLP+UC-MSC+Saline, [n=9]) or CLP procedure with 1x10<sup>6</sup>

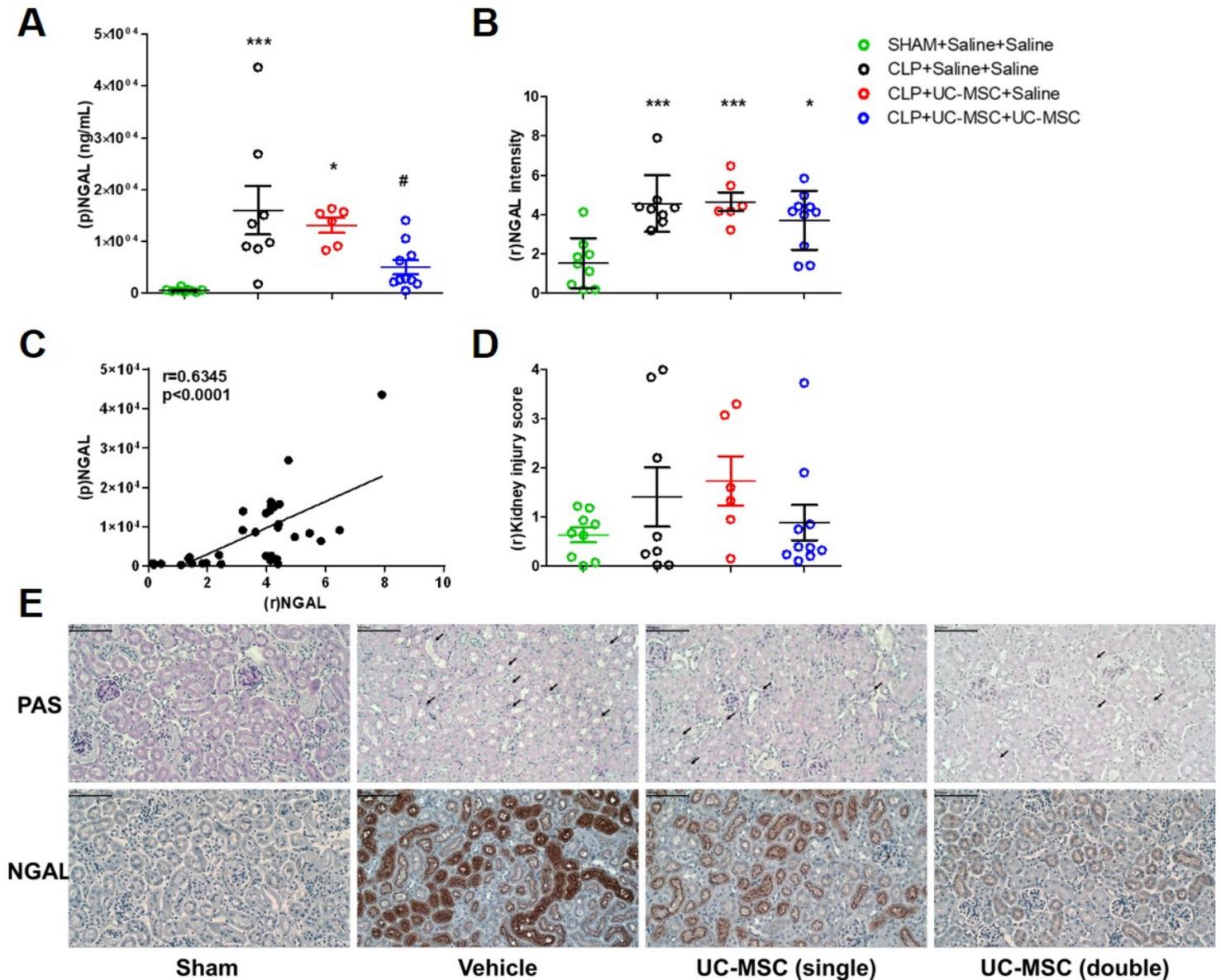
hUC-MSC at 4 hours and at 28 hours (CLP+UC-MSC+UC-MSC, [n=9]). Animals were monitored for 168 hours following procedures. B) Serum concentrations of inflammatory cytokines and chemokines at 48 hours following the same combinations of procedures and treatments. Groups sizes for the multiplex assay analyses were SHAM+Saline+Saline (n=6), CLP+Saline+Saline (n=7) and CLP+UC-MSC+Saline (n=6) or CLP+UN-MSC+UC-MSC (n=10). Statistical analysis: A) Long-rank T test of Kaplan Meier survival curve B) Non-parametric Kruskal-Wallis test with Dunn's multiple comparison test,  $p < 0.05$ . Follows Gaussian distribution: One-way ANOVA with Bonferroni multiple comparison post-test and with 95% confidence interval. \*Significantly different from SHAM group ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ).



**Figure 3**

Results of serum biochemistry assays at 48 hours following Sham procedure or CLP procedure with injections of saline or hUC-MSC: Results for serum biochemical assays at 48 hours post-procedure are shown for the following groups: sham procedure with saline injections at 4 and 28 hours (SHAM+Saline+Saline, [n=8]), CLP procedure with saline injections at 4 and 28 hours (CLP+Saline+Saline, [n=7]), CLP procedure with 1x10<sup>6</sup> hUC-MSC at 4 hours and saline injection at 28 hours (CLP+UC-

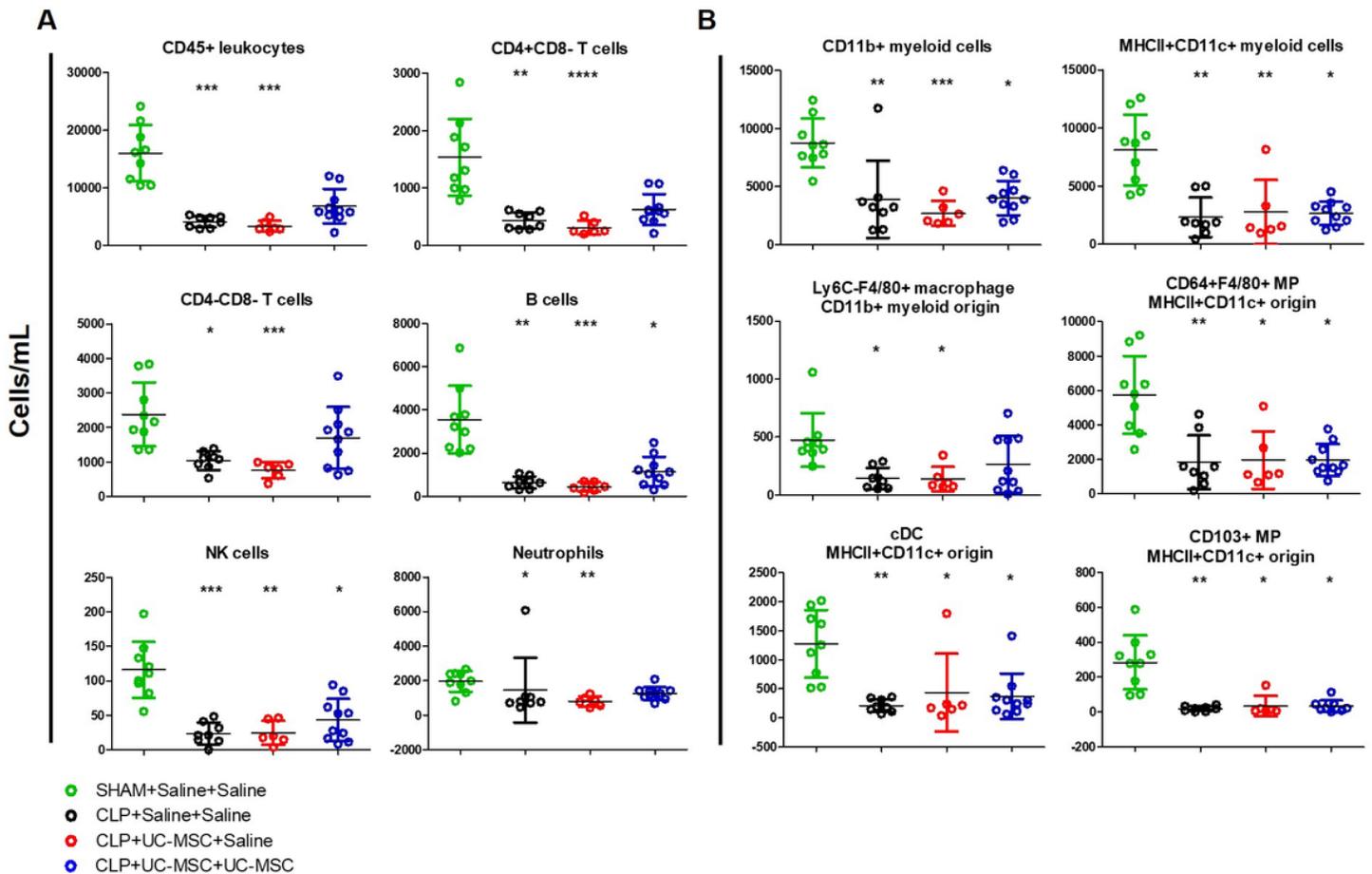
MSC+Saline, [n=6]) or CLP procedure with  $1 \times 10^6$  hUC-MSC at 4 hours and at 28 hours (CLP+UC-MSC+UC-MSC, [n=10]). Statistical analysis: Non-Gaussian distribution: Non-parametric Kruskal-Wallis test with Dunn's multiple comparison test,  $p < 0.05$ . Gaussian distribution: One-way ANOVA with Bonferroni multiple comparison post-test and with 95% confidence interval. \*Significantly different from SHAM group ( $*p < 0.05$ ,  $**p < 0.01$ ). Abbreviations: AST=Aspartate Aminotransferase; ALT=Alanine Aminotransferase; GGT=Gamma-Glutamyl Transferase; ALP=Alkaline Phosphatase.



**Figure 4**

Markers of acute kidney injury 48 hours following Sham or CLP procedures with saline, single-dose hUC-MSC and double-dose hUC-MSC: A) Plasma (p)NGAL concentrations. B) Semi-quantitative scoring of renal tubular (r)NGAL staining intensity in kidney sections C) Scatter plot illustrating the correlation between 48-hour (p)NGAL and (r)NGAL for individual animals from all CLP groups. D) Total kidney injury scores, representing the sum of individual semi-quantitative scores for tubular dilatation, cast formation

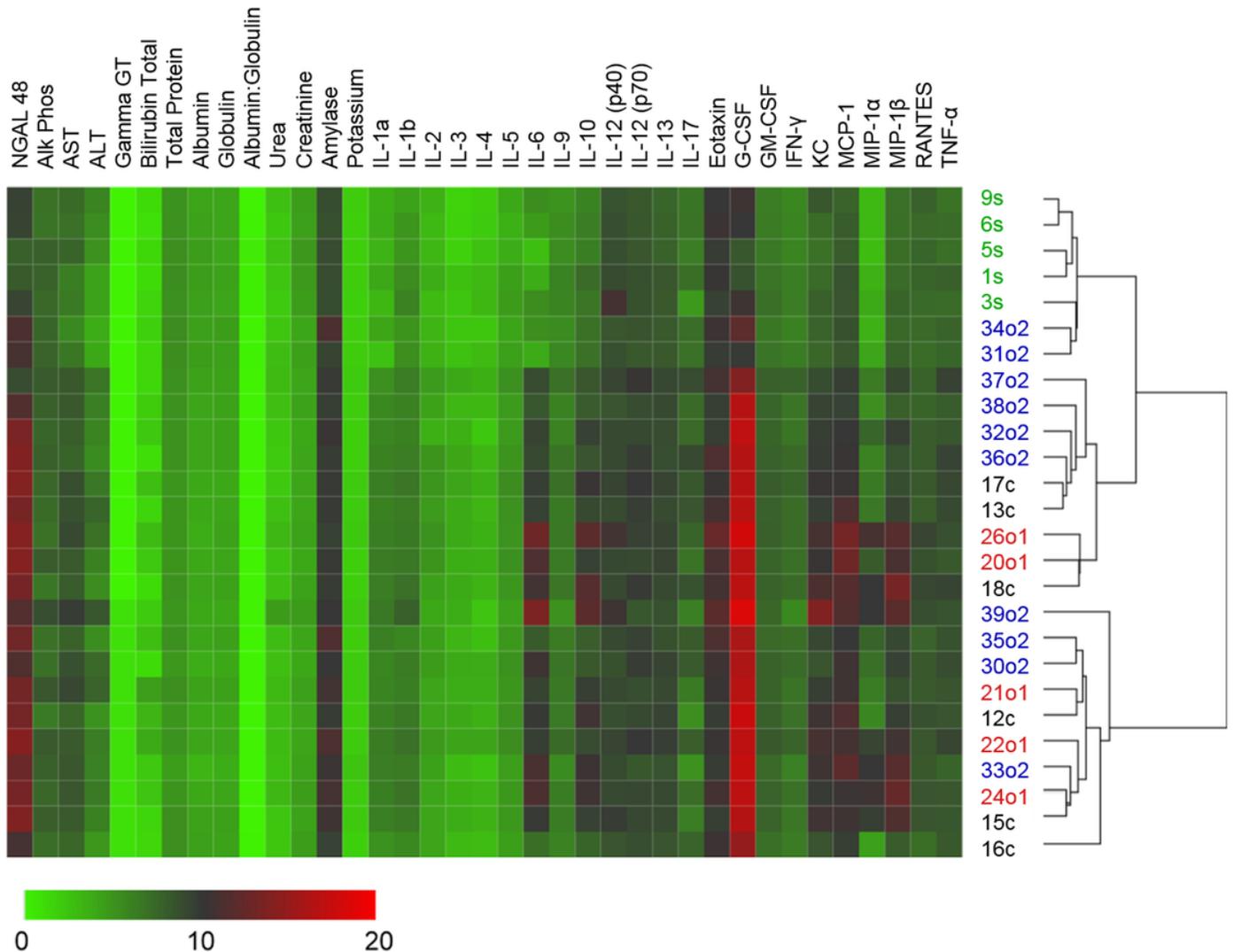
and necrosis in PAS-stained 48-hour kidney tissue sections. E) Representative examples of PAS and NGAL staining of 48-hour kidney sections. Arrows show tubular dilatation, cast and necrosis. NGAL expression showed peritubular appearance in the cortex of the kidney. Magnification 10X. Bar=100µm. Group sizes: SHAM+Saline+Saline (n=9), CLP+Saline+Saline (n=8), CLP+UC-MSC+Saline (n=6) and CLP+UC-MSC+UC-MSC (n=10). Statistical analysis: (A-B) One-way ANOVA with Bonferroni multiple comparison post-test and with 95% confidence interval (C) Two-tailed Spearman correlative testing with regression line (D) Kruskal-Wallis non-parametric test, Dunn's multiple comparison test,  $p < 0.05$ . \*Significantly different from SHAM group ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ). #Significantly different from CLP group ( $p < 0.05$ ).



**Figure 5**

Flow cytometric analysis of intra-renal immune cell subpopulations at 48 hours following Sham or CLP procedures with saline, single-dose hUC-MSC and double-dose hUC-MSC: Total cell numbers of intra-renal A) lymphoid and B) myeloid cell subpopulations quantified by multi-colour flow cytometry of CD45-enriched cell suspensions from collagenase/DNase-digested kidneys prepared at 48 hours post-procedure from four groups of mice: SHAM+Saline+Saline (n=9), CLP+Saline+Saline (n=8), CLP+UC-MSC+Saline (n=6) and CLP+UC-MSC+UC-MSC (n=10). Statistical analysis: Non-Gaussian distribution: Non-parametric Kruskal-Wallis test with Dunn's multiple comparison test,  $p < 0.05$ . Gaussian distribution: One-way ANOVA with Bonferroni multiple comparison post-test and with 95% confidence interval.

\*Significantly different from SHAM group (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001). Abbreviations: MP=mononuclear phagocytes.



**Figure 6**

Unsupervised hierarchical clustering of response patterns of control and hUC-MSC-treated animals: Average linkage Euclidean distance clustering of data from Sham (green, s, n=5), CLP (black, c, n=6) and UC-MSC-treated animals with single (red, o1, n=5) or double dose (blue, o2, n=10) of 1x10<sup>6</sup> UC-MSC. The lowest level is represented by green, while highest level is represented by red. All data except NGAL and Albumin:Globulin subjected to Log<sub>2</sub> transformation prior to clustering.

## Supplementary Files

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