**ONLINE DATA SUPPLEMENT**

**Title**: Human Umbilical Cord Mesenchymal Stromal cells attenuate systemic sepsis in part by enhancing peritoneal macrophage bacterial killing via Heme Oxygenase-1 induction in rats.

### Authors: Mirjana Jerkic1 , Stéphane Gagnon1, Razieh Rabani1, Taylor Ward-Able 1, Claire Masterson1,2, Gail Otulakowski3, Gerard F. Curley1,4, John Marshall1,6, Brian P. Kavanagh, MD7,8, John G. Laffey1,2,4,8

**Affiliations**: 1Keenan Research Centre for Biomedical Science, St. Michael’s Hospital, University of Toronto, Toronto, ON, Canada, 2Regenerative Medicine Institute (REMEDI) at CÚRAM Centre for Research in Medical Devices, Biomedical Sciences Building, National University of Ireland Galway, Galway, Ireland; 3Translational Medicine, Hospital for Sick Children, Toronto; 4Department of Anesthesia and Critical Care Medicine, St. Michael’s Hospital, Toronto, ON, Canada, 5Department of Anaesthesia and Critical Care, Royal College of Surgeons in Ireland, 6Departments of Surgery and Interdepartmental Division of Critical Care, University of Toronto, 7Department of Critical Care Medicine, Hospital for Sick Children, Toronto; 8Departments of Anesthesia, Physiology and Interdepartmental Division of Critical Care, University of Toronto; CANADA.

**Correspondence:** John G. Laffey, Keenan Research Centre for Biomedical Science, St. Michael’s Hospital, University of Toronto, Toronto, Canada; Regenerative Medicine Institute (REMEDI) at CÚRAM Centre for Research in Medical Devices, Biomedical Sciences Building, School of Medicine, National University of Ireland Galway, Galway, IRELAND

Email: [john.laffey@nuigalway.ie](mailto:John.laffeyj@nuigalway.ie) Phone: 1-353-91-495662

**MATERIALS AND METHODS**

All work was approved by the Animal Care and Use Committee of the Keenan Research Centre for Biomedical Science of St Michael’s Hospital, Toronto (ACC648) and conducted under license from Health Canada. All studies on human peripheral blood mononuclear cells were approved by the Research Ethics Board of St Michael’s Hospital, Toronto (REB: 14-278). Specific-pathogen-free adult male Sprague Dawley rats (Charles River Laboratories, Quebec, Canada) weighing between 350–450g were used in all in vivo experiments.

**Human Mesenchymal Stromal Cells**

Human umbilical cords were obtained from full-term, consenting donors undergoing caesarean section at Mount Sinai Hospital, Toronto, Canada using the protocol approved by research ethics boards at both the University of Toronto and Mount Sinai Hospital’s Research Centre for Women’s and Infants’ Health (RCWIH). UC-MSCs were non-enzymatically extracted by a proprietary methodology and provided by Tissue Regeneration Therapeutics (TRT) Inc., Toronto, Canada 1,2. Cells harvested by this process were cultured in Lonza TheraPEAK™ MSCGM-CD serum-free complete medium (Cedarlane, Burlington, ON, Canada) supplemented with antibiotics (28 µM Penicillin G, 104 µM Gentamycin, and 324 nM Amphotericin B) on fibronectin-coated flasks, passaged twice and cryopreserved. Routine flow cytometry on identically processed lots revealed highly positive expression of CD90, CD73, CD105, CD140b and CD166 and lack of CD31, CD34, CD45 and MHC-II. The adipogenic, osteogenic and chondrogenic differentiation potential of these cells has previously been reported by our group 3.

Cryopreserved UC-MSCs were thawed, cultured for 3-5 days in serum-free complete medium, lifted from the flask on the day of administration, using TrypLE™ Express (ThermoFisher Sci., Burlington, ON, Canada), washed with PBS by centrifugation, counted and resuspended in PBS vehicle for delivery.

**Rodent *fecal sepsis* protocol**

***Cecal slurry preparation:*** A stock of the cecal slurry (CS) was prepared using a modified previously reported protocol 4. Briefly, rats were anesthetized using isoflurane (3.5%) and then sacrificed by exsanguination. The whole cecum was dissected from each rat, the collected cecal contents were combined, mixed with sterile water and sequentially filtered through sterile meshes (380µm and 190µm), and an equal volume of 30% glycerol in phosphate buffered saline (PBS) was added while continuously stirring on a plate with a magnetic stir bar. A small aliquot of CS was kept separately for bacterial CFU estimation and microbiome sequencing, while the rest of the stock was aliquoted into 5 ml cryovials. All aliquots were then placed in a cryogenic freezing container (ThermoFisher Sci Nalgene, Burlington, ON, Canada) to ensure slow freezing of the bacteria and stored at -80°C.

***Fecal sepsis induction:*** The animals were anesthetized by inhalational induction with isoflurane (5%) and after confirmation of depth of anesthesia by paw clamp were quickly transferred to the warming plate and 3-3.5% isoflurane delivered via nose cone. The lower abdomen was shaved, cleaned with alcohol followed by betadine solution, and an 18G-catheter (BD Biosciences, Mississauga, ON, Canada) was inserted about 1 cm deep through the skin and muscle at a 45° angle. The stylet was removed and 4 mL of previously thawed CS was instilled into the abdominal space, and injection site was cleaned with 3% H2O2. Buprenorphine (0.05 mg/kg) and lactated Ringers solution (20 mL/kg) were given subcutaneously preoperatively and every 12 hours post-instillation, to minimize discomfort and prevent animal dehydration, respectively.

***Experimental Design*:** Preliminary experiments determined the cecal slurry dose required to produce sepsis over a 48 hour period. ***Series 1***, determined the effects of UC-MSCs in severe fecal sepsis. Animals were randomized to undergo intraperitoneal instillation of cecal slurry (4ml slurry in 15% glycerol) or sham procedure (intra-peritoneal vehicle, 4ml 15% glycerol in PBS). Animals undergoing fecal sepsis were randomized to treatment with 10 million/kg UC-MSCs or to vehicle. In ***series 2***, the dose response characteristics of UC-MSCs (2, 5 and 10 million UC-MSCs/kg) was determined, using a different cecal slurry batch that produced moderate fecal sepsis. In ***series 3***, the potential for Zinc protoporphyrin (ZnPP), an inhibitor of heme oxygenase-1 activity, to block the effects of UC-MSCs, was examined. Animals were randomized to intra-peritoneal injection of ZnPP 50 μmol/kg or vehicle 1 day prior to fecal instillation, and to treatment with UC-MSCs or vehicle, respectively. In all *in vivo* experiments, UC-MSCs or vehicle was administered via tail vein (in 0.5 mL of sterile PBS), 4h after sepsis induction.

***Assessment of Septic Injury:***After 48 hours, animals were anesthetized with intraperitoneal 50 mg/kg ketamine (Vetalar, Bioniche, Belleville, ON, Canada) and 10 mg/kg Xylazine (Rompun, Bayer, Montreal, Que, Canada). Intravenous access was established via tail vein and anesthesia maintained with Alfaxan (Alfaxadone 0.9% and alfadadolone acetate 0.3%, Jurox, Rutherford, Australia). A tracheostomy tube was inserted and intra-arterial access secured in the carotid artery. Muscle relaxation was induced with cisatracurium besylate (Alveda Pharma, Toronto, ON, Canada) and the lungs were mechanically ventilated (CWE SAR 830 AP, CWE Inc, Pennsylvania, USA) as previously described 3,5-11. Systemic arterial blood pressure and peak airway pressure were continually measured. Body temperature was maintained at 36° – 37.5°C. Lung static compliance and arterial blood gas analysis were measured after 20 minutes 5.

***Ex vivo Analyses:*** Animals were then killed by exsanguination under anesthesia. 20 mL of sterile PBS was instilled into the peritoneal cavity and peritoneal lavage fluid (PLF) fluid recovered. The liver, spleen and the heart-lung block were then dissected from the animal and bronchoalveolar lavage (BAL) performed. Total and differential leukocyte counts were estimated in PLF and BAL fluid. Lung, hepatic, and splenic tissue were collected for bacterial colony count estimation (1 mL PBS per 100 mg of tissue), snap frozen for later protein extraction, and stored in 10% neutral buffered formalin (Sigma-Aldrich, Oakville, ON, Canada) for histologic assessment. PFL and BAL fluid were centrifuged, and the supernatant was snap frozen and stored at -80°C. Lung, hepatic, and splenic concentrations of TNF-α, and IL-10 were determined using Enzyme Linked Immunosorbent Assay (ELISA; R&D Systems, Minneapolis, MN, USA) and protein concentrations measured (Bio-Rad protein assay, Hercules, CA, USA) in tissue lysates and PLF.

***Western blot procedure:***Western blot analysis was performed according to an established protocol 12. Briefly, tissues were homogenized in TNE buffer (0.05 M Tris/HCl, pH7.4, 0.1 M NaCl, 1 mM EDTA) supplemented with 1% Triton X-100 and protease/phosphatase inhibitors and equal protein amounts were fractionated on 4-12% gradient NuPAGE gels (Invitrogen, Burlington, ON, Canada) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corp, Bedford, MA, USA). After blocking with 5% milk in Tris-Buffered Saline and Tween 20 (TBS-T), the blot was incubated with primary antibody for 2 hours or overnight, followed by a secondary antibody conjugated with horseradish peroxidase for 1 hour. The following primary antibodies were used: heme oxygenase-1 (HSP-32), 1:1,000 (mouse IgG1; Enzo Life Sci, Farmingdale, NY, USA or rabbit IgG AvivaSystems Biology San Diego CA, USA); NF-κB p65, 1:1,000, phospho-p65 NF-κB 1:500, and IKBa, 1:1,000 (all three rabbit IgG, Cell Signaling Inc., Danvers, MA, USA). Signals were detected using an ECL-Plus kit (Amersham Biosci, Piscataway, NJ, USA). Band intensities were quantified and expressed relative to that of β-actin, 1:10,000 (mouse IgG1, Sigma-Aldrich, Oakville, ON, Canada).

***Mφ Phagocytosis and Superoxide assays:*** Rat peritoneal macrophages were isolated from sham or septic animals that received UC-MSC or vehicle therapy, by Ficoll gradient. Isolated macrophages then were seeded (50,000 cells/well) and left overnight without treatment or were exposed to: heme oxygenase-1 inducer hemin (10 μM; Sigma-Aldrich, Oakville, ON, Canada) or to the inhibitor of heme oxygenase-1 activity, Zn protoporphyrin (ZnPP; 10 μM; Alexis Biochem, Cedarlane, Burlington, ON, Canada). Their phagocytic capacity was determined using two assays: the Cyto Select Phagocytosis colorimetric Kit (Cell BioLabs Inc., San Diego, CA, USA) and the Zymosan/Nitroblue tetrazolium (NBT) assay 13, by which phagosomal superoxide production was also estimated. In the NBT assay, Mφ phagocytic capacity was determined using Alexa-488-conjugated serum opsonized zymosan, while dark formazan deposits formed from NBT in presence of reactive oxygen species (ROS) production, were indicative of phagosomal superoxide production.

Human monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy volunteers and from patients with clinical criteria for sepsis (in the first 48h of diagnosis) with consent obtained. Lympholyte H cell media (Cedarlane, Burlington, ON, Canada) was used for separation. Monocytes attached to the culture plates became macrophages over 5 days. UC-MSCs (or vehicle) were added to the macrophages (50 000 cells/well, 12 well plate or 100 000 cells/well, 6 well plate) for an additional 2 days. Macrophage phagocytic capacity was determined using the Zymosan assay, while their killing capabilities were estimated using live *E. coli* (DH5-α, Thermo Fisher Sci, Toronto, ON, Canada). Prostaglandin E2 inhibitor (10µM, CAY10526, Cayman chemical, Cedarlane, Burlington, ON, Canada) and 15-lipoxygenase (15-LO) inhibitor (2µM, PD146176, Cayman chemical, Cedarlane, Burlington, ON, Canada) were (or vehicle) added to the macrophages immediately after their treatment with UC-MSCs was introduced.

The colorimetric phagocytosis assay (three experiments done in duplicates) was done in accordance with manufacturer’s instructions (Cytoselect kit, Cell Biolabs, Inc., San Diego, CA, USA). Briefly, enzyme-labelled *E. coli* particles were co-incubated with macrophages for 4h (at 37°C). Extracellular particles were removed by washes and incubation with blocking agent, macrophages were then permeabilized, incubated with the substrate and absorbance was measured at 450 nm.

The Zymosan assay (four experiments done in duplicates) was performed as described previously 13. Briefly, Alexa-488-conjugated serum opsonized zymosan (SOZ; Sigma-Aldrich, Oakville, ON, Canada) were added to cells growing on 18-mm coverslips in 12-well plates at the concentration of 0.125 mg/ml. The 12-well plate was then centrifuged for 1 min (1500 rpm) to rapidly bring the SOZ in contact with the cells. The cells were then incubated at 37 ̊C for 30 min. Excess particles were removed by three washes with PBS and the cells were fixed in 4% paraformaldehyde in PBS for 15 min. The coverslips were mounted on the slides using mounting medium (Dako Manufacturing Ltd., Woodbridge, ON, Canada) and stored under dark cold conditions. Cells were visualized by confocal microscopy using laser scanning Zeiss LSM700 microscope equipped with a single pinhole (Carl Zeiss Microscopy GmbH, Peabody, MA, USA) and ZEN software (2012 blue edition). Counting of engulfed (green) particles was done in 8-10 randomly chosen fields/slide using Image-J (1.48a, NIH, USA) software. The phagocytic index was calculated as the average number of particles ingested per phagocyte.

***Mφ Bacterial killing assay:*** Human macrophages were cultured in 12 well plates in duplicate and *E. coli* bacteria (unopsonized) (~8.6 x 106 cells/ml) were re-suspended and added to the macrophage cells. The plates were spun down (1500 rpm; 60 sec), placed in a 37°C incubator for 20 min. and then washed 3 times with PBS. DMEM media (with 5% FBS and 100 g/ml gentamycin) was added for 25 min at 37°C. The cells were then washed 3 times with PBS. The cells in plate #1 were then lysed with 1%TX-100/PBS and the lysates were plated onto LB plates. In plate #2 antibiotic-free DMEM media (with 5% FBS) was added and the plate was incubated for 1h at 37oC. Plate #2 was then lysed and the lysate plated onto LB plates as described above. The next day bacterial colonies were counted and the percentage killing determined.

***Elucidation of Prostaglandin*** ***E2, Lipoxin*** ***A4 and heme oxygenase-1*** ***roles:*** Rat peritoneal and human monocyte derived macrophages were incubated with hUC-MSCs in the presence and absence of a Prostaglandin E2 inhibitor (10 µM, CAY10526) 14 and 15-lipoxygenase (15-LO) inhibitor (2µM, PD146176) 15, respectively (both from Cayman chemical, Cedarlane, Burlington, ON, Canada), and phagocytosis assessed. The potential for exogenous Prostaglandin E2 (0.2 µM, CAY 14010) or lipoxin A4 (0.1 µM, CAY 90410) (both from Cayman chemical) to directly enhance Mφ phagocytosis and bacterial killing determined. Prostaglandin E2 and lipoxin A4 were assayed in cell supernatants using Prostaglandin E2 (R&D Systems. Inc., Minneapolis, MN, USA) and lipoxin A4 (USBiological Life Sci, Swampscott, MA, USA) ELISA kits.

Human monocyte derived macrophages were transfected with heme oxygenase-1 siRNA or non-related (NR) siRNA (Thermo Fisher Sci, Mississauga, ON, Canada) using NeonTM transfection system (Thermo Fisher Sci, Mississauga, On, Canada) in media without antibiotics. Manufacturer protocol for transfection was followed and parameters optimized (1200 V, 1 pulse, with width of 40). Macrophages were left undisturbed for 30-32h and then treated or not with UC-MSCs or lipoxin A4 (0.1 μM) for 48h. Cells were fixed, permeabilized and immuno-fluorescence (IF) staining was done using heme oxygenase-1 1 primary antibodies (rabbit IgG AvivaSystems Biology San Diego CA, USA ) and appropriate Fab fragment secondary Ab conjugated with Alexa 488 (Jackson ImmunoResearch Labs, Inc., West Grove, PA, USA). Cells were also stained with Hoechst dye to reveal the location of nuclei. Images were taken using Zeiss LSM700 confocal microscope and cytosolic mean fluorescent intensity (MFI) in green channel (to estimate heme oxygenase-1 expression) was quantified using Imaris 8.02 software. The effects of heme oxygenase-1 blockade on macrophage phagocytosis, and ROS production were then assessed as described earlier.

**Statistical Analysis**

The sample size was based on our previous experience with this design and on pilot studies results. We anticipated a mortality in the range of 30% in untreated animals, but the mortality rate in the UC-MSC treated animals was unknown. Our primary measurements were bacterial colony counts, and we estimated (for an ANOVA test, 0.05, 1-= 0.8, effect size 1x104, expected standard deviation 0.75x104) that this required 12 surviving animals for each of 3 groups. To minimize animal numbers, we randomly allocated 12 animals to each group in series 1, and decided a priori to replace non-surviving animals. For series 2, and 3 we recalculated sample sizes each time, which reduced the numbers of surviving animals needed to 8 in each of 4 groups in series 2, and to 5 in each of 2 groups for series 3.

The data are expressed as mean ± SD, or presented as whisker plots (Q1 to Q3 ± 1.5 IQR).

Data were analyzed using GraphPad Prism (GraphPad® software, La Jolla, CA, USA). The distribution of all data was tested for normality using Kolmogorov-Smirnov tests. Data were analyzed by one-way ANOVA (using between-subjects factors), or ANOVA on Ranks with *post hoc* testing using Newmann-Keuls, multiple comparison test or Dunnet’s tests as appropriate. Chauvenet’s (for normal distribution of the data) or Pierce’s (non-normal distribution of the data) criterion were used for data exclusion 16. The only excluded values are 1 data point per septic animal group for Figure 1 panels D-F and the values are listed in the eTable 1. For animal survival the log rank test was used and there were no censored data during the observation period. A two-tailed *P* value of <0.05 was considered significant.

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