Online Supplemental

Title: Human mesenchymal stromal cells decrease the severity of acute lung injury induced by *E.coli* in the Rat.

Authors: Dr. James Devaney, PhD [1,2]
Ms. Shahd Horie, BSc, MSc [1,2]
Dr. Claire Masterson, PhD [1,2]
Dr. Steve Elliman, PhD [3]
Prof. Frank Barry, PhD [2]
Prof. Timothy O'Brien, MD, PhD [2]
Dr. Gerard F. Curley, MB, FCARCSI, PhD [4]
Dr. Daniel O' Toole, PhD [1,2]
Prof. John G. Laffey, MD, FCARCSI [4]

Institution: [1]Anaesthesia, School of Medicine, Clinical Sciences Institute, National University of Ireland, Galway, IRELAND; [2]Regenerative Medicine Institute, National University of Ireland, Galway, IRELAND; [3]Orbsen Therapeutics Ltd, National University of Ireland, Galway, Ireland; [4]Department of Anesthesia, Critical Illness and Injury Research Centre, Keenan Research Centre for Biomedical Science, St Michael's Hospital, University of Toronto, CANADA.

Corresponding Author: Prof John G Laffey, Department of Anesthesia, Keenan Research Centre for Biomedical Science, St Michael's Hospital, University of Toronto, CANADA. E-mail: laffeyj@smh.ca Phone: 1-416-864-5071

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Abbreviated Title/ Running Head: MSCs decrease *E. coli* induced acute lung injury

MATERIALS AND METHODS

All experiments were approved by the Animal Ethics Committee at the National University of Ireland, Galway and were performed under license from the Department of Health and Children, Ireland. Specific-pathogen–free adult male Sprague–Dawley rats (Charles River Laboratories, Kent, United Kingdom) weighing between 350 and 450 g were used in these studies.

Preparation of Cells and Conditioned Medium

Cell Isolation and Culture: The human MSCs used in these studies were provided by Orbsen Therapeutics Ltd (Galway, Ireland). Bone marrow was isolated from human, and plated into tissue culture flasks, as previously described [1]. Adherent cells were grown until 80% confluent and then trypsinized and culture expanded to passage 4, whereupon they were used for experiments. MSCs were characterized according to the international guidelines. Human fibroblasts MRC5 were used as control cells (American Type Culture Collection (ATCC), VA USA). The U937 monocytic/macrophage cell line were also obtained from the ATCC.

Cryopreserved MSCs: Human MSC were cultured until 80% confluent and then cryopreserved in 10% DMSO/FBS freezing solution in 5 x10^6 aliquots. When thawed for subsequent experiments, the cells were either administered immediately, in the cryopreservation medium (‘Thawed hMSC’), or were washed twice in medium and resuspended in PBS prior to administration (‘Washed hMSC’). The dose was adjusted for trypan blue cell viability to ensure that all animals received the same number of viable cells.

Conditioned Medium: Human MSC (4×10^6) were cultured in serum-free media for either 24 hours (CM-24hr) or 48 hours (CM-48hr) as previously described [2]. After replacement of the medium, the subsequently harvested serum-free medium was used as the conditioned medium. Fifteen milliliters of this medium was centrifuged through a 3,000 kD filter (Amicon, Billerica, MA, USA) to reduce the volume to 300 µl.

Experimental model and Series Design

E. coli Instillation: The E. coli used in these experiments was originally isolated from the urine of a female infant, labelled E5162 (serotype: O9 K30 H10) and was supplied by the National Collection of Type Cultures, Central Public Health Laboratory, London, England. The E. coli were stored on preservative beads (Protect, Lancashire, England) at -80°C. Beads were placed in 3-ml vials of peptone water (Cruinn Diagnostics, Dublin, Ireland) and incubated at 37°C for 18 hours to allow bacterial concentrations to reach a plateau. The bacterial suspension was then centrifuged, washed in phosphate-buffered saline, re-centrifuged, and resuspended in phosphate-buffered saline to produce the inoculum. The bacterial load in each inoculum was determined by plating serial dilutions on agar plates.

Animals were anesthetized by inhalational induction with isoflurane and an intraperitoneal injection of 40 mg/kg ketamine (Pfizer, Kent, UK). Following confirmation of anesthesia depth, 2×10^9 E.coli in a 300 µl PBS suspension was instilled into the trachea under direct vision, and the animals allowed to recover for one hour.

A preliminary series of experiments was performed to determine the bacterial load of intra-tracheal E. coli required to produce a severe lung injury over a 48 hour period. An inoculum of 2 x 10^9 colonies of E. coli produced a severe ALI over a 48 hour period, compared with non-inoculated controls.

Experimental Design: Following E. coli inoculation, animals were entered into one of the following four animal series: ***Series 1*** determined the efficacy of hMSC therapy in attenuating E. coli induced lung injury. Animals were randomized one hour post injury to intravenous administration of: (i) vehicle (PBS, 300μL); (ii) 1x10^7 fibroblasts/kg; (iii) 1x10^7 hMSCs/kg or (iv) 2x10^7 hMSCs/kg. ***Series 2*** evaluated the lowest effective MSC doses. Animals were randomized one hour post injury to intravenous administration of: (i) vehicle (PBS, 300μL); (ii) 1x10^7 hMSCs/kg; (iii) 5x10^6 hMSCs/kg or (iv) 2x10^6 hMSCs/kg. ***Series 3*** compared the efficacy of intra-tracheal versus intra-venous hMSC administration. Animals were randomized one hour post injury to administration of: (i) IV vehicle (PBS, 300μL); (ii) IV 1x10^7 hMSCs/kg; (iii) IT 1x10^7 hMSCs/kg or (iv) IT vehicle (PBS, 300μL). ***Series 4*** examined the efficacy of cryopreserved hMSCs. Animals were randomized one hour post injury to intravenous administration of: (i) vehicle (PBS, 300μL); (ii) 1x10^7 fresh hMSCs/kg; (iii) 1x10^7 'thawed' hMSCs/kg (i.e. cryopreserved hMScs thawed and administered); and (iv) 1x10^7 'washed' hMSCs/kg (i.e. cryopreserved hMScs thawed and washed prior to use). ***Series 5*** examined the efficacy of the hMSC secretome. Animals were randomized to receive: (1) IV vehicle (PBS, 300μL); (ii) IV 1x10^7 hMSCs/kg; (iii) medium hMSC conditioned for 24hrs (CM-24hr); and (iv) medium hMSC conditioned for 48hrs (CM-48hr).

Injury Assessment: Animals entered into each experiment were monitored closely for forty eight hours following E. coli and hMSC administration. Animals were caged separately in individually ventilated cages. After 48 hours animals were re-anesthetized with intraperitoneal 80 mg/kg ketamine and 8 mg/kg xylazine. Intravenous access was secured via the tail vein and anesthesia maintained with repeated intravenous boli of Alfaxin® (Alfaxadone 0.9% and alfadadolone acetate 0.3%; Schering Plough, Welwyn Garden City, UK). A tracheostomy tube (1mm internal diameter) was inserted and intra-arterial access (22 gauge cannulae; Becton Dickinson, Franklin Lakes, NJ, USA) was sited in the carotid artery. Cis-atracurium besylate 0.5mg.kg^{-1} (GlaxoSmithKline, Dublin, Ireland) was administered intravenously and the lungs were mechanically ventilated (Model 683; Harvard Apparatus, Holliston, MA, USA) at a respiratory rate of 90/min, tidal volume 6 ml/kg and positive end-expiratory pressure of 2cm H_2O. To minimize lung derecruitment, a recruitment manoeuvre consisting of positive end-expiratory pressure 15cm H_2O for 20 breaths was applied at the start of the protocol. All animals were ventilated with an inspired gas mixture of FiO_2 = 0.3, and FiN_2 = 0.7, for 20 min. Systemic arterial pressure, peak airway pressures and temperature were continuously measured, arterial blood samples were drawn for analysis (ABL 710; Radiometer, Copenhagen, Denmark) and static inflation lung compliance measured [3]. Animals were then ventilated for a further 15 minutes with an inspired gas mixture of FiO_2 = 1.0 and arterial blood samples were again drawn for analysis.

Ex vivo Analyses

Ex vivo analyses were restricted to animals that survived the experimental protocol. At the end of the protocol, Heparin (400 U/kg) was administered intravenously, and the animals sacrificed by exsanguination under anesthesia and the heart–lung block was dissected. Blood was centrifuged, and the serum was snap frozen for analysis. Immediately postmortem, the heart–lung block was dissected from the thorax, and bronchoalveolar lavage (BAL) was performed. BAL was performed by intratracheal instillation of three aliquots (5 ml each) of normal saline and collection of the returned fluid by free drainage. Total leukocyte numbers per ml in the BAL fluid were counted, and differential cell counts were performed after staining with Hema-Gurr Rapid Staining set for Hematology (BDH Laboratory Supply, Poole, UK). Samples of BAL fluid were centrifuged, and the supernatant was snap frozen in liquid nitrogen and stored at -80°C for cytokine analysis. The concentration of bacteria in the BAL fluid was determined by plating serial dilutions on agar plates and performing a colony count 24 hours later.

ELISA Analyses: The concentration *IL-10*, *KGF* (R&D Systems, Abingdon, UK) and *LL37* (Hycult Biotech, Uden, The Netherlands) in the BAL was determined using ELISA. The Micro BCA^{TM} Protein assay kit (Pierce, Rockford, IL), was utilized to determine total BAL protein levels [4].

In vitro LL37 assay: Human MSC were seeded on a 6 well plate and exposed for 4 hours to an increasing number of *E.coli* bacteria recovered from the animal BAL. The concentration of LL37 secreted by the MSC was determined by an LL37 ELISA kit (Hycult Biotech).

In vivo phagocytosis: Peripheral blood monocytes (PBMC) were obtained by Ficoll-Hypaque and Percoll gradient separation of whole blood from animals that received

hMSC or vehicle therapy. PBMCs were suspended in 20 ml of DMEM and placed in 75-cm^2 tissue culture flasks at 37 °C. After 1 hour non-adherent cells including lymphocytes were removed by washing at least five times with DMEM. Adherent cells were then detached by incubation for 15 min with Trypsin/EDTA/DMEM. The monocytes/macrophages were seeded in 6 well plates overnight and their phagocytic potential was determined using the Vybrant Phagocytosis Kit (Life Technologies, NY USA).

In vitro phagocytosis: U937 cells, differentiated into macrophages via exposure to phorbol myristate acetate (PMA) 1μg/ml for 72 hours, were seeded in 6 well plates with or without human MSC and exposed to E.coli bacteria for four hours. Their phagocytic potential was then determined using the Vybrant Phagocytosis Kit (Life Technologies, NY USA).

Histologic Analysis: The left lung was isolated and fixed [3 5], and the extent of histologic lung damage determined using quantitative stereological techniques [6]. Briefly, the pulmonary artery was cannulated, the left atrium was incised, and the pulmonary circulation was perfused with normal saline at a constant hydrostatic pressure of 35 cm H_2O until the left atrial effluent was clear of blood. The left lung was then inflated through the tracheal catheter using paraformaldehyde (4% wt/vol) in phosphate-buffered saline (300 mOsmol) at a pressure of 25 cm H2O. Paraformaldehyde was then instilled through the pulmonary artery catheter at a pressure of 62.5 cm H_2O. The left atrium was then tied off to prevent pulmonary venous inflow into the atrium, creating a constant distending pressure across the pulmonary vasculature, and maximally distending the pulmonary vessels. After 30 min, the pulmonary artery and trachea were ligated, and

the lung was stored in paraformaldehyde for 24 hours. The vertical axis of the lung was identified, and the lung was cut perpendicular to this axis into 4-mm-thick slices with a sharp blade beginning at a position chosen by random number within the first slice. These tissue slices were then embedded in paraffin and sections (7 μm) from each slice mounted on slides and stained with hematoxylin and eosin.

An image of each complete lung section was captured as previously described [3 5]. A point-counting grid was superimposed on the image of each section to estimate its area, and the number of randomly chosen visual fields sampled from any section was proportionate to its area. Each field was examined under light microscopy (× 10 objective; Leica, Laboratory Instruments, Wetzlar, Germany). The images were acquired as described and then imported into Stereology Toolbox (Morphometrix, Davis, CA, USA) at a final magnification of × 60. The intra-acinar tissue was defined as all tissues within the gas exchange portion of the lung, i.e., respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli, including blood vessels contained within their walls. The intra-acinar airspace was defined as all airspaces within the lumen of respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. The volume fractions of intra-acinar tissue and intra-acinar airspace were then determined by the use of a point-counting grid [6].

Data Presentation and Analysis

Continuous responsive variables are summarized using mean (SD) and median (interquartile range, IQR) as necessary. Animal survival was analysed using the log rank test, with combined hMSC treated groups compared to combined control groups within each series. There was no evidence against the normality and equal variance assumptions

for the response variables for each time-treatment combination. In series 1, 2 and 4. Data were analyzed by one-way ANOVA, with post hoc testing using Dunnets test, with the vehicle group as the comparison group or by Kruskal-Waliss ANOVA on ranks with post hoc testing using Dunn's method for non-normally distributed data. In series 3, which examined the efficacy of different routes of hMSC administration, a two-way ANOVA was used, with treatment (hMSC versus Vehicle) and route of administration (IV versus IT) as the 2 factors. Subsequent between-group analyses, where indicated, were restricted to comparisons of hMSC versus vehicle for each route of administration. Underlying model assumptions were deemed appropriate on the basis of suitable residual plots. A two-tailed P value of <0.05 was considered significant.

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