Supplemental Digital Content 1: Materials and Methods

All work was approved by the Animal Ethics Committee of the National University of Ireland, Galway and conducted under license from the Department of Health, Ireland. Specific-pathogen-free adult male Sprague Dawley rats (Charles River Laboratories, Kent, United Kingdom) weighing between 350–450g were used in all experiments. The methods are similar to those described in our recent publications,[1] with a number of modifications.

Human MSC (hMSC) Isolation and Culture

The human MSCs used in these studies were provided by Orbsen Therapeutics Ltd. (Galway, Ireland). Briefly, bone marrow was aspirated from the iliac crests of healthy human volunteers as previously described.[2] The bone marrow was then filtered using a 70 μm cell strainer (BD Labware, Franklin Lakes, NJ) prior to centrifugation at 400 g for 10 min. Cell pellets were resuspended in media consisting of MEM-α (Gibco, Paisley, United Kingdom), supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% streptomycin and penicillin (Gibco), and cultured in 175 cm^2 flasks at 37°C in a humidified atmosphere containing 5% CO_2. At day four, the cultures were washed with PBS to remove the non-adherent cells and further expanded until >80% confluence, when they were harvested and expanded in 175 cm^2 flasks. After subculture, these cells were designated as passage 1.

For passage, media was aspirated off and cells washed remove any remaining serum with sterile phosphate buffered saline. 8mls 0.25% trypsin/EDTA solution

was added to the cells, which were incubated for 5 min at 37°C. The enzymatic reaction was stopped by adding the same volume of MSC media to cells. Cells were centrifuged at 400g for 5 min. Media was aspirated, and the cell pellet was resuspended in 1ml and a haemocytometer count was undertaken. Cells were expanded to passage 4, whereupon they were used for experiments.

Characterization of human MSCs

hMSCs were characterized according to international guidelines.[3] hMSC cell surface marker profile was determined by FACS analysis. hMSCs were negative for hematopoietic markers CD34, CD133, CD45 and c-kit. hMSCs expressed high levels of CD13, CD105 and CD73 and were low for STRO-1. hMSCs were also negative for HLA-DR (MHC class II), but positive for HLA-ABC (MHC-class I).

Fibroblast isolation and Culture

Primary human lung fibroblasts were purchased from ATCC (American Type Culture Collection) and frozen, following culture in aliquots of 1 million cells per cryovial in liquid nitrogen. When thawed from liquid nitrogen, the fibroblasts were seeded onto T175 polystyrene filtered lid flasks (Greiner®, Monroe, NC) at a seeding density of between 800,000-1 million cells per flask. The medium (MEM-α modification with 10% Hyclone FCS, Pen-strep and L-glutamine) was refreshed every 3-4 days. When the fibroblasts had reached a confluence of 70%, they were trypsinized with 0.25%Trypsin, resuspended with medium and centrifuged at 400g for 5 min. The supernatant was then aspirated, and the

fibroblasts were resuspended in PBS and a cell count was performed using a haemocytometer. Aliquots of 4 million cells were made up in 300µL of PBS for intravenous administration to the animals

Rodent Ventilator-induced Injury Protocol

We utilized our established model of repair from ventilation induced lung injury (VILI).[4] Adult male Sprague Dawley rats were anaesthetized with intraperitoneal ketamine 80 $mg.kg^{-1}$ (Ketalar, Pfizer, Cork, Ireland) and xylazine 8 $mg.kg^{-1}$ (Xylapan, Vétoquinol, Dublin, Ireland). After confirmation of depth of anesthesia by paw clamp, intravenous access was obtained *via* tail vein, laryngoscopy was performed and the animals were intubated with a size 14G intravenous catheter (BD Insyte®, Becton Dickinson Ltd., Oxford, United Kingdom). The lungs were ventilated using a small animal ventilator (CWE SAR 830 AP, CWE Inc., Ardmore, PA). Anesthesia was maintained with repeated boli of Saffan® (Schering Plough, Welwyn Garden City, United Kingdom) and paralysis with cisatracurium besylate $0.5mg.kg^{-1}$ (GlaxoSmithKline, Dublin, Ireland). Animals were then randomly allocated to undergo either injurious or protective ventilation depending on the specific experimental series.

In animals subjected to injurious mechanical ventilation, the following ventilator settings were used: Fi_{O2} of 0.3, P_{insp} 35 cmH_2O, respiratory rate 18 min^{-1}, and PEEP 0 cmH_2O. When respiratory static compliance had decreased by 50% the animals were allowed to recover.[4] The protective ventilation protocol comprised of mechanical ventilation for 90 min with the following settings: Fi_{O2} of 0.3, respiratory rate $80.min^{-1}$, tidal volume 6 $ml.kg^{-1}$ and positive end-expiratory pressure of 2cm H_2O.[4]

Experimental Series

Series 1A examined the efficacy of hMSC therapy in VILI. Animals were subjected to injurious ventilation, and following recovery, were randomly allocated to intravenous administration of: (i) vehicle (PBS, 300μL); (ii) human lung fibroblasts ($1x10^7$ cells/Kg); or (iii) hMSCs ($1x10^7$ cells/kg) and the extent of recovery from VILI assessed at 24 hours. ***Series 1B*** examined the effects of hMSC therapy in the setting of non-injurious ventilation. *A*nimals underwent protective ventilation, and following recovery, were randomly allocated to intravenous administration of vehicle, human lung fibroblasts or hMSC therapy as for *series* 1A. ***Series 2*** examined the efficacy of lower hMSC doses in animals following VILI. Following induction of VILI, animals were randomized to intravenous administration of: (i) vehicle (PBS, 300μL); (ii) $1x10^6$ cells/kg hMSCs; (iii) $2x10^6$ cells/kg hMSCs; (iv) $5x10^6$ cells/kg hMSCs; or (v) $1x10^7$ cells/kg hMSCs; and the extent of recovery from VILI assessed at 24 h. ***Series 3*** compared the efficacy intratracheal and intraperitoneal administration of $1x10^7$ cells/kg hMSC to the intravascular route. ***Series 4*** examined the efficacy of delayed hMSC administration when given at 0.25 h, 6 h, and 24 h following VILI. The recovery period was extended to 48 h in this series, and the later treatment points represented 12.5% and 50% of the recovery period respectively.

Assessment of Injury and Repair

At 24 or 48 h following VILI induction (depending on the experimental series), animals were reanesthetized. A tracheostomy was performed, and arterial blood gases and static inflation lung compliance were measured as previously

described.[5,6] After 20 min, the inspired gas was altered to a Fi_{O2} of 1.0 for 15 min, and a final arterial blood sample was taken. Heparin (400 $IU.kg^{-1}$, CP Pharmaceuticals, Wrexham, United Kingdom) was then administered intravenously, and animals were euthanized by exsanguination. Immediately post-mortem, the heart–lung block was dissected and bronchoalveolar lavage (BAL) collection was performed.[7,8] BAL differential cell counts were obtained. Protein concentration was determined using a Micro BCA™ Protein assay kit (Pierce, Rockford, IL).[9] BAL CINC-1, IL-6, IL-10 and KGF concentrations were determined using quantitative sandwich enzyme-linked immunosorbent assays (R&D Systems, Abingdon, United Kingdom). Wet:dry lung weights were determined using the lowest lobe of the right lung.[10] The left lung was isolated and fixed for morphometric examination, and the extent of histologic lung damage was determined using quantitative stereological techniques.[7,10]

Statistical Analysis

Data was analyzed using Sigma Stat (SYSTAT® software, Richmond, CA). The normality of the distribution of all data was tested using Kolmogorov-Smirnov tests. Results are expressed as mean (± SD) for normally distributed data, and as median (interquartile range) where non-normally distributed. Data were analyzed by one-way ANOVA, followed by Student-Newman-Keuls, or by Kruskalis-Wallis followed by Mann-Whitney U test with the Bonferroni correction for multiple comparisons, as appropriate. Underlying model

assumptions were deemed appropriate on the basis of suitable residual plots. A two-tailed *p* value of <0.05 was considered significant.

References

1. Curley GF, Hayes M, Ansari B, Shaw G, Ryan A, Barry F, O'Brien T, O'Toole D, Laffey JG: Mesenchymal stem cells enhance recovery and repair following ventilator-induced lung injury in the rat. Thorax 2012; 67:496-501
2. McAuley DF, Curley GF, Hamid UI, Laffey JG, Abbott J, McKenna DH, Fang X, Matthay MA, Lee JW: Clinical grade allogeneic human mesenchymal stem cells restore alveolar fluid clearance in human lungs rejected for transplantation. Am J Physiol Lung Cell Mol Physiol 2014; 306:L809-15
3. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E: Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8:315-7
4. Curley GF, Contreras M, Higgins B, O'Kane C, McAuley DF, O'Toole D, Laffey JG: Evolution of the inflammatory and fibroproliferative responses during resolution and repair following ventilator-induced lung injury in the rat. ANESTHESIOLOGY 2011; 115:1022-32
5. Costello J, Higgins B, Contreras M, Chonghaile MN, Hassett P, O'Toole D, Laffey JG: Hypercapnic acidosis attenuates shock and lung injury in early and prolonged systemic sepsis. Crit Care Med 2009; 37:2412-20
6. Higgins BD, Costello J, Contreras M, Hassett P, O'Toole D, Laffey JG: Differential effects of buffered hypercapnia *versus* hypercapnic acidosis on shock and lung injury induced by systemic sepsis. ANESTHESIOLOGY 2009; 111:1317-26

7. O'Croinin DF, Hopkins NO, Moore MM, Boylan JF, McLoughlin P, Laffey JG: Hypercapnic acidosis does not modulate the severity of bacterial pneumonia-induced lung injury. Crit Care Med 2005; 33:2606-12
8. O'Croinin DF, Nichol AD, Hopkins N, Boylan J, O'Brien S, O'Connor C, Laffey JG, McLoughlin P: Sustained hypercapnic acidosis during pulmonary infection increases bacterial load and worsens lung injury. Crit Care Med 2008; 36:2128-35
9. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC: Measurement of protein using bicinchoninic acid. Anal Biochem 1985; 150:76-85
10. Laffey JG, Honan D, Hopkins N, Hyvelin JM, Boylan JF, McLoughlin P: Hypercapnic acidosis attenuates endotoxin-induced acute lung injury. Am J Respir Crit Care Med 2004; 169:46-56