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 [1,2], with a number of modifications.

Rodent Mesenchymal Stromal Cells Isolation and Culture

Rodent mesenchymal stromal cells (rMSCs) were isolated from rat femora and tibiae under sterile conditions as previously described [3] with additional modifications [1]. Briefly, male 8-12 week old Sprague Dawley rats were euthanized *via* carbon dioxide inhalation. Incisions were made on both lower limbs to expose the tibiae and femora. Both bones were removed from the hind limbs and placed in ice cold sterile Tyrode's solution (Sigma, St. Louis, MO). The marrow was then flushed into a dish containing rMSC complete culture medium (MEM-α Media (Gibco, Paisley, United Kingdom), F12-Ham Media (Gibco), 10% foetal bovine serum (PAA, Somerset, United KIngdom), 1% antibiotic/antimycotic (Sigma) and dispersed into a cell suspension. After centrifugation and filtration through a 100 μm nylon mesh, cells were counted, and then transferred to a 175 cm^2 flasks containing 30 mls of rMSC complete medium, at a density of $9x10^5$ cells/cm^2. On day 3 of culture in an atmosphere of 5% CO_2/90% humidity at 37^{o}C, nonadherent cells and medium were decanted and fresh medium was added to each flask. When colonies began to exhibit a compact appearance and multilayered growth or when the loosely formed

colonies became largely (<90%) confluent, they were subcultured (usually after 16-17 days).

Thereafter, cells were ready to be passaged after 6/7 days culture, at 80% confluence. For passage, media was aspirated off and cells washed remove any remaining serum with sterile phosphate buffered saline. 8 mls 0.25% trypsin/EDTA solution was added to the cells, which were incubated for 5 min at 37^{o} C. The enzymatic reaction was stopped by adding the same volume of rMSC 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 MSCs

MSC characterization was performed in accordance with minimal criteria [4]. Osteogenic differentiation was induced by culturing rMSCs for up to 4 weeks in rMSC Complete Medium supplemented with dexamethasone, ascorbic acid and β-glycerophosphate as previously described [5]. Alizarin Red stain (Sigma) was used to demonstrate calcium deposition. Adipogenic differentiation was induced by culturing rMSCs for 4 weeks in medium containing dexamethasone and insulin; adipocytes were discerned by staining with Oil Red O (Sigma). Chondrogenic capacity was assessed by addition of tumor necrosis factor-α (Invitrogen, Dublin, Ireland) and staining with Toluidine blue (See Supplemental Digital Content 2).

Phenotypic characterization of MSCs was performed using the following antibodies: anti-rat CD29-FITC, anti-rat CD90-PE, anti-rat CD44H-FITC, anti-rat CD73-PE, anti-rat CD45-FITC, anti-rat CD71-PE, anti-rat CD80-PE, anti-CD106-PE (all from BD Biosciences, San Jose, CA), anti-rat MHC class I-FITC (AbD Serotec, Kidlington, United Kingdom), anti-rat MHC class II-PE (AbD Serotec). Cells were washed with flourescence actiavated cell sorting (FACS) buffer (phosphate buffered saline containing 2 % fetal calf serum and 0.1 % sodium azide (NaN_3), all from Sigma) and incubated for 5 min on ice with anti-rat CD32 (Fcg receptor; BD Biosciences) to reduce unspecific binding prior to staining. The monoclonal antibodies were then added and the cells incubated for 30 - 45 min on ice. Finally, unbound reagents were removed by washing twice with FACS buffer and the cells were re-suspended in FACS buffer for analysis using a FACS Canto (BD Biosciences). Some cells were fixed by adding 2 % paraformaldehyde in FACS buffer. Cells were labeled with monocloncal antibodies against CD31, CD34, CD44, CD45, CD54, CD73, and CD90 (Santa Cruz Biotechnology, Santa Cruz, CA) and analyzed with a FACScan (Becton Dickinson, Franklin Lakes, NJ) and CellQuest software (Becton Dickinson) as described [1] (See Supplemental Digital Content 3).

Fibroblast isolation and Culture

Adult male Sprague Dawley rats were euthanized by carbon dioxide inhalation. The ventral surface of the rat was shaved and sprayed with 70% ethanol [1]. Skin and subcutaneous tissue was removed and placed into 70% ethanol for 30 s. Fat and subcutaneous tissue was removed and the skin strips were placed in 0.25% trypsin (Sigma) overnight. The epidermis was then peeled

from the dermal layer, and the dermal layer was placed on a scored 6 well plate (Sarstedt, Wexford, Ireland) in F-12/MEM-α medium (Gibco) supplemented with fetal calf serum (10%, PAA) and penicillin/streptomycin (1%, Sigma).

Conditioned Medium

Allogeneic rodent MSC's (4 x 10^6) were washed with PBS, cultured without serum for 24 h, then washed. The medium was then replaced, and the subsequent serum-free medium used as the conditioned medium. All conditioned medium was sterile filtered through a 22 μm filter to remove cellular debris. Fifteen mls of this medium was concentrated using a 3000 kDa centrifugal concentrating filter (Amicon, Billerica, MA) to give 300 μL.

Rodent Ventilator-induced Injury Protocol

We utilized our established model of repair from Ventilator-induced lung injury [2]. Anesthesia was induced with intraperitoneal ketamine 80 $mg.kg^{-1}$ (Ketalar, Pfizer, Cork, Ireland) and xylazine 8 $mg.kg^{-1}$ (Xylapan, Vétoquinol, Dublin, Ireland). Depth of anesthesia was confirmed by paw clamp, intravenous access was secured in the tail vein, and the animals were intubated under direct laryngoscopy with a size 14G intravenous catheter (BD Insyte®, Becton Dickinson Ltd., Oxford, United Kingdom). The lungs were ventilated using a Charles Ward SAR 830 AP ventilator (CWE Inc., Ardmore, PA). Repeated boli of Saffan® (alfaxadone 0.9% and alfadadolone acetate 0.3%; Schering Plough, Welwyn Garden City, United Kingdom) were used to maintain anesthesia and paralysis was achieved with cis-atracurium besylate $0.5mg.kg^{-1}$ (GlaxoSmithKline, Dublin, Ireland). The animals were then subjected to injurious

mechanical ventilation (Fi_{O2} 0.3, inspiratory pressure 35 cmH_2O, respiratory rate 18 min^{-1}, and zero positive end-expiratory pressure). High stretch ventilation was discontinued once static compliance decreased by 50%, and animals were extubated, allowed to regain consciousness, and entered into the treatment protocol.

Treatment Protocol

After recovery, animals were randomized to receive (i) no therapy, (ii) intratracheal vehicle (Phosphate buffered saline, 30 0 μL), (iii) intratracheal fibroblasts (4 x 10^6), (iv) intratracheal MSCs (4 x 10^6), (v) intratracheal conditioned medium (300 μL) or (vi) intravenous MSCs (4 x 10^6). All treatments were suspended in 300 μL phosphate buffered saline.

Assessment of Injury and Repair

At 48 h after stretch-induced injury, animals were reanesthetized, tracheostomized and carotid arterial access established (22G, BD Insyte). The lungs were mechanically ventilated at a respiratory rate of 80 min^{-1}, tidal volume 6 $ml.kg^{-1}$ and positive end-expiratory pressure 2 cmH_2O [2,6,7]. Peak airway pressures, intraarterial blood pressure, and rectal temperature were continuously recorded. Static inflation lung compliance was measured as previously described [2,6,7]. After 20 min, the inspired gas was increased to 100% O_2 for 15 min, and arterial oxygenation reassessed. Animals were killed by exsanguination under anesthesia following heparinization (400 $IU.kg^{-1}$, CP Pharmaceuticals, Wrexham, United Kingdom).

The heart–lung block was excised immediately postmortem and bronchoalveolar lavage collection was performed as previously described [7,8]. Bronchoalveolar lavage differential cell counts were performed. Protein concentration was measured using a Micro BCA™ Protein assay kit (Pierce, Rockford, IL).[9] Bronchoalveolar lavage tumor necrosis factor-α, interleukin-6, and interleukin-10 concentrations were determined using enzyme-linked immunosorbent assays (R and D Systems, Abingdon, United Kingdom) [10]. The detection limit for these assays was 62·5 pg/ml for the interleukin-6 assay and 31·2 pg/ml for tumor necrosis factor-α and interleukin-10 assays.

Wet:dry lung weight ratios were determined using the lowest lobe of the right lung [11]. The left lung was isolated and fixed for histologic examination, and the extent of lung damage and repair was determined using quantitative stereology as previously described [7,11].

Analysis of lung MSC distribution patterns

MSC labeling: Rat MSCs were tryspinized, counted in a hemocytometer, and 4 x 10^6 cells pelleted in a 15 mL tube. Fluorescent labeling was performed with a PKH Red Fluorescent Cell Linker Kit (Sigma). rMSCs were resuspended in 400 mL of kit Diluent C, and mixed with 4 mM PKH26 label in 400 mL Diluent C to give a final concentration of 2 mM. Preliminary studies had indicated that labelling at this concentration did not affect cell viability over 4 h as assessed by trypan blue dye exclusion. Every minute for 5 min, the suspension was gently mixed by pipetting. Labeling was halted through addition of 800 mL of 1% (w/v) bovine serum albumin in phosphate buffered saline followed by 1,600 mL of

complete MSC medium. Cells were pelleted and washed three further times with 3 mL of complete medium before final resuspension in 300 mL of 1% fetal bovine serum (v/v) in phosphate buffered saline. 1 mL of the prepared cells was examined under fluorescent microscopy to ensure successful labeling before administration to the animal.

Flow cytometry*:* At 1, 4, and 24 h post-MSC administration, lungs from rats were excised and digested to obtain single-cell suspensions. Briefly, after mincing, lung samples were digested with collagenase (Sigma) and DNase (Sigma), and subsequently filtered to obtain single-cell suspensions. As negative controls, single-cell suspensions from naive rats were run in parallel. From each sample, 500,000 cells were analyzed and the number of events falling in the PKH26 fluorescence channel (Phycoerythrin – PE channel) was recorded. No positive events were found in the PKH26 fluorescence window with negative control cells. The total number of $PKH26^+$ cells in each tissue was calculated by relating the number of $PKH26^+$ events in 500,000 cells to the total number of cells in each tissue and the percentage of $PKH26^+$ MSC/total MSC infused was calculated as follows: number of $PKH26^+$ cells in each tissue/total number of infused PKH26-labeled MSC (500,000) × 100. On FACS analysis, dead cells were excluded using Sytox dead cell stain (Invitrogen). Flow cytometry was performed using FACScan (Becton Dickinson) and CellQuest - Pro software (Becton Dickinson).

Statistical Analysis

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 nonnormally 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 G, Hayes M, Shaw G, Ryan A, Barry F, O'Brien T, O'Toole T, Laffey JG: Mesenchymal stem cells enhance recovery and repair following ventilator induced lung injury in the rat. Thorax 2012; 67:496-501
2. 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 after ventilator-induced lung injury in the rat. ANESTHESIOLOGY 2011; 115:1022-32
3. Meirelles Lda S, Nardi NB: Murine marrow-derived mesenchymal stem cell: Isolation, *in vitro* expansion, and characterization. Br J Haematol 2003; 123:702-11
4. 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
5. Phinney DG, Kopen G, Isaacson RL, Prockop DJ: Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: Variations in yield, growth, and differentiation. J Cell Biochem 1999; 72:570-85
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. Adamson IY, Bakowska J: Relationship of keratinocyte growth factor and hepatocyte growth factor levels in rat lung lavage fluid to epithelial cell regeneration after bleomycin. Am J Pathol 1999; 155:949-54
11. 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