Combined Mesenchymal Stromal Cell Therapy and ECMO in ARDS: A Controlled Experimental Study in Sheep


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Combined mesenchymal stromal cell therapy and extracorporeal membrane oxygenation in Acute Respiratory Distress Syndrome: A controlled experimental study in sheep


Online Supplement

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A. Sheep preparation

Female sheep (*Ovis aries*, Border Leicester Cross), aged 1-3 years, were selected at random from a flock of healthy farm reared animals. Sheep were transferred to the animal research facility at least two weeks before use. They could graze freely in an outdoor enclosure and underwent veterinary inspection, including testing for common ovine pathogens (e.g. Q fever). On the day before experimentation, animals were housed in an indoor pen with free access to water and were denied solid feed for 12 hours. On the morning of experimentation, sheep were transported to the operating theatre in a specially modified sling.

Under local anesthesia (5-10 mL 2% lignocaine, Pfizer, Sydney, NSW, Australia), a quadrilumen central venous catheter (Arrow, Teleflex Medical Australia, Mascot, NSW, Australia) was inserted into the left external jugular vein (EJV), after which baseline blood samples were obtained. An 8 Fr percutaneous introducer sheath (Arrow, Teleflex Medical Australia, Mascot, NSW, Australia) was inserted cephalad to the central venous catheter in the left EJV. Two 8 Fr percutaneous introducer sheaths were then inserted, approximately 8 cm apart, in the right EJV. All lines were secured with 1-0 silk sutures. Once vascular access was secured, animals were pre-oxygenated for 3 minutes using a facemask and a Mapleson C anesthetic circuit set to deliver 15 L O<sub>2</sub> min<sup>-1</sup>. General anesthesia was then induced as described below. Animals were transferred to an operating table and positioned supine. A surgical tracheostomy was performed, by midline longitudinal incision exposing the space between the 2<sup>nd</sup>/3<sup>rd</sup> tracheal rings. A size 9.0 Portex tracheostomy tube (Smith’s Medical Australia, Sydney, NSW, Australia) was inserted, and positioning was confirmed using a flexible video bronchoscope (aScope™, Ambu, Ballerup, Denmark). Thereafter, the tracheostomy was suctioned hourly using an in-line suction catheter.
B. Supportive care protocol

Experienced medical and nursing staff, with training in the prevision of critical care and ECMO, were present for the duration of each experiment. Expert large animal veterinarian advice was available upon request.

Anesthesia/analgesia

General anesthesia was induced by intravenous injection of midazolam (0.5 mg/Kg, Pfizer, Sydney, NSW, Australia) and ketamine (5 mg/kg, Troy Laboratories, Sydney, NSW, Australia). Animals were orotracheally intubated (size 9.0-10.0 ID Portex endotracheal tube, Smith’s Medical Australia, Sydney, NSW, Australia) and connected to a mechanical ventilator. Maintenance of anesthesia/analgesia was achieved by intravenous infusion of midazolam (0.5-0.8 mg/kg/hr, Pfizer, Sydney, NSW, Australia), ketamine (5-7.5 mg/kg/hr, Troy Laboratories, Sydney, NSW, Australia), and fentanyl (5-10 mcg/kg/hr, Hameln Pharmaceuticals, Hameln, Germany). Depth of anesthesia was assessed prior to neuromuscular blockade by abolition of the eyelash reflex, after which attention was paid to clinical indicators such as heart rate and salivation. Total intravenous anesthesia was maintained until euthanasia.

Once maintenance anesthesia was established, animals were paralysed with an intravenous bolus of vecuronium (50 mcg/kg, Pfizer, Sydney, NSW, Australia). Adequacy of neuromuscular blockade (NMB) was assessed using the train of four (TOF) response to peripheral nerve stimulation. NMB was maintained through the course of the experiment by an intravenous infusion of vecuronium (50 mcg/kg/hr, Pfizer, Sydney, NSW, Australia).

Mechanical ventilation

In the period between induction of anesthesia and the commencement of VV-ECMO, animals were ventilated according to a protocolized lung-protective strategy (Table A). In a volume-controlled mode, the ventilator was initially set to achieve a tidal volume ($V_t$) of 6
mL/kg actual body weight (ABW). One of, a Hamilton Galileo (Hamilton Medical, Bonaduz, Switzerland) or a Puritan Bennett 840 (Puritan Bennett, Medtronic, Dublin, Ireland) ventilator was used for the full course of each study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
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<tr>
<td>Mode</td>
<td>Volume controlled</td>
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<tr>
<td>Tidal Volume ($V_t$)</td>
<td>4 - 6 mL/kg</td>
</tr>
<tr>
<td>Respiratory Rate</td>
<td>$\leq$ 35 breaths per minute$^a$</td>
</tr>
<tr>
<td>FiO$_2$</td>
<td>Lowest required for SpO$_2$ 88-93%$^b$</td>
</tr>
<tr>
<td>PEEP</td>
<td>Adjusted to maintain $P_{plat}$ 28-30 cmH$_2$O$^c$</td>
</tr>
<tr>
<td>Plateau Pressure ($P_{plat}$)</td>
<td>$\leq$ 30 cmH$_2$O</td>
</tr>
<tr>
<td>I:E Ratio</td>
<td>1:1 to 3</td>
</tr>
</tbody>
</table>

**Table A.** Pre-ECMO ventilatory strategy.

$^a$ Adjusted to maintain pH 7.30-7.45. Permissive hypercapnia was tolerated to a minimum pH of 7.15.

$^b$ PaO$_2$ 55 – 80 mmHg. If despite an FiO$_2$ of 1.0 oxygenation targets were not met, PEEP was increased, maintaining $P_{plat} \leq 32$ cmH$_2$O.

$^c$ Total PEEP (extrinsic PEEP + intrinsic PEEP) did not exceed 20 cmH$_2$O. PEEP was permitted to be reduced to 5 cmH$_2$O to maintain $P_{plat} \leq 30$ cmH$_2$O. If despite a PEEP of 5 cmH$_2$O, $P_{plat} > 30$ cmH$_2$O, $V_t$ was reduced in 1 mL/kg steps until set at 4 mL/kg.

FiO$_2$ – Fraction of inspired oxygen, SpO$_2$ – Peripheral oxygen saturation, PEEP – Positive end-expiratory pressure, $P_{plat}$ – Airway plateau pressure, I:E Ratio – Inspiratory to expiratory ratio

**Fluid and electrolyte management**

After the induction of general anesthesia, a maintenance infusion of crystalloid (1-2 mL/kg/hr, compound sodium lactate, Baxter, Sydney, NSW, Australia) was commenced for
the duration of the study. Fluid therapy was indicated at other times to support hemodynamics or ECMO flows, in this case 100 mL boluses of compound sodium lactate were titrated to effect.

Electrolyte levels were assessed 4-hourly. Serum potassium levels were maintained > 3.5 mmol/L by i.v. infusion of 10-20 mmol potassium chloride (AstraZeneca, Sydney, NSW, Australia). In the event of hyperkalemia (K⁺ > 5.5 mmol/L), supplemental potassium administration was ceased and 10 mL 10% calcium gluconate (B. Braun Melsungen AG, Melsungen, Germany) was given by i.v. bolus injection. Hyperchloremia (Cl⁻ >103) was avoided. All large volume gastric losses were returned via the orogastric tube. Blood glucose levels were measured hourly and maintained > 1.5 mmol/L by infusion of 50% glucose (Baxter, Sydney, NSW, Australia).

**Monitoring and hemodynamic management**

All animals were monitored according to the ‘*Recommendations for standards of monitoring during anaesthesia and recovery 2015: Association of Anaesthetists of Great Britain and Ireland*’, including; pulse oximetry, 3-lead electrocardiogram, and continuous waveform capnography. In addition, the central venous catheter (CVC) was transduced as a measure of central venous pressure.

Following induction of general anesthesia and repositioning to the operating table, invasive arterial blood pressure monitoring was established by cannulation of the left facial artery (20G Leadercath arterial, Vygon, Écouen, France). Using the 8 Fr percutaneous sheath introducer positioned in the left EJV, a pulmonary artery catheter (PAC) was inserted (7.5 Fr Swan-Ganz CCOmbo, Edwards Life Sciences, Irvine, CA, USA). The catheter was inserted to a depth of 15 cm after which the flotation balloon was inflated. The catheter was directed to the pulmonary artery, the balloon was deflated, and the catheter secured. Positioning was
confirmed by obtaining a satisfactory waveform. Continuous cardiac output monitoring (Vigilance monitor, Edwards Lifesciences, Irvine, CA, USA) was instituted, with cardiac index estimated by; cardiac output/BSA [weight (kg)\(^{0.67}\) x 0.0842]. Continuous measurements of core body temperature were also obtained from the PAC.

A 14 Fr urinary catheter (Gildana Healthcare, Oakleigh, VIC, Australia) was inserted and connected to an hourly urometer to allow for accurate quantification of urine output. A 16 Fr orogastric tube (ConvaTec, Glen Waverley, VIC, Australia) was inserted and left on free drainage.

A mean arterial pressure ≥ 65 mmHg was targeted. In the face of sustained hypotension, repeated 100 mL boluses of compound sodium lactate (Baxter, Sydney, NSW, Australia) were given until; CVP ≥ 8 mmHg and/or the animal was no longer fluid responsive (failure to increase stroke volume > 10% by PAC). Thereafter, noradrenaline (80 mcg/mL in 5% dextrose, Hospira, Lake Forrest, IL, USA) was commenced at 80 mcg/min and the dose titrated at 5-minute intervals. If noradrenaline requirements reached 2400 mcg/min, vasopressin (PPC, Richmond Hill, Canada) was commenced at 0.8 units/hr and increased to a maximum of 1.8 units/hr.

**Euthanasia**

At the end of each study, animals were euthanised by i.v. injection of phenobarbitone (142.5 mg/kg, Aspen Pharma, Dandenong, NSW, Australia). After death was confirmed (absence of cardiac electrical activity, blood pressure, and cardiac output monitoring), organs were retrieved surgically. Animal carcasses were stored and subsequently disposed of by incineration.
C. Model of experimental ARDS

After instrumentation, in a supine position, animals were injured using a combination of intravenous oleic acid (OA) and endobronchial \textit{E. coli} lipopolysaccharide (LPS).

\textbf{Oleic acid preparation}

A total dose of 0.06 ml/kg OA was used to induce injury. Firstly, 0.03 mL/kg OA (O1008, Sigma-Aldrich, Castle Hill, NSW, Australia) was suspended in 20 mL arterial blood and 150 IU porcine heparin (Pfizer, Sydney, NSW, Australia). This mixture was administered via the distal port of the right EJV central venous catheter, followed by a flush of 50 mL 0.9% sodium chloride (Baxter, Sydney, NSW, Australia). The animal could recover and after 15 minutes this procedure was repeated. When 15 minutes elapsed from the second dose, arterial blood gas analysis, at an FiO$_2$ of 1.0 and a PEEP of 10 cmH$_2$O, was used to confirm a PaO$_2$/FiO$_2$ ratio <100 mmHg.

\textit{E. coli} lipopolysaccharide preparation

Immediately after a PaO$_2$/FiO$_2$ ratio <100 mmHg was confirmed, 50 mcg \textit{E. coli} LPS (O55:B5, Sigma-Aldrich, Castle Hill, NSW, Australia), diluted in 10 mL 0.9% sodium chloride (Baxter, Sydney, NSW, Australia), was administered, via a designated video bronchoscope, to each main bronchus.
Online supplement – Combined MSC therapy and ECMO in experimental ARDS  JE Millar et. al.

D. Veno-venous ECMO

Equipment

ECMO was performed using a Maquet ROTAFLOW centrifugal pump (Maquet, Getinge Group, Rastatt, Germany) along with a Maquet Permanent Life Support (PLS) set (Maquet, Getinge Group, Rastatt, Germany). Circuits were primed in a sterile fashion (prime volume ~ 585 mL) with compound sodium lactate (Baxter, Sydney, NSW, Australia).

Cannulation

Cannulation attempts were commenced after injury was confirmed (T0). Two 19 Fr Bio-medicus single-stage venous catheters (Medtronic, Dublin, Ireland) were used in a right sided V_EJ – V_EJ configuration. The access cannula was inserted by first re-wiring the proximal right EJV percutaneous sheath and was then positioned in the inferior vena cava at the level of the diaphragm. The return cannula was inserted by first re-wiring the distal right EJV percutaneous sheath and was then positioned with the tip at the cavo-atrial junction. Cannula insertion and positioning were guided by fluoroscopy and real-time intra-cardiac and intra-cannular echocardiography. Cannulae were flushed with heparinised 0.9% sodium chloride (10 IU/mL porcine heparin, Pfizer, NSW, Australia). Cannulae were secured by suturing to the skin. Animals were then placed in sternal recumbency for the remaining duration of the study.

Management

Once cannulation was successfully completed and positioning was confirmed, VV-ECMO was commenced (T1). Pump speeds were slowly increased to 1000 rpm before clamp release. Pump speeds were then adjusted to achieve flow rates of ~2/3 CO (typically between 60-90 mL/kg). Fresh gas flow (FGF) was initially set at 80% of pump flow and adjusted based on arterial blood gas analysis. The FGF blender was set to deliver 100% O_2 at all times. During ECMO, PaO_2 will be maintained ≥ 65 mmHg and PaCO_2 ≤ 45 mmHg. Hyperoxia (PaO_2 > 150
mmHg) will be avoided. A water heater-cooler unit was connected to the membrane oxygenator and set to 38°C for the duration of the ECMO run.

At the beginning of VV-ECMO, animals received a bolus dose of unfractionated heparin (20 IU/kg porcine heparin, Pfizer, Sydney, NSW, Australia), when the activated clotting time (ACT) fell below 160 s an infusion was commenced at 4 IU/kg/hr. An ACT of 180 s-210 s was targeted. ACT was measured prior to ECMO, at 1 hour, 2 hours, and every 2 hours thereafter. The unfractionated heparin infusion was titrated as per a pre-defined protocol (Table B).

<table>
<thead>
<tr>
<th>ACT</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 130</td>
<td>Bolus 10 IU/kg and increase infusion by 1.5 IU/kg/hr</td>
</tr>
<tr>
<td>130 - 150</td>
<td>Increase infusion by 1 IU/kg/hr</td>
</tr>
<tr>
<td>150 - 180</td>
<td>Increase infusion by 0.5 IU/kg/hr</td>
</tr>
<tr>
<td>180 - 210</td>
<td>No change</td>
</tr>
<tr>
<td>210 – 250</td>
<td>Decrease infusion by 1 IU/kg/hr</td>
</tr>
<tr>
<td>&gt; 250</td>
<td>Cease infusion for 1 hr and recheck ACT, restart when ACT &lt; 210 AND decrease infusion by 1.5 IU/kg/hr</td>
</tr>
</tbody>
</table>

Table B. ECMO anti-coagulation titration protocol.

**Mechanical ventilation during ECMO**

Once VV-ECMO was established, mechanical ventilation was adjusted to target an ultra-protective ventilatory strategy, consistent with that described in the EOLIA trial (1). Neuromuscular blockade was maintained throughout. Ventilator settings and targets are detailed further in Table C.
Termination and return to pre-ECMO mechanical ventilation

VV-ECMO was halted 23 hours after injury (T23). Pump speeds were gradually reduced to zero and both cannulae were clamped. Pump blood was returned by unclamping the return cannula and gently flushing the circuit with Compound Sodium Lactate (~585 mL blood returned). Clamped cannulae were left in situ until termination of the study. Immediately before cessation of VV-ECMO, a standardised recruitment manoeuvre was performed. The recruitment manoeuvre involved a 40 s breath hold at an airway pressure of 40 cmH\textsubscript{2}O, in a pressure-controlled mode, with an FiO\textsubscript{2} of 1.0. After which animals were returned to standardised pre-ECMO ventilator settings (with an FiO\textsubscript{2} of 1.0) for 60 minutes at which time the trial was terminated.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>Volume controlled</td>
</tr>
<tr>
<td>Tidal Volume</td>
<td>(\leq 4) ml/kg</td>
</tr>
<tr>
<td>Respiratory Rate</td>
<td>8 breaths per minute</td>
</tr>
<tr>
<td>FiO\textsubscript{2}</td>
<td>0.30</td>
</tr>
<tr>
<td>PEEP</td>
<td>(\geq 10) cmH\textsubscript{2}O</td>
</tr>
<tr>
<td>Pplat</td>
<td>(\leq 25) cmH\textsubscript{2}O</td>
</tr>
<tr>
<td>I:E Ratio</td>
<td>1:1 to 1:3</td>
</tr>
</tbody>
</table>

**Table C.** ECMO ventilatory strategy.
E. Human mesenchymal stromal cells

Induced pluripotent stem cell (iPSC) derived human mesenchymal stromal cells (hMSCs) were obtained from Cynata Therapeutics Ltd. (CYP-001; Cynata Therapeutics Ltd., Melbourne, VIC, Australia). Cells were manufactured under Good Manufacturing Practices (GMP) conditions and were > 99% positive for CD-73, CD-90, and CD-105, but negative for CD-31 and CD-45. iPSC-derived hMSCs were between passage 3 and 5. iPSC derived hMSCs were presented in discrete batches of 1 x 10^8 cells, suspended in 20 mL of a carrier vehicle (57.5% Plasmalyte-A, 40% Flexbumin 25%, 2.5% Dimethyl sulfoxide). Cells were shipped to our laboratory stored in the vapour phase of liquid nitrogen at -80°C where this was maintained until use.

Cell preparation

Two hours before administration, cryopreserved cells in their carrier vehicle were thawed in a water bath at 37.5°C, agitated to prevent clumping, and decanted into a sterile container. Batches were sampled and assessed for viability (> 95%) prior to use.

Cell administration

Animals allocated to the treatment group received a fixed endobronchial dose of 3 x 10^8 cells (suspended in 60 mL of carrier vehicle), via a flexible video bronchoscope (aScope™, Ambu, Ballerup, Denmark). Those allocated to the control group, received an equal volume of cell-free carrier vehicle, administered in an identical fashion. Common to both groups, 25 mL of solution was administered to the left diaphragmatic lobar bronchus and 5 mL to the left apico-cardiac lobar bronchus, with a further 25 mL administered via the right diaphragmatic lobar bronchus and 5 mL via the right cardiac lobar bronchus. The right apical lobar bronchus, which arises in sheep directly from the trachea at approximately the level of the third rib (2),
was untreated. After each instance of cell delivery, the channel of the bronchoscope was flushed with 5 mL of 0.9% sodium chloride (Baxter, Sydney, NSW, Australia).
F. Clinical measurements

Hemodynamic and ventilatory data (including data derived from the PAC) were continuously monitored and automatically recorded at 5-minute intervals using a data monitoring system (Solar 8000, GE Healthcare, Waukesha, WI, USA) coupled with custom software. Pressure readings across the membrane oxygenator were made using a silicone-based pressure transducer (Omega Engineering, Norwalk, CT, USA), these data were recorded continuously using LabVIEW software (National Instruments, Austin, TX, USA). ECMO flow readings and pump revolutions were read and recorded on an hourly basis from the ROTAFLOW console (Maquet, Getinge Group, Rastatt, Germany). Urine and oro-gastric outputs were recorded on a pre-piloted observation chart on an hourly basis, while continuous temperature data were taken from the PAC.
G. Blood and bronchoalveolar lavage fluid analyses

Blood

Whole blood was sampled from the facial artery catheter. Arterial blood gas analysis was undertaken on at least an hourly basis (ABL800 FLEX, Radiometer, Copenhagen, Denmark). At baseline, T-1, T0, T1-4, T6, T14, T23, and T24, blood was sampled for routine laboratory hematological and biochemical testing. These tests were undertaken in a blinded fashion by an accredited veterinary pathology laboratory (IDEXX Laboratories Pty. Ltd., Brisbane, QLD, Australia). At baseline, T-1, T0, T1-4, T6, T14, T23, and T24, blood was sampled for plasma cytokine measurements. The concentration of IL-6, IL-1β, IL-8 and IL-10 in plasma and bronchoalveolar lavage (BAL) fluid was quantified by in-house ELISAs using methods published previously (3-5). Positive internal controls were used to ensure that inter- and intra-plate variability was < 10% and confirm the precision and accuracy of all ELISA assays.

Bronchoalveolar lavage fluid

BAL was undertaken by an experienced bronchoscopist using a video bronchoscope (aScope™, Ambu, Ballerup, Denmark). At each examination the right and left middle and lower lobes were sampled. Each lobe was injected with 20 mL sterile 0.9% sodium chloride (Baxter, Sydney, NSW, Australia) and gentle suction was applied. The lavage fluid was collected in a sterile universal container. BAL fluid was centrifuged, and the supernatant collected for ELISA analysis. Aliquots were also cytopun onto slides for inflammatory cell counts.
H. Histological analyses and lung tissue gene expression

After the confirmation of death, the heart and lungs were retrieved en bloc via a midline sternotomy. Lung tissue was set on ice and portions of left and right lower and upper lobe lung tissue were fixed in 10% buffered formalin for at least 24 hours, processed and embedded in paraffin. All sections (5 µm) were stained with haematoxylin and eosin. Sections were examined by an independent, blinded, veterinary pathologist and scored for features of lung injury (6).

Total RNA was isolated from lung tissue using the Invitrogen PureLink® RNA Mini Kit (Thermo Fisher Scientific, VIC, Australia). All samples were DNase treated (Invitrogen DNA-free™ DNA Removal kit; Thermo Fisher Scientific) and the concentration of RNA determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). First strand cDNA was synthesized from 1µg of RNA (iScript™ Select cDNA Synthesis kit; Bio-Rad, NSW, Australia).

A non-exhaustive panel of genes associated with epithelial, endothelial, and coagulative function were analysed. These genes have been associated with dysfunction in ARDS. In addition, a panel of matrix metalloproteinase genes and their regulators were analysed. Realtime Quantitative PCR was performed using primers for MMP1, MMP2, MMP7, TIMP1, TIMP2, AGER, VEGFA, ANGPT2, F3, vWF and SFTPD (PrimerDesign, Southampton, UK) with PrecisionFAST qPCR SYBR Green Master Mix with low ROX (Primer Design). Reactions consisted of 10 µL SYBR Green Master Mix, 1 µL mixed primers (300 nM), and 5 µL of cDNA (equivalent to 50 ng) and nuclease free water to a final volume of 20 µL. The cycling conditions were as follows: cDNA was denatured at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. Melt curve analysis was programmed at the end of the run, 60–90°C with increments rising by 0.5°C each step and a 5 s hold at each degree to
determine reaction specificity and the absence of contamination, mispriming and primer dimer. A no-template control was included for each primer set. An ovine specific SYBR Green reference gene assay (Primer Design) was used to evaluate the stability of six candidate normalization genes (GAPDH, RPL19, β-2M, ACTB, RPS26, YWHAZ) using real-time quantitative PCR. The Qbase PLUS program was used to identify the most stably expressed housekeeping genes and all data was subsequently normalized to geomean of GAPDH and RPL19.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Forward primer (5'→3')</th>
<th>Reverse primer (5'→3')</th>
<th>Amplicon size (bp)</th>
<th>PCR Efficiencya</th>
</tr>
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<tbody>
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<td>MMP1</td>
<td>Matrix metallopeptidase 1</td>
<td>CGTGACTCAGTTGTCCCTACTCC</td>
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<td>1.95</td>
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<tr>
<td>MMP7</td>
<td>Matrix metallopeptidase 7</td>
<td>TCCCAACCAGATATAAGAACAATG</td>
<td>GGAGACGCATAGATAGTAAACAGA</td>
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<td>1.99</td>
</tr>
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<td>TIMP1</td>
<td>Tissue inhibitor of metallopeptidase 1</td>
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<td>GCAGGGGTGTAAGATGAATCG</td>
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<td>Tissue inhibitor of metallopeptidase 2</td>
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<td>CTGGTCAGGTCCTTTGAACAT</td>
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<td>AGER</td>
<td>Advanced glycosylation end-product specific receptor</td>
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<td>GGCAGGTGTTTACTCATCACTTTTC</td>
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<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
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<td>Angiopoietin 2</td>
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<td>Amplicon size (bp)</td>
<td>PCR Efficiency&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>F3</td>
<td>Coagulation factor III, tissue factor</td>
<td>AAGCCAGATTA TCTAAGGAAAG ACAAA</td>
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<td>von Willebrand factor</td>
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<td>CCAGGTAAGG GACAGAGACC A</td>
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<td>Surfactant protein D</td>
<td>CCTCCATGTCT ACCAACCATCG</td>
<td>CTGAGCAGCC AGGAAGATAA GAA</td>
<td>109</td>
<td>2.08</td>
</tr>
</tbody>
</table>

Table D. Primers used in qPCR analysis.

<sup>a</sup> PCR efficiencies were determined using the formula $10^{-1/slope}$
I. Analyses of membrane oxygenators

At the cessation of VV-ECMO (T23) the circuit was disassembled and the membrane oxygenator (MO) was recovered. The MO was rinsed with 0.9% sodium chloride (Baxter, Sydney, NSW, Australia) until the effluent was observed to be clear, after which the MO was infused with 500 mL formaldehyde and then sealed. MOs were stored at 5°C overnight and then opened using a commercially available band saw. The fiber bundle was extracted, and approximate 5 cm x 5 cm sections were taken from the heat and gas exchange sections. These were stored in sterile containers in Phosphate Buffered Saline (PBS) until they were analyzed.

For immunohistochemical detection of MSCs, approximately 1 cm x 1 cm fiber sections were blocked for 3 hours in 2% HISS (heat inactivated sheep serum) and 0.5% triton X-100 in PBS. The samples were washed 3 times in PBS for 5 minutes each, followed by overnight incubation with primary antibodies at 4°C. After primary incubation, the fiber sections were washed 3 times in PBS for 5 minutes each, incubated in secondary antibody solution for 1 hour at room temperature, and then washed an additional 3 times in PBS for 5 minutes each. Primary antibodies: mouse Ab to CD105-FITC (ab18278, 1:10), rabbit Ab to CD73 (ab175396, 1:50), goat Ab to CD90 (ab189367, 1:100). Secondary antibodies: donkey anti-mouse 488 (Invitrogen, 1:100), donkey anti-rabbit 647 (Invitrogen, 1:100), donkey anti-goat 568 (Invitrogen, 1:100). Nuclei were counterstained with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich, NSW, Australia) during secondary incubation. All antibodies were diluted in blocking solution during primary and secondary incubations. A widefield Nikon deconvolution microscope was used to acquire images using both 4X and 20X objectives. Final images were processed with ImageJ software.
J. Sample size calculation

Prior to the study commencing, a sample size calculation was undertaken based on a primary outcome of improvement in PaO₂/FiO₂ ratio. Using data from two previous trials of MSCs in ovine models of ARDS (7, 8), we calculated that 7 animals per group were required to detect a 100 mmHg difference in PaO₂/FiO₂ ratio (alpha 0.05, power 0.9, outcome SD 42 mmHg, paired test).
J. Results supplement

<table>
<thead>
<tr>
<th></th>
<th>Overall (n=14)</th>
<th>Carrier vehicle (n=7)</th>
<th>hMSCs (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At time of injury (T0)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/L)</td>
<td>106.7 ± 8.3</td>
<td>112.6 ± 7.1</td>
<td>100.9 ± 4.3</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.322 ± 0.030</td>
<td>0.339 ± 0.032</td>
<td>0.306 ± 0.010</td>
</tr>
<tr>
<td>White cell count (x10^9/L)</td>
<td>1.8 (1.0-2.3)</td>
<td>1.6 (1.0-2.1)</td>
<td>2.0 (1.1-3.0)</td>
</tr>
<tr>
<td>Neutrophil count (x10^9/L)</td>
<td>0.4 (0.2-0.9)</td>
<td>0.3 (0.2-0.4)</td>
<td>0.9 (0.2-1.4)</td>
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<tr>
<td>Lymphocyte count (x10^9/L)</td>
<td>1.1 (0.9-1.4)</td>
<td>1.1 (0.8-1.3)</td>
<td>1.0 (0.9-1.5)</td>
</tr>
<tr>
<td>Monocyte count (x10^9/L)</td>
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<td>0.2 (0.1-0.3)</td>
<td>0.1 (0-0.1)</td>
</tr>
<tr>
<td>Prothrombin time (s)</td>
<td>15.3 ± 1.6</td>
<td>16.3 ± 1.3</td>
<td>14.5 ± 1.3</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (s)</td>
<td>24.6 ± 2.5</td>
<td>24.0 ± 3.0</td>
<td>25.2 ± 2.0</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>139 ± 3</td>
<td>140 ± 2</td>
<td>137 ± 3</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>3.6 (3.3-3.9)</td>
<td>3.6 (3.3-4.0)</td>
<td>3.5 (3.4-3.8)</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>106 (105-106)</td>
<td>106 (105-107)</td>
<td>105 (103-106)</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>5.7 ± 1.1</td>
<td>5.9 ± 1.0</td>
<td>5.5 ± 1.3</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>0.07 (0.06-0.07)</td>
<td>0.07 (0.06-0.07)</td>
<td>0.06 (0.06-0.07)</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>26 ± 2</td>
<td>26 ± 2</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>3 (1-4)</td>
<td>3 (3-5)</td>
<td>1 (1-3)</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>102 ± 26</td>
<td>89 ± 22</td>
<td>114 ± 23</td>
</tr>
<tr>
<td>Aspartate aminotransferase (IU/L)</td>
<td>83 (72-97)</td>
<td>82 (72-95)</td>
<td>84 (73-103)</td>
</tr>
<tr>
<td>Creatinine kinase (IU/L)</td>
<td>205 (205-242)</td>
<td>242 (200-299)</td>
<td>191 (162-255)</td>
</tr>
</tbody>
</table>

Supplementary Table E1. Baseline hematological and biochemical characteristics.
<table>
<thead>
<tr>
<th></th>
<th>Overall (n=14)</th>
<th>Carrier vehicle (n=7)</th>
<th>hMSCs (n=7)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/L)</td>
<td>78.9 ± 14.1</td>
<td>78.6 ± 18.0</td>
<td>79.3 ± 7.1</td>
<td>0.930</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.249 ± 0.040</td>
<td>0.247 ± 0.050</td>
<td>0.252 ± 0.023</td>
<td>0.855</td>
</tr>
<tr>
<td>White cell count (x10^9/L)</td>
<td>1.1 (0.8-1.4)</td>
<td>1.1 (0.9-2.2)</td>
<td>1.2 (0.7-1.3)</td>
<td>0.606</td>
</tr>
<tr>
<td>Neutrophil count (x10^9/L)</td>
<td>0.2 (0.2-0.5)</td>
<td>0.2 (0.2-0.9)</td>
<td>0.4 (0.1-0.5)</td>
<td>0.494</td>
</tr>
<tr>
<td>Lymphocyte count (x10^9/L)</td>
<td>0.7 ± 0.3</td>
<td>0.8 ± 0.4</td>
<td>0.6 ± 0.2</td>
<td>0.243</td>
</tr>
<tr>
<td>Monocyte count (x10^9/L)</td>
<td>0.1 (0.1-0.3)</td>
<td>0.1 (0.1-0.3)</td>
<td>0.2 (0.1-0.2)</td>
<td>0.779</td>
</tr>
<tr>
<td>Prothrombin time (s)</td>
<td>28.3 ± 5.7</td>
<td>27.8 ± 5.3</td>
<td>29.1 ± 6.3</td>
<td>0.755</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (s)</td>
<td>45.5 ± 7.0</td>
<td>44.3 ± 7.9</td>
<td>47.8 ± 4.3</td>
<td>0.482</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>140 (137-141)</td>
<td>141 (138-142)</td>
<td>140 (136-141)</td>
<td>0.331</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>4.4 ± 0.7</td>
<td>4.4 ± 0.6</td>
<td>4.3 ± 0.7</td>
<td>0.857</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>111 (109-112)</td>
<td>112 (110-113)</td>
<td>111 (109-112)</td>
<td>0.331</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>4.5 ± 1.2</td>
<td>4.7 ± 1.5</td>
<td>4.2 ± 0.8</td>
<td>0.493</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>0.11 ± 0.03</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.654</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>16 ± 2</td>
<td>16 ± 2</td>
<td>15 ± 2</td>
<td>0.355</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>18 ± 5</td>
<td>19 ± 6</td>
<td>17 ± 4</td>
<td>0.436</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>54 ± 15</td>
<td>56 ± 20</td>
<td>53 ± 8</td>
<td>0.735</td>
</tr>
<tr>
<td>Aspartate aminotransferase (IU/L)</td>
<td>171 (144-207)</td>
<td>200 (155-247)</td>
<td>143 (138-196)</td>
<td>0.259</td>
</tr>
<tr>
<td>Creatinine kinase (IU/L)</td>
<td>7498 ± 4079</td>
<td>8990 ± 4233</td>
<td>6006 ± 3303</td>
<td>0.482</td>
</tr>
</tbody>
</table>

**Supplementary Table E2.** Hematological and biochemical characteristics at the end of ECMO (T23).

* P values corrected for multiple comparison.
<table>
<thead>
<tr>
<th></th>
<th>Overall (n=14)</th>
<th>Carrier vehicle (n=7)</th>
<th>hMSCs (n=7)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haemoglobin (g/L)</strong></td>
<td>87.5 ± 13.6</td>
<td>86.0 ± 12.8</td>
<td>89.0 ± 14.2</td>
<td>0.733</td>
</tr>
<tr>
<td><strong>Haematocrit</strong></td>
<td>0.268 ± 0.040</td>
<td>0.265 ± 0.039</td>
<td>0.270 ± 0.041</td>
<td>0.846</td>
</tr>
<tr>
<td><strong>White cell count (x10^9/L)</strong></td>
<td>1.2 (1.0-1.6)</td>
<td>1.6 (1.1-2.0)</td>
<td>1.2 (0.9-1.3)</td>
<td>0.266</td>
</tr>
<tr>
<td><strong>Neutrophil count (x10^9/L)</strong></td>
<td>0.4 (0.1-0.6)</td>
<td>0.4 (0.2-0.8)</td>
<td>0.4 (0.1-0.6)</td>
<td>0.381</td>
</tr>
<tr>
<td><strong>Lymphocyte count (x10^9/L)</strong></td>
<td>0.7 (0.7-1.0)</td>
<td>1.0 (0.8-1.1)</td>
<td>0.7 (0.6-0.7)</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>Monocyte count (x10^9/L)</strong></td>
<td>0.1 (0.1-0.1)</td>
<td>0.2 (0-0.3)</td>
<td>0.1 (0.1-0.1)</td>
<td>0.433</td>
</tr>
<tr>
<td><strong>Prothrombin time (s)</strong></td>
<td>29.5 ± 5.0</td>
<td>31.5 ± 5.9</td>
<td>27.9 ± 3.3</td>
<td>0.346</td>
</tr>
<tr>
<td><strong>Activated partial thromboplastin time (s)</strong></td>
<td>47.3 ± 4.5</td>
<td>47.7 ± 3.3</td>
<td>47.0 ± 5.2</td>
<td>0.831</td>
</tr>
<tr>
<td><strong>Sodium (mmol/L)</strong></td>
<td>139 (138-141)</td>
<td>140 (138-142)</td>
<td>139 (135-140)</td>
<td>0.139</td>
</tr>
<tr>
<td><strong>Potassium (mmol/L)</strong></td>
<td>4.4 ± 0.5</td>
<td>4.5 ± 0.5</td>
<td>4.4 ± 0.6</td>
<td>0.886</td>
</tr>
<tr>
<td><strong>Chloride (mmol/L)</strong></td>
<td>111 ± 4</td>
<td>112 ± 5</td>
<td>110 ± 2</td>
<td>0.392</td>
</tr>
<tr>
<td><strong>Urea (mmol/L)</strong></td>
<td>4.3 ± 1.0</td>
<td>4.4 ± 1.2</td>
<td>4.2 ± 0.8</td>
<td>0.839</td>
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<tr>
<td><strong>Creatinine (mmol/L)</strong></td>
<td>0.11 ± 0.03</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.656</td>
</tr>
<tr>
<td><strong>Albumin (g/L)</strong></td>
<td>17 ± 2</td>
<td>17 ± 2</td>
<td>17 ± 1</td>
<td>0.896</td>
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<tr>
<td><strong>Bilirubin (µmol/L)</strong></td>
<td>19 ± 8</td>
<td>19 ± 9</td>
<td>18 ± 7</td>
<td>0.960</td>
</tr>
<tr>
<td><strong>Alkaline phosphatase (IU/L)</strong></td>
<td>57 ± 18</td>
<td>53 ± 24</td>
<td>58 ± 10</td>
<td>0.992</td>
</tr>
<tr>
<td><strong>Aspartate aminotransferase (IU/L)</strong></td>
<td>190 (162-222)</td>
<td>185 (164-217)</td>
<td>191 (161-221)</td>
<td>0.931</td>
</tr>
<tr>
<td><strong>Creatinine kinase (IU/L)</strong></td>
<td>8008 ± 3351</td>
<td>8858 ± 2734</td>
<td>7157 ± 3679</td>
<td>0.415</td>
</tr>
</tbody>
</table>

**Supplementary Table E3.** Hematological and biochemical characteristics at T24.
* P values corrected for multiple comparison.
<table>
<thead>
<tr>
<th></th>
<th>Carrier vehicle (n=7)</th>
<th>hMSCs (n=7)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAL Monocytes/macrophages</strong> (n/200 cells ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-1</td>
<td>194 ± 4</td>
<td>193 ± 5</td>
<td>0.005</td>
</tr>
<tr>
<td>T0</td>
<td>189 ± 8</td>
<td>186 ± 10</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>148 ± 37</td>
<td>163 ± 13</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>100 ± 54</td>
<td>118 ± 36</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>38 ± 28</td>
<td>74 ± 52</td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>33 ± 26</td>
<td>37 ± 28</td>
<td></td>
</tr>
<tr>
<td>T14</td>
<td>37 ± 38</td>
<td>14 ± 13</td>
<td></td>
</tr>
<tr>
<td>T23</td>
<td>21 ± 10</td>
<td>11 ± 12</td>
<td></td>
</tr>
<tr>
<td><strong>BAL Neutrophils</strong> (n/200 cells ± SD)</td>
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<td></td>
<td></td>
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<tr>
<td>T-1</td>
<td>4 ± 4</td>
<td>6 ± 5</td>
<td>0.008</td>
</tr>
<tr>
<td>T0</td>
<td>9 ± 6</td>
<td>12 ± 9</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>51 ± 37</td>
<td>36 ± 9</td>
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<tr>
<td>T3</td>
<td>96 ± 54</td>
<td>80 ± 34</td>
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<tr>
<td>T4</td>
<td>156 ± 29</td>
<td>123 ± 51</td>
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</tr>
<tr>
<td>T6</td>
<td>162 ± 26</td>
<td>158 ± 28</td>
<td></td>
</tr>
<tr>
<td>T14</td>
<td>160 ± 37</td>
<td>184 ± 13</td>
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</tr>
<tr>
<td>T23</td>
<td>178 ± 11</td>
<td>186 ± 13</td>
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</tr>
<tr>
<td><strong>BAL Lymphocytes</strong> (n/200 cells ± SD)</td>
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<td>0.169</td>
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<td>2 ± 3</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>2 ± 3</td>
<td>0 ± 1</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>2 ± 2</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>2 ± 3</td>
<td>0 ± 1</td>
<td></td>
</tr>
<tr>
<td>T14</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>T23</td>
<td>0 ± 1</td>
<td>2 ± 2</td>
<td></td>
</tr>
<tr>
<td><strong>BAL Eosinophils</strong> (n/200 cells ± SD)</td>
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</tr>
<tr>
<td>T3</td>
<td>2 ± 3</td>
<td>2 ± 2</td>
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</tr>
<tr>
<td>T4</td>
<td>4 ± 4</td>
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</tr>
<tr>
<td>T6</td>
<td>3 ± 4</td>
<td>1 ± 2</td>
<td></td>
</tr>
<tr>
<td>T14</td>
<td>3 ± 3</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>T23</td>
<td>2 ± 2</td>
<td>1 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

**Supplementary Table E4.** Bronchoalveolar lavage fluid cell counts.

Pooled BAL from right and left lower lobes. Two hundred cells per count. * P values presented for mixed model group:time interaction.
Supplementary figure E1. Bronchoscopic hMSC delivery

Volumes of carrier fluid instilled and location of delivery within the ovine pulmonary tree.
Figure E2. ECMO management

a. ECMO system variables and gas exchange on ECMO. Points represent individual observations. Dashed lines represent lower (PaO₂) and upper (PaCO₂) targets. b. Anticoagulation during ECMO. Activated partial thromboplastin time (APTT) ratio and heparin dose are presented as mean (± 95% confidence interval).
Figure E3. Lung tissue qPCR

**a.** Matrix metalloproteinases (MMPs) and inhibitors (TIMPS). **b.** Tissue factor (F3). **c.** Epithelial markers. AGER – receptor for advanced glycation endpoints, SPFTD – surfactant protein D. **d.** Endothelial markers. ANGPT2 – angiopoietin-2, VEGFA – vascular endothelial growth factor A, vWF – von Willebrand factor. Results are presented as mean (± 95% confidence interval).
Figure E4. Bronchoalveolar lavage fluid cytokines

Data presented as mean (± 95% confidence interval). Where error bars intersect the x axis the 95% CI includes zero. * - p <0.05.
Figure E5. Plasma cytokines and leukocytes
**a.** Plasma cytokine concentrations. **b.** Leukocyte counts in whole blood. Data presented as mean (± 95% confidence interval). Where error bars intersect the x axis the 95% CI includes zero. * - p < 0.05.
Figure E6. Immunofluorescence of explanted oxygenator fibers.

DAPI - 4',6-diamidino-2-phenylindole.

K. Supplement references


measurements of experimental acute lung injury in animals. *Am J Resp Cell Mol*

2011; 44: 725-738.
