Supporting Information

Lee et al. 10.1073/pnas.0907996106

SI Text

Selection Criteria for Ex Vivo Perfused Human Lung Preparation. Our laboratory received 1–2 human lungs from brain-dead donors from the Northern California Transplant Donor Network weekly. Previously, we found the primary reasons for rejection of these lungs for transplantation were hypoxemia (PaO_2/FiO_2 <300), smoking history >20 pack-years, evidence of infection or aspiration or chest radiographic infiltrates. However, 41% of these rejected lungs were potentially suitable for transplantation by physiological, microbiological and histological methods (1). Consequently, the initial set of exclusion criteria for these experiments were as follows: A lung was rejected for these studies if 1) the total ischemic time exceeded 48 h, 2) the lung contained areas of hemorrhage or consolidation or 3) there was evidence of ALI based on radiographic and oxygenation criteria. The final exclusion criterion was based on whether or not the lung demonstrated normal alveolar fluid clearance after a test solution of an instillate (see below) was instilled into the upper lobe of the lung to be used for these experiments. Approximately 50% of the lungs were excluded based on the exclusion criteria alone.

Ex Vivo Perfused Human Lung and Measurement of Alveolar Fluid Clearance (AFC), Lung Endothelial Permeability to Protein and Wet/Dry Ratio. Either lung (35% right, 65% left) was used. The lung to be used was first separated and the pulmonary artery was cannulated by passing a Foley catheter 2–3 cm into the surgical stump, securing it in place with a purse-string suture. The Foley catheter was then connected to a peristaltic pump via PVC tubing (Nalge). To measure pulmonary artery pressure, a pulmonary artery (PA) catheter (Cook) was passed through a side port in the tubing and advanced to the end of the Foley catheter. The right or left mainstem bronchus was then intubated with the tip of a 7.0 endotracheal tube. The lung preparation was weighed and suspended within a sealed acrylic container from a mass transducer (Harvard Apparatus). The container was surrounded by a heated (38 °C) water bath in which the inner container served as a reservoir for the perfusate solution (DME H-21 with 5% albumin, 900 mL). The lung preparation was slowly warmed over 1 h with the peristaltic pump until a cardiac output of 0.3–0.4 L/min was achieved, giving an average mean pulmonary artery pressure of 10–12 mmHg. Venous drainage or left atrial pressure (LAP) was passive (0 mmHg). When the temperature of the venous drainage reached 36 °C, the lung was slowly inflated with continuous positive airway pressure (CPAP) at 10 cmH_2O with 95% O_2 and 5% CO_2. Pulmonary artery pressure and lung weight were then continuously monitored using a computer-integrated data acquisition system (Biopac). Perfusate pH, PO_2 and PCO_2 tensions were measured hourly with a blood gas machine (Bayer RapidLab 248) (Fig. 1). One hour following perfusion and ventilation (CPAP), the right (RUL) or left (LUL) upper lobe was cannulated with a PE catheter (BD, 240 tubing) and advanced until gentle resistance was encountered. Warmed normal saline (NS) with 5% albumin (125 mL, alveolar fluid solution) were instilled into the RUL or LUL. AFC was measured by the change in protein concentration of a 5% albumin instillate in the lung lobe over 1 h and expressed as mean AFC ± SD (%/h per 150 mL alveolar fluid) for each condition using the equation, as in our previous experiments (2, 3): AFC (%/hr) = (1-Ci/Cf) × 100 (Ci = protein concentration at time = 0 and Cf = protein concentration after 1 h). If the AFC of the upper lobe was greater than or equal to 10%/h (final exclusion criteria), 100 mL fresh whole human blood containing 267 ± 147 million (mean ± SD) neutrophils was added to the perfusate. Then, 10 mL normal saline solution containing 5% albumin and 6 mg endotoxin (0.1 mg/kg, *E. coli* 0111:B4, Sigma-Aldrich) was instilled into the right middle lobe (RML) or left lower lobe (LLL). After 4 h, AFC in the endotoxin-injured RML or LLL was measured. The endotoxin dose was based on our previous work measuring AFC and lung endothelial permeability in sheep (4). In a separate set of experiments, control AFC was also measured at 4 h to account for any effect of whole blood. All subsequent comparisons between endotoxin-injured and treated lung lobes were performed with the control AFC rate at 0 h. Endothelial permeability for each lung lobe was determined as follows: ^{125}I-albumin, a vascular protein tracer, was injected into the perfusate at the beginning of the experiment. Then, the total counts of ^{125}I-albumin that collected in the instilled lung lobe were measured in the homogenate of that lung lobe. The fraction of the ^{125}I-albumin in the plasma of that lobe was subtracted, and remaining count in the homogenate was divided by the mean counts in the perfusate at the end of the experiments. The perfusate volume within the lung lobe was determined by measuring the hemoglobin in the supernatant of the homogenized lung lobe and in the plasma. Total lung water was measured by determining the wet/dry ratio of the lung lobes at the end of the experiments. The W/D ratio was determined for each lung lobe. The volume of alveolar fluid solution added intra-bronchially was equal between control, endotoxin-injured, MSC-treated and MSC-CM treated lung lobes and subtracted from the total wet weight of the lung lobe before the calculation.

Whole Blood for Ex Vivo Perfused Human Lung. Whole blood (100 mL) was removed from normal healthy volunteers and immediately added to the perfusate (final hematocrit, 4%). The whole blood was not cross-matched or type-specific with the donor human lung's blood type. The human lungs contained almost no residual blood, and the lung was flushed initially during reperfusion with the perfusate solution. In addition, almost the entire set of experiments was performed with blood donated by volunteers who were AB, Rh+. There were no adverse hemodynamic or pulmonary effects associated with the addition of the whole blood to the perfusate or any changes in the AFC or endothelial permeability to the control lung lobes. The donation of whole blood by volunteers was approved by the University of California, San Francisco Committee on Human Research.

Intrapulmonary Delivery of Mesenchymal Stem Cells. For experiments requiring intrapulmonary delivery of MSCs, 5×10^6 human MSCs grown in tissue culture was instilled into the RML or LLL 1 h after the instillation of 6 mg endotoxin. We used allogeneic human mesenchymal stem cells from the NIH repository, Tulane Center for Gene Therapy. The adult stem cells from Tulane meets all of the criteria for the classification as MSCs as defined by the International Society of Cellular Therapy (5). For controls, 5×10^6 normal adult human lung fibroblasts (PromoCell) were used. Only cells with the total passage less than or equal to 10 were used in the experiments. The concentration of MSCs was based on an approximate extrapolation from in vivo mouse experiments (6). For experiments involving the conditioned medium or siRNA treated conditioned medium, the conditioned medium of 1×10^6 MSC with and without pretreatment with the siRNA for the gene of interest, in approximately 15 mL, was instilled into the RML or

LLL 1 h after endotoxin-induced lung injury to test the contribution of the targeted soluble factor in restoring AFC.

Conditioned Medium With and Without KGF siRNA Pretreatment. Allogeneic human MSC (1×10^6 cells) were cultured without serum for 24 h. The medium was then replaced, and the subsequent medium without serum for the next 24 h was used as the CM. The number of MSCs and the time period for the incubation of the medium was based on preliminary results of total growth factor levels secreted by 5×10^6 MSCs over 4 h. For siRNA experiments, MSCs were cultured on 24-well plates, 100,000 cells/well, pretreated with siPORT NeoFX (a lipid based reverse transfection agent, Ambion) and the siRNA for keratinocyte growth factor (KGF, Ambion) for 24 h. The medium was then replaced, and the subsequent medium for 10 wells were collected over the next 24 h and used as the CM pretreated with the siRNA. Five different siRNA for KGF was tested individually. Total secreted KGF protein levels were measured using a standard ELISA (R&D Systems). The siRNA (#10818 for KGF, Ambion) had the most significant knockdown at the protein levels and was used for all subsequent experiments. For experiments using recombinant KGF (rhKGF, R&D Systems), 100 ng rhKGF was added to the CM pretreated with KGF siRNA and given intra-bronchial into the lung lobe 1 h after endotoxin-induced injury.

Measurement of Inflammatory Cytokines in the Bronchoalveolar Lavage Fluid. The alveolar fluid collected during the measurement of AFC in both the control and the endotoxin-injured lung lobes was used to measure the levels of the pro-inflammatory cytokines, IL-1β and TNFα, and IL-8, the anti-inflammatory cytokines, IL-10 and IL-1RA, and growth factors KGF/FGF7 and KGF2/FGF10 using ELISA kits (R&D Systems, Antigenix America).

Histology. Separate experiments were carried out for histological analysis of lung injury. Following 4 h of endotoxin injury or control or endotoxin with MSCs or MSC-CM, tissue samples from the anterior lobes of the RUL or LUL (control) and RML or LLL (endotoxin-injured) were stained with hematoxylin and eosin (H&E).

Isolation of Primary Cultures of Human Alveolar Epithelial Type II Cells. Type II epithelial cells were isolated from human donor lungs (preserved at 4 °C for 4–8 h), as previously described (7). The alveolar epithelial type II cells were plated on collagen I-coated 24-well Transwell plates (0.4-μm pore size, PTFE Membrane, CoStar, Corning) at 5% CO_2, at 37 °C at a concentration of 1.0×10^6 cells/well. The cells were exposed to media, DMEM-H21 and F-12 Ham's (1:1), with antibiotics and 10% FBS for 72 h and without FBS for 48 h. Following 120 h from the isolation, the type II cells were exposed to cytomix at 50 ng/mL for 24 h. For experiments with MSCs, allogeneic MSCs (250,000 cells/well) were added to the bottom chamber of the Transwell plate simultaneously with the cytomix.

Fluid Transport Across Human Alveolar Epithelial Type II Cells. Net fluid transport was measured across human type II cells on Transwell plates (0.4-μm pore size and collagen I-coated, CoStar, Corning) in a humidified tent within a 37 °C, 5% CO_2 incubator with 100% humidity. Measurement of fluid transport from the apical to basolateral membrane of the type II cell monolayers was done at 48 h after the air-liquid interface was achieved (120 h following the initial isolation); previously, the transmembrane electrical resistance peaked (1,530 $\Omega \cdot cm^2$) at 96 h with morphological evidence of tight junctions (7). The cells were first exposed to 150 μL cytomix (containing 0.3 μCi/ml ^{131}I-albumin) in the apical chamber of the Transwell with and without MSCs in the bottom chamber. After 5 min, 20 μL medium was then aspirated as the initial sample. After 24 h, another 20 μL was aspirated from the upper compartment of the Transwell as the final sample. Each sample was weighed, and radioactivity was counted in a gamma counter (Packard MINAXI 5000 series). Net fluid transport ($\mu L/cm^2/h$) was calculated as previously described: = [1–(Radioactivity in the initial sample/weight of the initial sample)/(Radioactivity in the final sample/weight of the final sample)] $\times$ 150 μL (volume in the upper chamber)/0.33 cm^2 (surface area of the Transwell plate)/24 h.

Biotinylation of the Apical Membrane Proteins and Western Blotting. Apical membrane proteins were isolated from primary cultures of human alveolar epithelial type II cells exposed to control medium, cytomix, and cytomix with MSCs grown in the bottom chamber of the Transwell plate using the Cell Surface Protein Isolation Kit (Pierce). Ten wells were needed per condition to yield sufficient protein for a western blot. The protein was extracted from the cells using 0.15 mL lysis buffer per well containing 1% Triton X-100, 20 mM Tris Base (ph 8.0), 137 mM NaCl, 10% glycerol, 2 mM vanadate, 2 μg/mL aprotinin, 5 μg/mL leupeptin, and 1 mM pefabloc and homogenized. Protein content was measured by the bicinchoninic acid method (Pierce). Each sample was first reduced and denatured with sample buffer and run on a 4–12% gradient Bis-Tris gel (Invitrogen), 10–20 μg proteins per lane for total cell lysis, using a MOPS SDS buffer (Invitrogen) at 100 V for roughly 2 h. The proteins were then transferred onto a nitrocellulose membrane and blocked with 5% milk in Tris-buffered saline with Tween-20 (TBS-T) for 1 h. The nitrocellulose membrane was then exposed to the primary antibody overnight at 4 °C (αENaC, Calbiochem). The protein bands were visualized with a chemiluminescence's agent, ECL+ (Amersham), and quantitated with the NIH software, ImageJ.

Conditioned Medium with Amiloride. Amiloride hydrochloride (Sigma-Aldrich) was added to both the MSCs conditioned medium and the alveolar fluid and instilled into the lung lobe at a final concentration of 5×10^{-4} M.

Results

Inhibition of KGF Secretion from Mesenchymal Stem Cells. Allogeneic mesenchymal stem cells were found to constitutively secrete KGF into the medium (82 $\pm$ 0.5 pg/mL in a volume of 15 mL). However, we were not able to detect KGF2 or FGF10, a growth factor with similar properties to KGF. Using three different siRNA for KGF (Ambion), the secretion of KGF was markedly diminished by 24 h. At 48 h, the siRNA #10818 (Ambion) reduced KGF secretion by 95% (Fig. S1). A non-specific, non-targeting siRNA (Ambion) had no effect on KGF secretion. In addition, transfection with the same control siRNA had no effect on other constitutively expressed proteins.

Cytokine Levels in the Alveolar Fluid in the Endotoxin-Injured Lung Lobe. Allogeneic human MSC had no effect on the alveolar fluid levels of the pro-inflammatory cytokines, IL-1β, TNFα, and IL-8. The MSC-CM significantly reduced IL-1β levels but did not reduce the levels of TNFα or IL-8. There was also no effect on the levels of the anti-inflammatory cytokines, IL-1RA, nor IL-10 (Table S3).

1. Ware LB, et al. (2002) Assessment of lungs rejected for transplantation and implications for donor selection. *Lancet* 360:619–620.

2. Sakuma T, et al. (1996) Preservation of alveolar epithelial fluid transport mechanisms in rewarmed human lung after severe hypothermia. *J Appl Physiol* 80:1681–1686.

3. Frank JA, Briot R, Lee JW, Ishizaka A, Uchida T, Matthay MA (2007) Physiological and biochemical markers of alveolar epithelial barrier dysfunction in perfused human lungs. *Am J Physiol Lung Cell Mol Physiol* 293:L52–59.
4. Wiener-Kronish JP, Albertine KH, Matthay MA (1991) Differential responses of the endothelial and epithelial barriers of the lung in sheep to *Escherichia coli* endotoxin. *J Clin Invest* 88:864–875.
5. Dominici M, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8:315–317.
6. Gupta N, Su X, Popov B, Lee JW, Serikov V, Matthay MA (2007) Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol* 179:1855–1863.
7. Lee JW, et al. (2007) Acute lung injury edema fluid decreases net fluid transport across human alveolar epithelial type II cells. *J Biol Chem* 282:24109–24119.

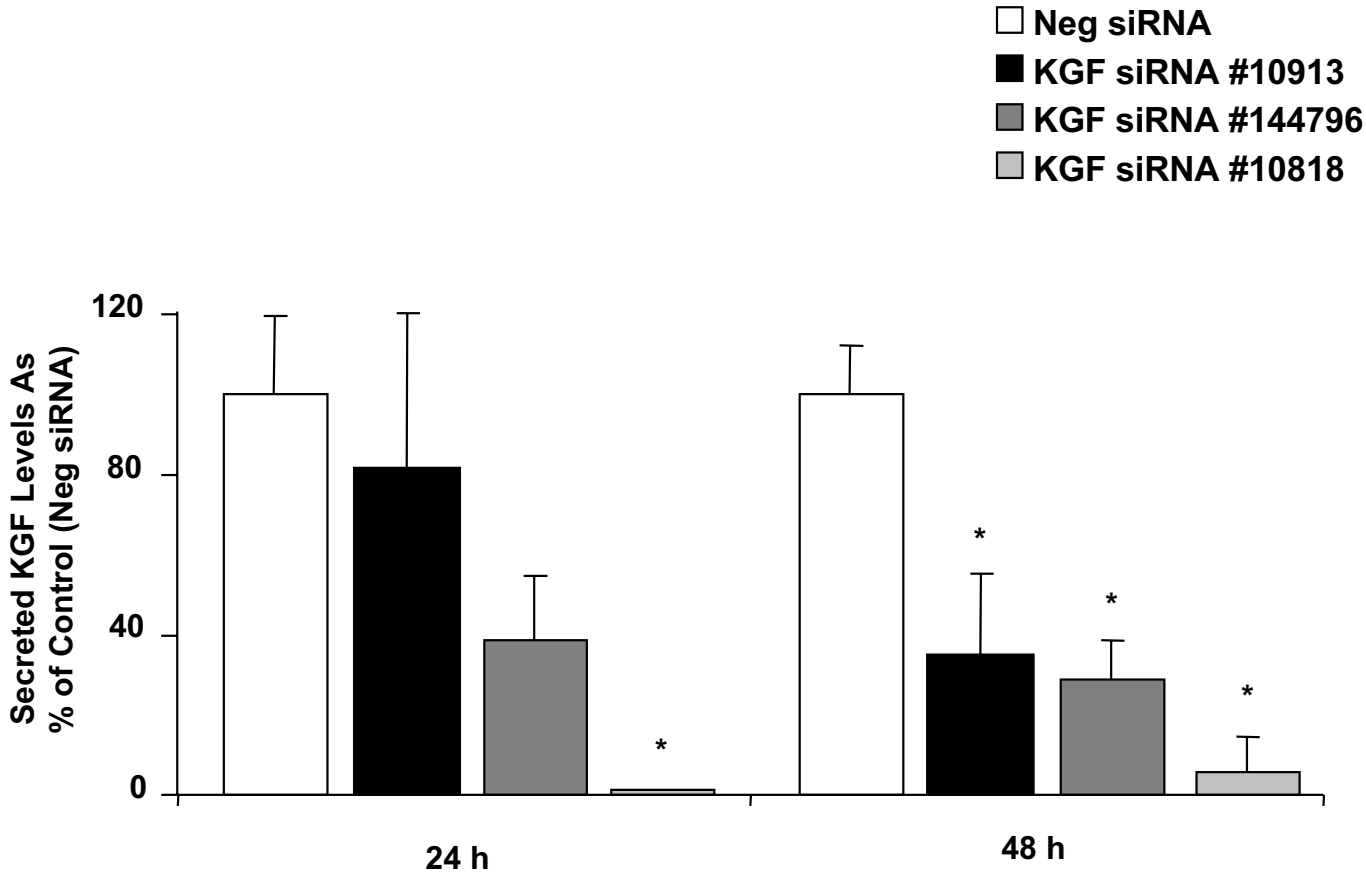

Fig. S1. Secretion of keratinocyte growth factor by mesenchymal stem cells transfected with siRNA for KGF. Human mesenchymal stem cells were transfected separately with three different siRNA (Ambion) for KGF. The secreted KGF protein in the medium was measured by ELISA (R&D Systems) for up to 72 h. By 48 h, MSCs transfected with KGF siRNA #10818 had >95% reduction in the secreted protein. The control for the experiment was a non-specific, non-targeting siRNA. *, $P < 0.0007$ vs. negative control siRNA, $n = 3$, at 24 h and *, $P < 0.0003$ vs. negative control siRNA, $n = 3$, at 48 h, by ANOVA (Bonferroni). The negative control siRNA was arbitrarily set at 100% for comparison.

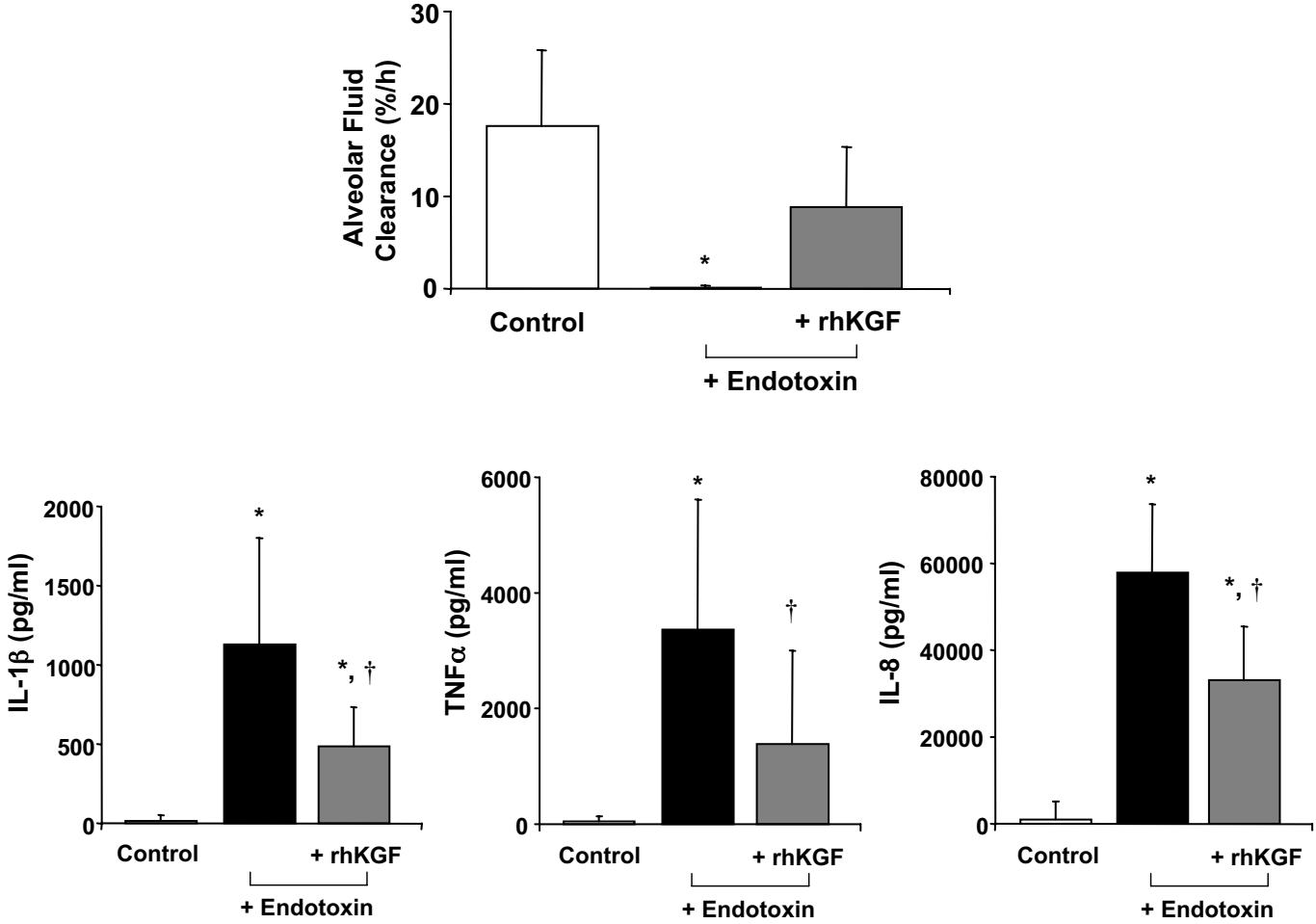

Fig. S2. Effect of recombinant KGF on alveolar fluid clearance and inflammation in the endotoxin-injured lung lobe. Instillation of recombinant KGF (rhKGF) into the endotoxin-injured lung lobe was associated with a partial restoration in (*A*) alveolar fluid clearance (AFC) and (*B*) inflammatory cytokine secretion. (*A*) AFC in the endotoxin-injured lung lobe treated with rhKGF was 50% of the control AFC level. AFC was measured by the change in protein concentration of a 5% albumin instillate in the lung lobe over 1 h and expressed as mean AFC (%/h per 150 mL alveolar fluid) ± SD. For each condition, $n = 3$–6; *, $P < 0.0001$ vs. control AFC by ANOVA (Bonferroni). (*B*) The addition of rhKGF into the endotoxin-injured lung lobe was associated with a partial restoration in IL-1β, TNFα, and IL-8 levels in the alveolar fluid compared to the control lobe at 4 h. $n = 3$; *, $P < 0.0074$ vs. control, †, $P < 0.0015$ vs. endotoxin-injured for IL-1β; *, $P < 0.0001$ vs. control, †, $P < 0.0046$ vs. endotoxin-injured for TNFα; *, $P < 0.0001$ vs. control, †, $P < 0.0001$ vs. endotoxin-injured for IL-8 by ANOVA (Bonferroni).

Table S1. Clinical characteristics of the 38 donor lungs in the study

Clinical Characteristics	Results
Age (years)	48 ± 13
Male/Female (%)	45/55
Total Ischemia Time (h)	21 ± 13
First perfusate blood gas with perfusion	
pH	7.6 ± 0.1
pCO_2 (mmHg)	32 ± 7
pO_2 (mmHg)	295 ± 91

Data are shown as mean ± SD

Table S2. Cell counts of whole blood added to the perfusate

Cell Type	Values
Hematocrit (%)	41 ± 3
Total WBC (x 10^3 cells/μl)	5.3 ± 1
Neutrophils (%)	49 ± 21
Lymphocytes (%)	27 ± 6
Macrophages (%)	7 ± 5
Platelets (x 10^3 cells/μl)	298 ± 98

Data are shown as mean ± SD

Table S3. Cytokine levels in the alveolar fluid

	Median [25%–75% percentile]	Overall *P*
IL-1β (pg/ml)		
Control	0 [0, 0]	0.0001
Endotoxin	1,076 [795, 1,399]	
+ MSC	1,273 [757, 2,033]	
+ MSC CM	95 [0, 186]	*
TNFα (pg/ml)		
Control	0 [0, 27]	0.0001
Endotoxin	3,262 [1,417, 4,193]	
+ MSC	1,646 [1,436, 4,301]	
+ MSC CM	1,253 [4, 11,619]	
IL-8 (pg/ml)		
Control	0 [0, 0]	0.0001
Endotoxin	56,306 [46,770, 72,971]	
+ MSC	59,165 [44,960, 71,833]	
+ MSC CM	52,139 [4,769, 63,068]	
IL-1RA (pg/ml)		
Control	148 [0, 1,552]	0.0001
Endotoxin	54,199 [30,995, 77,812]	
+ MSC	31,770 [16,272, 66,330]	
+ MSC CM	33,092 [2,636, 51,672]	
IL-10 (pg/ml)		
Control	0 [0, 0]	0.0001
Endotoxin	1,476 [703, 2,714]	
+ MSC	1,067 [689, 1,635]	
+ MSC CM	969 [20, 1,665]	

We used nonparametric Kruskal-Wallis tests for overall *P* values and Mann–Whitney *U* tests with Bonferroni corrections. For all cytokines, the treatment groups (endotoxin with and without MSC or MSC CM) were significantly greater than controls except IL-1β. *, For IL-1β, endotoxin treated with MSC CM was not different than the control. For all other comparisons, there were no significant differences between treatments groups and each other.