Allogeneic human mesenchymal stem cells for treatment of E. coli endotoxin-induced acute lung injury in the ex vivo perfused human lung

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Recent studies have suggested that bone marrow-derived multipotent mesenchymal stem cells (MSCs) may have therapeutic applications in multiple clinical disorders including myocardial infarction, diabetes, sepsis, and hepatic and acute renal failure. Here, we tested the therapeutic capacity of human MSCs to restore alveolar epithelial fluid transport and lung fluid balance from acute lung injury (ALI) in an ex vivo perfused human lung preparation injured by E. coli endotoxin. Intra-bronchial instillation of endotoxin into the distal airspaces resulted in pulmonary edema with the loss of alveolar epithelial fluid transport measured as alveolar fluid clearance. Treatment with allogeneic human MSCs or its conditioned medium given 1 h following endotoxin-induced lung injury reduced extravascular lung water, improved lung endothelial barrier permeability and restored alveolar fluid clearance. Using siRNA knockdown of potential paracrine soluble factors, secretion of keratinocyte growth factor was essential for the beneficial effect of MSCs on alveolar epithelial fluid transport, in part by restoring amiloride-sensitive epithelial sodium channels. In summary, treatment with allogeneic human MSCs or the conditioned medium restored normal fluid balance in an ex vivo perfused human lung injured by E. coli endotoxin.

alveolar fluid clearance | keratinocyte growth factor | pulmonary edema | acute respiratory distress syndrome

Despite extensive research into the pathogenesis of acute lung injury and the acute respiratory distress syndrome (ALI/ARDS), mortality remains high at approximately 40% (1, 2). Current treatment is supportive with lung protective ventilation and a fluid conservative strategy (3, 4). Pharmacologic therapies that reduce the severity of lung injury in experimental studies have not yet been translated to effective clinical treatment options. Therefore, innovative therapies are needed.

Experimentally and clinically, the ability of the lung epithelium to remove alveolar edema is quantified as alveolar fluid clearance (AFC). Impaired AFC in patients with ALI/ARDS is associated with higher morbidity and mortality (5, 6). In the alveolar environment, basal AFC is determined predominantly by amiloride-sensitive and insensitive epithelial sodium channels (ENaC) on the apical membrane as well as Na-K ATPase, located on the basolateral membrane, in both alveolar epithelial type I and II cells (7, 8). Several stimuli can up-regulate AFC including cAMP-dependent and -independent mechanisms. In the mouse, rat and human lung, cAMP dependent alveolar epithelial fluid transport is dependent on ENaC, CFTR, and Na-K ATPase (9). Catecholamine-independent pathways can also up-regulate AFC including growth factors, thyroid hormone, and glucocorticoids (7, 10, 11). Multiple pathways can reduce alveolar epithelial fluid transport and impair the resolution of alveolar edema, including hypoxia, high tidal volume ventilation, and pro-inflammatory cytokines in the pulmonary edema fluid (7, 12–16).

Recent studies have suggested that bone marrow-derived multipotent mesenchymal stem cells (MSCs) may have therapeutic applications in several clinical disorders including myocardial infarction (17), diabetes (18), sepsis (19), hepatic (20), and acute renal failure (21). Recently, allogeneic MSC have been studied in several in vivo models of lung disease (22–25). Despite initial interest in their multipotent properties (26, 27), engraftment in the lung does not appear to play a major role. The beneficial effects of MSCs in the other organ systems derive from their capacity to secrete paracrine soluble factors that modulate immune responses as well as alter the responses of endothelium or epithelium to injury through the release of growth factors (19, 28–31).

Currently, little is known regarding the effect of bone marrow-derived MSCs in experimental models of ALI and pulmonary edema. However, we recently reported that intrapulmonary (via the trachea) treatment with MSCs 4 h after endotoxin delivery to the lung improved survival and reduced pulmonary edema formation in endotoxin-induced ALI in mice, although the exact mechanisms of benefit were not identified in the study (23).

Here, we tested the therapeutic role of human allogeneic MSCs in resolving pulmonary edema in an ex vivo perfused human lung preparation (32) injured by E. coli endotoxin. Intra-bronchial instillation of endotoxin into the distal airspaces resulted in an increase in extravascular lung water and a complete loss of AFC. The addition of allogeneic human MSCs or its conditioned medium 1 h following endotoxin-induced lung injury fully restored AFC. Using siRNA knockdown of potential paracrine soluble factors, we found that the secretion of keratinocyte growth factor (KGF) was essential for the restorative effect of MSCs.

Results

Ex Vivo Human Lung Preparation. Human lungs donated by the Northern California Transplant Donor Network were used for perfusion and experimentation once the exclusion criteria were met. Baseline demographic data and ischemia time for the 38 donor lungs are listed in Table S1, including the initial blood gas values with perfusion. The details of the preparation and the protocol for the experiments are provided in Fig. 1. The cell counts of the whole blood added are listed in Table S2.

Effect of Endotoxin on Alveolar Fluid Clearance With and Without Fresh Human Blood. Following rewarming, the right middle lobe (RML) or left lower lobe (LLL) was injured with the intra-bronchial instillation of endotoxin at a dose of 0.1 mg/kg. For the initial experiments shown in Fig. 2, human lungs were perfused with no. 38

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from the endotoxin-injured lung lobe at 4 h. For the control lung lobe at 4 h, there was small increase in the proinflammatory cytokines (Fig. 2B).

**Lung Histology and Neutrophil Counts.** Representative histology from a lung lobe injured by endotoxin demonstrated an increase in edema and cellularity compared to histology from a control lobe. Histology from a representative sample of a lung lobe treated with the instillation of either $5 \times 10^6$ allogeneic human MSCs or MSC-conditioned medium (CM) given 1 h after instillation of endotoxin showed a reduction in the inflammatory cell infiltration and septal thickening at 4 h (Fig. 3). The number of white blood cells and neutrophils in the alveolar fluid of the endotoxin-injured lung lobe with and without treatment with MSCs or MSC-CM are shown (Fig. 3). There was a significant increase in the influx of neutrophils in the endotoxin-injured lung lobe compared to the control lobes. Although the mean number of neutrophils was reduced in the MSCs and MSC-CM-treated lobes, these differences did not quite achieve statistical significance when the Bonferroni adjustment was applied for multiple comparisons.

**Lung Endothelial Permeability and Lung Water Content in Endotoxin-Injured Lung Lobe.** Instillation of allogeneic human MSCs or the MSC-CM 1 h following endotoxin-induced injury restored lung endothelial permeability to control levels (Fig. 4A). Instillation of endotoxin increased the water content (expressed as the wet/dry ratio of g H$_2$O/g dry lung) by 21% ($P < 0.002$). The addition of MSCs or MSC-CM reduced the water content to control levels (Fig. 4B).

**Alveolar Fluid Clearance in the Endotoxin-Injured Lung Lobe.** Either allogeneic human MSCs or the CM of MSCs instilled 1 h following endotoxin-induced lung injury normalized AFC. Instillation of $5 \times 10^6$ normal adult human lung (NHL) fibroblasts given 1 h following endotoxin-induced injury had no effect on AFC (Fig. 5A).

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**Fig. 1.** Schematic diagram of the ex vivo perfused human lung and experimental protocol.

only with a medium (DME H-21) containing 5% albumin; for the remainder of the experiments, perfusion was carried out with the same solution plus fresh whole human blood. AFC was measured at 4 h following endotoxin-induced lung injury. AFC was significantly reduced for the endotoxin-injured lung lobe only in the presence of fresh human blood. In this model, impaired AFC can reflect both an alteration in paracellular permeability as well as a decrease in transcellular ion and fluid transport. There was no significant difference in control AFC rates, measured at 0 and 4 h to reflect the lung lobes before and after the addition of whole blood (Fig. 2A). In addition, instillation of endotoxin into one lung lobe with whole human blood in the perfusate led to a sharp increase in IL-1β, TNFα, and IL-8 levels in the alveolar fluid.

**Fig. 2.** Effect of endotoxin on alveolar fluid clearance and inflammation. Instillation of endotoxin into the lung lobe was associated with a significant decrease in (A) alveolar fluid clearance (AFC) and (B) an increase in inflammatory cytokine secretion. (A) The decrease in AFC in the endotoxin-injured lung lobe was dependent on the presence of fresh whole human blood in the perfusate. AFC in the endotoxin-injured lung lobe with fresh whole blood was significantly decreased compared to control AFC at 0 or 4 h measurements without endotoxin or to experiments with endotoxin without blood in the perfusate at 4 h. 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 (0 h); †, $P < 0.0013$ vs. control AFC (4 h) by ANOVA (Bonferroni). (B) The addition of endotoxin into the lung lobe was associated with a significant increase in IL-1β, TNFα, and IL-8 levels in the alveolar fluid compared to the control lobe at 0 or 4 h. $n = 3–6$; *, $P < 0.0001$ vs. control (0 h); †, $P < 0.0001$ vs. control (4 h) for IL-1β, TNFα, and IL-8 by ANOVA (Bonferroni).

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- Surgical preparation of one lung
- Begin perfusion without blood
- Add 100 ml Fresh Whole Blood to Perfusate
- Instill 0.1 mg/kg of LPS
- Measure AFC
- Instill allogeneic human MSC or MSC conditioned medium to RML or LLL
- Measure AFC over 1 h in RML or LLL (endotoxin)
- Lung Temp 36°C
- Apply CPAP
- Rate: 0.32 liters/min
- Pressure = 10 - 12 mm Hg
- CPAP 10 cm H$_2$O
- 95% O$_2$, 5% CO$_2$
Permeability and LPS

ANOVA (Bonferroni), P values are shown for the comparisons between LPS vs. presence of the MSC themselves. Although not quite statistically significant by Measurement of WBC in MSC-treated lung lobes was confounded by the endotoxin exposure. There was a trend toward endotoxin-induced lung injury. The median control level was 0.0 and wet/dry (W/D) ratio (B)

injured RML or LLL 1 h later restored lung endothelial permeability to protein 6% AFC/h for the CM alone, mean rate 

The CM of MSCs Pretreated with KGF siRNA and AFC. When the MSCs were pretreated with the KGF siRNA (Fig. S1), 80% of the protective effect of the MSC-CM on AFC in endotoxin-injured lung lobes was abolished (Fig. 5B). The CM of MSCs pretreated with a non-targeting, non-specific siRNA (Neg Control #1, Ambion) maintained the protective effect of the CM alone on AFC (24 ± 12% for MSC-CM pretreated with negative control siRNAs vs. 18 ± 6% AFC/h for the CM alone, mean rate ± SD% AFC/h, P<NS).

Alveolar Fluid KGF Levels. KGF levels were measured in the alveolar fluid with and without MSCs or MSC-CM treatment following endotoxin-induced lung injury. The median control level was 0.0 pg/mL [ (0, 2), 25%–75% percentile] and increased to 3.5 pg/mL (0, 212) following endotoxin exposure. There was a trend toward increased levels of KGF in the alveolar fluid of endotoxin-injured lungs treated with MSCs or MSC-CM. KGF was 33 pg/mL (4, 156) with MSCs and 12 pg/mL (0, 35) with MSC-CM.

Effect of Recombinant KGF. The addition of recombinant KGF (100 ng, R&D Systems) to the MSC-CM (4 ng/mL in 25 mL instillate), pretreated with KGF siRNA before instillation into the lung, restored the protective effect of the CM alone on AFC in the endotoxin-injured lung lobes compared to MSC-CM pretreated with the KGF siRNA (Fig. S8). RhKGF by itself partially restored the decrease in the AFC rate as well as the increase in the levels of the inflammatory cytokines, suggesting the presence of other soluble factors with an effect on net fluid clearance (Fig. S2).

Effect of MSC on Net Fluid Transport in Primary Cultures of Injured Human Alveolar Epithelial Type II Cells. To test one mechanism that could account for the therapeutic effect of KGF in these experiments, we used an in vitro model of alveolar epithelial human type II cells, as in our prior studies (33). The addition of cytomix, a mixture of the most biologically active pro-inflammatory cytokines present in ALI pulmonary edema fluid (IL-β, TNFα, and IFNγ) (34), reduced net fluid transport by 70% in primary cultures of human alveolar epithelial type II cells. The simultaneous addition of allogeneic MSCs (250,000 cells per well) in the bottom chamber of the Transwell plate partially restored the loss of net fluid transport caused by the cytomix, whereas pretreatment of MSCs with a KGF siRNA abrogated the therapeutic benefit of MSCs on net fluid transport (Fig. 6A). These in vitro studies replicated the beneficial effects of human MSCs and KGF on alveolar fluid transport from the ex vivo perfused human lung experiments (Fig. 5B).
Effect of MSCs on the Apical Membrane Expression of ENaC. Because previous studies demonstrated that KGF increased αENaC expression at the mRNA level (35, 36), we tested the effect of MSCs on both trafficking of ENaC subunits to the apical surface using biotinylation and on total cellular protein levels in the cultured alveolar type II cells injured with cytokinx. Cytomix reduced both the total cellular level and the apical membrane expression of αENaC by 41% and 50%, respectively, at 24 h in primary cultures of human alveolar epithelial type II cells. The addition of human MSCs in the bottom chamber of the Transwell plate partially restored both the total cellular level and the apical membrane expression of αENaC (72% and 83%, respectively, of control values, Fig. 6B). In contrast, there was no statistically significant difference in the apical membrane expression of γENaC. We were not able to detect βENaC at either the total cellular protein level or on the apical membrane.

Effect of Amiloride on the CM of MSCs on AFC. Because the in vitro experiments suggested a role for ENaC in the KGF-mediated restoration of AFC in the endotoxin-injured lung lobe, we tested the effect of amiloride, an inhibitor of ENaC, in the perfused human lung. The protective effect of MSC-CM on AFC in the lung lobe injured by endotoxin was significantly reduced when amiloride (5 \times 10^{-4} \text{M}) was added to the medium. Overall, AFC was decreased by 56% compared to the MSC-CM alone (Fig. 7).

Discussion
The major findings of these experiments can be summarized as follows: (1) Following *E. coli* endotoxin-induced acute lung injury, treatment with either allogeneic human MSCs or human MSC-CM reduced pulmonary edema, improved lung endothelial barrier integrity and normalized alveolar epithelial fluid transport in an ex vivo perfused human lung; (2) the effect was mediated in part by the secretion of KGF; and (3) the beneficial effect of KGF was mediated in part by restoring sodium dependent alveolar fluid transport.

To study the potential therapeutic effect of human allogeneic MSCs, we developed a reproducible *E. coli* endotoxin-induced ALI model in an ex vivo human lung preparation perfused partially with whole blood. Instillation of endotoxin into the distal airspaces of one lung lobe resulted in acute pulmonary edema, an increase in lung vascular permeability and an almost complete loss of AFC. These effects were associated with an acute neutrophilic inflammatory response within the injured alveolus, as well as elevated levels of IL-1β, TNFα, and IL-8, the most biologically active cytokines found in ALI pulmonary edema fluid (1). The presence of fresh whole blood was essential for these effects (Fig. 2). PMNs in the fresh whole blood were likely responsible for the effect of endotoxin-induced lung injury although monocytes and platelets in the blood could also play a role. By histology, endotoxin increased the degree of edema and cellularity within the injured alveolus (Fig. 3). Lung endothelial permeability and the wet/dry ratio (a measure of lung water) increased as well following endotoxin-induced injury (Fig. 4). Instillation of either allogeneic human MSCs or its CM 1 h following endotoxin-induced lung injury fully restored normal fluid balance by three criteria: Alveolar lung endothelial permeability to protein was normalized (Fig. 4A), the wet/dry ratio returned to control levels (Fig. 4B) and alveolar epithelial fluid transport was restored to a normal level (Fig. 4D).

As suggested by recent mouse injury models [intra-tracheal LPS (23), intra-tracheal bleomycin (28), cecal ligation and puncture (19)], the secretion of pro- and anti-inflammatory cytokines, such as IL-1RA and IL-10, may account for some beneficial effects of MSCs. In the alveolar fluid of the LPS injured lung lobe, the instillation of allogeneic human MSCs or its CM had no statistically significant effect on the levels of TNFα and IL-8, although the level of IL-1β was decreased to control levels in MSC-CM experiments (Table S3). The levels of the anti-inflammatory cytokines, IL-1RA and IL-10, were not elevated in the LPS-injured lung lobe treated with MSCs or MSC-CM, similar to the findings of Ortiz et al. in bleomycin-injured mouse lungs treated with MSCs (28). These
results suggest that the therapeutic benefit of MSCs or its CM does not derive primarily from an anti-inflammatory effect. However, in this ex vivo human lung model, the levels of circulating monocytes and neutrophils are significantly lower than in an intact animal (19, 23). In addition, the low levels of IL-1RA and IL-10 measured may reflect an early anti-inflammatory effect or a lung in recovery. Nevertheless, it is interesting that the beneficial effect of the MSCs or MSC-CM in this model was not primarily mediated by effects on the levels of pro- or anti-inflammatory cytokines.

Because MSCs are known to produce several epithelial specific growth factors, we tested the capacity of allogeneic human MSCs to secrete keratinocyte growth factor, the seventh member of the fibroblast growth factor family. We were particularly interested in KGF because of work from our group as well as other investigators who have reported that KGF can reduce lung injury in small animal models of pulmonary edema. Recombinant KGF pretreatment reduced mortality following intra-tracheal instillation of hydrochloric acid (37), bleomycin (38), hyperoxia (39) and *Pseudomonas aeruginosa* (40). In models of acute permeability edema such as α-naphthylthioiurea (41), *P. aeruginosa* (40) or ventilator-induced lung injury (42), KGF reduced lung edema and bronchoalveolar lavage protein levels. In addition, KGF improved alveolar fluid transport in part by up-regulating αENaC gene expression (35) and Na-K ATPase activity (36) in rat lung. We found that cultured allogeneic human MSCs produced substantial quantities of KGF.

To determine the potential contribution of KGF secretion by MSC in these studies, we pretreated MSCs with KGF siRNA (Fig. S1) and instilled the CM into the endotoxin-injured ex vivo perfused human lung. The therapeutic benefit of MSC-CM on AFC was reduced by 80% when the MSCs were pretreated with a KGF siRNA, thus eliminating KGF secretion (Figs. 5B). The addition of rhKGF to the same CM (as rescue therapy) restored the therapeutic effect on AFC to the endotoxin-injured lung lobe. These results indicate that secreted KGF is an important paracrine soluble factor that mediates the therapeutic effect of MSCs on AFC. More significantly, MSCs or rhKGF can be given following the injury in the ex vivo perfused human lung and still maintain therapeutic efficacy. The addition of rhKGF itself partially restored AFC following endotoxin-induced lung injury, suggesting the presence of other important soluble factors with a therapeutic effect on AFC as well (Fig. S2).

To further test potential mechanisms of benefit of MSCs on alveolar fluid transport, we exposed primary cultures of human alveolar epithelial type II cells grown on a Transwell plate with an air-liquid interface to cytomix (33), a mixture of the most biologically active cytokines in ALI pulmonary edema fluid (34). Cytokine reduced vectorial fluid transport across epithelial type II cells by 70% over 24 h, an effect that was not associated with an increase in apoptosis or necrosis of the type II cells, as previously measured by flow cytometry (33). The addition of human MSCs to the lower chamber restored the cytokine-induced decrease in net fluid transport through a cell contact independent mechanism. As in the perfused human lung studies, pretreatment of MSCs with the KGF siRNA before its addition to the Transwell plate reduced the therapeutic benefit of MSCs on cytomix-injured net fluid transport in epithelial type II cells, suggesting an important contribution of this growth factor on AFC (Fig. 6A).

Several properties of KGF could explain the therapeutic effect of human MSCs on restoring AFC, including alveolar epithelial type II cell hyperplasia, surfactant production (43), anti-apoptotic effects (10) and increased transcription and/or translation of the major sodium and chloride transport proteins (35). Because the effect of MSC therapy in the *E. coli* endotoxin-induced lung injury in the ex vivo perfused human lung occurred over a 3 h time period, the therapeutic benefit of KGF is less likely explained by type II cell hyperplasia or transcriptional effects. Alternatively, we and other investigators have found that increases in vectorial fluid transport across the alveolar epithelium can be mediated by an increase in trafficking of sodium transport proteins to the cell surface in a short time frame (12, 44). To test this hypothesis, we carried out biotinylation studies to determine the effect of MSCs on the delivery of ENaC to the apical membrane in alveolar epithelial type II cells treated with cytomix. The results indicated that MSCs partially restored apical membrane protein levels of αENaC, the most significant ENaC isoform, suggesting the beneficial effect of MSCs or its CM was mediated in part through increased sodium transport on the alveolar epithelium (Fig. 6B). Currently, it remains unclear what portion of the increase in apical membrane levels of αENaC reflect an increase in membrane trafficking or a restoration of total αENaC protein levels. In addition, it is difficult to extrapolate the in vitro mechanistic data to the perfused human lung due to the different models, form of injury (endotoxin vs. cytomix) and time points (4 h vs. 24 h) that were used. However, in support of the in vitro data, the addition of amiloride, a pharmacologic inhibitor of ENaC activity, to the CM reduced the therapeutic effect of the MSC-CM by 56% on AFC in endotoxin-injured lung lobe in the ex vivo perfused lung (Fig. 7). In addition to restoring normal sodium uptake across the apical membrane, it is also possible that the beneficial effect of MSCs or the CM could be also mediated by improved trafficking of Na-K ATPase subunits to the basolateral membrane (44).

MSCs may also normalize lung fluid balance and AFC through therapeutic effects on the lung endothelium. The integrity of the lung microvascular endothelium is essential to prevent the influx of protein-rich fluid from the plasma as well as inflammatory cells which may further aggravate the ability of the lung epithelium to remove alveolar edema. In these experiments, instillation of MSCs or its CM into an endotoxin-injured lung lobe normalized lung endothelial permeability to protein (Fig. 4A). The role of KGF is intriguing given the previous studies of ALL in animal models and a recent study by Murakami et al. (45) who reported that fibroblast growth factors (FGF) are key mediators responsible for the maintenance of endothelial barrier homeostasis. In addition, in this model, the impact of MSCs on paracellular permeability cannot be separated out from its impact on transcellular ion and fluid transport on AFC measurements. In the future, it will be important to understand the therapeutic role of MSCs on lung endothelial permeability in ALL.

There are some limitations to the current study. MSCs secrete multiple paracrine factors that may affect AFC (20, 29). In the experiments that used siRNA knockdown, AFC was abolished by 80% with the addition of CM pretreated with the KGF siRNA, indicating the other protective paracrine factors are present in the medium. This ex vivo perfused human lung preparation is short term (4 h) and does not include the influence of perfusion of other systemic organs such as the liver or spleen, which may mount a significant inflammatory response. For instance, the beneficial effect of human MSCs could also be explained in part by the secretion of interleukin-1 receptor antagonist (IL-1RA). Recently, Ortiz et al. (28) found that IL-1RA mediated the anti-inflammatory and anti-fibrotic effect of MSCs during bleomycin-induced lung injury in mice. It is also unclear why absolute neutrophil counts were decreased in the alveolar fluid in the endotoxin-injured lung lobe following MSC or CM treatment. Although FGF may be involved in lung endothelial permeability, the role of KGF or other paracrine soluble factors in neutrophil transmigration will need to be studied in the future.

In conclusion, intra-pulmonary instillation of human MSCs or its CM following *E. coli* endotoxin-induced lung injury restored normal alveolar epithelial fluid transport in an ex vivo perfused human lung preparation. The results demonstrate that (1) allogeneic MSCs or MSC-CM can be used to restore lung fluid balance in the setting of a clinically relevant lung injury (endotoxin) in an ex vivo perfused human lung; (2) the mechanism for the beneficial effect of human MSCs on AFC is mediated in large part through secretion of the epithelial specific growth factor,
KGF: (3) the MSC therapy was effective as a treatment strategy. Cell-based therapy with MSCs or its CM needs to be tested further for its potential value in treatment of human ALI.

**Methods**

**Ex Vivo Perfused Human Lung and Primary Cultures of Human Alveolar Epithelial Type II Cells.** A detailed description of the perfused human lung model (preparation, CM development) (32) as previously described and the measurement of net fluid transport (33) as previously described can be found in the SI Text. A detailed description of the Transwell model of human alveolar Type II cells and the measurement of net fluid transport (33) as previously described can be found in the SI Text. All other techniques (western blot, biotinylation, and histology) as well as reagents used are standard and can be found in the SI Text as well.

**Data Analysis Plan.** Results were expressed as the mean ± SD if the data were normally distributed. Comparisons between two groups were made using the unpaired, two-tailed Student’s t test. Comparisons with a sample over time were made by repeated measures of analysis of variance (ANOVA) using the Bonferroni correction for multiple-comparison testing using Statview (SAS Institute Inc.). For abnormally distributed data (Table S3), results were expressed as median with 25th and 75th confidence intervals, and, for statistical analysis, we used the Kruskal-Wallis tests for overall P value and the Mann-Whitney U tests with Bonferroni-corrections for pairwise comparisons.

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Supporting Information

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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 (PaO2/FiO2 <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 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 5–0.4 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 cmH2O with 95% O2 and 5% CO2. Pulmonary artery pressure and lung weight were then continuously monitored using a computer-integrated data acquisition system (Biopac). Perfusion gas (O2 and CO2) 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):

$$AFC = (1 - Ci/Cf) \times 100$$

where Ci is protein concentration after 1 h, Cf is protein concentration at time = 0 and AFC is 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 (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: 125I-albumin, a vascular protein tracer, was injected into the perfusate at the beginning of the experiment. Then, the total counts of 125I-albumin that collected in the instilled lung lobe were measured in the homogenate of that lung lobe. The fraction of the 125I-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 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-match 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
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 × 10^5 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% CO2, at 37 °C at a concentration of 1.0 × 10^4 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 cytomin 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 cytomin.

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 humified tent within a 37 °C, 5% CO2 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 Ω cm²) at 96 h with morphological evidence of tight junctions (7). The cells were first exposed to 150 μL cytokim (containing 0.3 μg/mL 131I-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 MI-NAXI 5000 series). Net fluid transport (μL/cm²/h) was calculated as previously described: = [(Radioactivity in the initial sample/weight of the initial sample)/(Radioactivity in the final sample/weight of the final sample)] × 150 μL (volume in the upper chamber)/0.33 cm² (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, cytomin, and cytomin 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 leupepin, and 1 mM peffabloc 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 Amlorilide. 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 ± 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).

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 < 0.0001 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

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48 ± 13</td>
</tr>
<tr>
<td>Male/Female (%)</td>
<td>45/55</td>
</tr>
<tr>
<td>Total Ischemia Time (h)</td>
<td>21 ± 13</td>
</tr>
<tr>
<td>First perfusate blood gas with perfusion</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>pCO₂ (mmHg)</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>pO₂ (mmHg)</td>
<td>295 ± 91</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD
Table S2. Cell counts of whole blood added to the perfusate

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

Data are shown as mean ± SD
Table S3. Cytokine levels in the alveolar fluid

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

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.