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Therapeutic Effects of Human Mesenchymal Stem Cells in *Ex Vivo* Human Lungs Injured with Live Bacteria

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***Rationale*: Mesenchymal stem cells secrete paracrine factors that can regulate lung permeability and decrease inflammation, making it a potentially attractive therapy for acute lung injury. However, concerns exist whether mesenchymal stem cells' immunomodulatory properties may have detrimental effects if targeted toward infectious causes of lung injury.**

***Objectives*: Therefore, we tested the effect of mesenchymal stem cells on lung fluid balance, acute inflammation, and bacterial clearance.**

***Methods*: We developed an *Escherichia coli* pneumonia model in our *ex vivo* perfused human lung to test the therapeutic effects of mesenchymal stem cells on bacterial-induced acute lung injury.**

***Measurements and Main Results*: Clinical-grade human mesenchymal stem cells restored alveolar fluid clearance to a normal level, decreased inflammation, and were associated with increased bacterial killing and reduced bacteremia, in part through increased alveolar macrophage phagocytosis and secretion of antimicrobial factors. Keratinocyte growth factor, a soluble factor secreted by mesenchymal stem cells, duplicated most of the antimicrobial effects. In subsequent *in vitro* studies, we discovered that human monocytes expressed the keratinocyte growth factor receptor, and that keratinocyte growth factor decreased apoptosis of human monocytes through AKT phosphorylation, an effect that increased bacterial clearance. Inhibition of keratinocyte growth factor by a neutralizing antibody reduced the antimicrobial effects of mesenchymal stem cells in the *ex vivo* perfused human lung and monocytes grown *in vitro* injured with *E. coli* bacteria.**

***Conclusions*: In *E. coli*–injured human lungs, mesenchymal stem cells restored alveolar fluid clearance, reduced inflammation, and exerted antimicrobial activity, in part through keratinocyte growth factor secretion.**

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Although bone marrow–derived mesenchymal stem cells (MSC) have been proposed as a novel treatment for acute lung injury, MSC possess potent immunomodulatory properties that might impair host defense against bacterial infection. Therefore, there is an important need to determine the effects of MSC on the pulmonary response to live bacterial infection and to test the effect of human MSC in acute bacterial-induced lung injury.

What This Study Adds to the Field

Using an *ex vivo* perfused human lung injured with live *Escherichia coli* bacteria, MSC treatment restored alveolar fluid clearance to a normal level, decreased inflammation, and reduced bacterial growth in the lung by increasing bacterial phagocytosis by alveolar macrophages, in part through secretion of keratinocyte growth factor. In cultures of blood monocytes *in vitro*, MSC also reduced the apoptosis of the monocytes, which expressed the keratinocyte growth factor receptor, and increased granulocyte-macrophage colony–stimulating factor secretion, further increasing the antimicrobial effect of MSC against *E. coli* bacteria.

Keywords: acute lung injury; bacterial pneumonia; cell-based therapy; keratinocyte growth factor

Despite extensive research into the pathogenesis of acute lung injury (ALI) and the acute respiratory distress syndrome, mortality remains high (1–3). Current treatment is supportive with lung protective ventilation and a fluid conservative strategy (4, 5). Innovative therapies are needed. Recent studies have suggested that mesenchymal stem or stromal cells (MSC) may have therapeutic applications in multiple models of lung disease (6–17). Despite initial interest in their multipotent properties, engraftment in the lung does not seem to play a major therapeutic role. The beneficial effect of MSC derives from their capacity to secrete paracrine factors that modulate immune responses and alter the responses of the endothelium or epithelium and inflammatory cells to injury (17–28). Because of the immunosuppressive properties of MSC, one safety concern is a potential deleterious effect on host defense against bacterial infection. Bacterial pneumonia and sepsis from a nonpulmonary cause are the two most common etiologies of ALI and acute respiratory distress syndrome (2). However, recent studies have provided evidence for the beneficial effects of MSC in the treatment of sepsis (17, 29), although the mechanism for enhanced bacterial clearance was not clearly identified.

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In the first part of this study, we hypothesized that clinical-grade, cryopreserved bone marrow–derived human allogeneic MSC from current Good Manufacturing Practices, thawed and washed as if in a clinical trial, would be as effective in restoring alveolar fluid clearance (AFC) as cultured human MSC in our *ex vivo* perfused human lung injured with *Escherichia coli* endotoxin. Clinical-grade MSC restored AFC rate to a normal level and decreased inflammation after endotoxin-induced ALI with similar efficacy as cultured human MSC (30).

In the second part of these studies, we hypothesized that these same clinical-grade human MSC would be effective in an infectious model of lung injury, *E. coli* pneumonia. Intrabronchial (IB) instillation of *E. coli* bacteria resulted in a marked decrease in AFC and an intense proinflammatory response. Human MSC administration after *E. coli* pneumonia–induced lung injury fully restored AFC, decreased inflammation, and reduced total bacterial load in the injured alveolus. During the period of this study, Wu and coworkers (31) reported in a mice pneumonia model that keratinocyte growth factor (KGF) stimulated the secretion of granulocyte-macrophage colony–stimulating factor (GM-CSF) by alveolar epithelial cells, which activated alveolar macrophages by STAT5 phosphorylation and increased the phagocytosis and killing of gram-negative bacteria. Previously, we reported that the secretion of KGF by MSC restored vectorial ion and fluid transport in injured human alveolar epithelial type II cells (30). To determine if KGF secretion by MSC was involved in bacterial killing, we conducted studies with MSC or KGF in the *ex vivo* perfused human lung and isolated blood monocytes exposed to *E. coli* bacteria. Some of the results of these studies have been previously reported in the form of an abstract (32).

METHODS

Ex Vivo Perfused Human Lung and *In Vitro* Monocyte Studies

We developed an *ex vivo* perfused human lung pneumonia model (30) from lungs declined for transplantation by the Northern California Transplant Donor Network to test the therapeutic effects of clinical-grade, cryopreserved human MSC (Figure 1). To injure the lung, either 10^9 or 10^{10} CFU of *E. coli* bacteria was instilled into the right middle lobe, and fresh human blood (100 ml) was added to the perfusate (crystalloid solution containing 5% albumin). The lungs were monitored for 6 or 10 hours, respectively. The lung was perfused using a roller pump with a cardiac output set at 0.2 L/min, giving a mean pulmonary artery pressure of less than 15 mm Hg, and oxygenated with continuous positive airway pressure at 10 cm H_2O with carbogen (a combination of 95% O_2 and 5% CO_2). One or 2 hours after the initiation of lung injury, 5 or 10 $\times$ 10^6 cryopreserved, clinical-grade human MSC, washed as if in a clinical trial, was instilled into the same right middle lobe. In separate experiments, recombinant human KGF (100 ng) or normal human lung fibroblasts (NHLF) as cellular controls was instilled. At 6 or 10 hours, the AFC rate of the injured right middle lobe was measured. The number of inflammatory cells, specifically neutrophils, and the levels of cytokines and the total bacterial load were measured in the alveolus. In separate experiments, 0.2 g of ampicillin was instilled into the perfusate at 1 hour, and the lungs were monitored for 10 hours to determine whether MSC instillation to antibiotic-treated *E. coli* bacteria-injured human lungs had any additive effect on AFC rate or total bacterial load in the injured alveolus or perfusate. The *E. coli* strain used is sensitive to ampicillin. To corroborate any findings in the *ex vivo* perfused human lung, human blood monocytes grown *in vitro* were exposed to *E. coli* bacteria with and without human MSC or KGF. We also measured various cytokines and antimicrobial proteins, such as GM-CSF, and intracellular proteins, such as phosphorylated STAT5 and phosphorylated AKT, to uncover potential mechanisms underlying the antimicrobial properties of MSC. The measurement of parameters, such as AFC, percent phagocytosis of alveolar macrophages, and total neutrophil and bacterial CFU counts in the alveolar fluid or conditioned medium can be found in the online supplement. All other techniques and reagents used are standard.

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 *t* test. Comparisons with a sample over time were made by repeated measures of analysis of variance using the Bonferroni correction for multiple-comparison testing using Statview (SAS Institute, Inc., Cary, NC).

RESULTS

MSC Restored Alveolar Fluid Clearance after *E. coli* Endotoxin–induced Acute Lung Injury

Similar to our prior work with cultured human MSC (30), instillation of 5 $\times$ 10^6 clinical-grade human MSC processed as if in

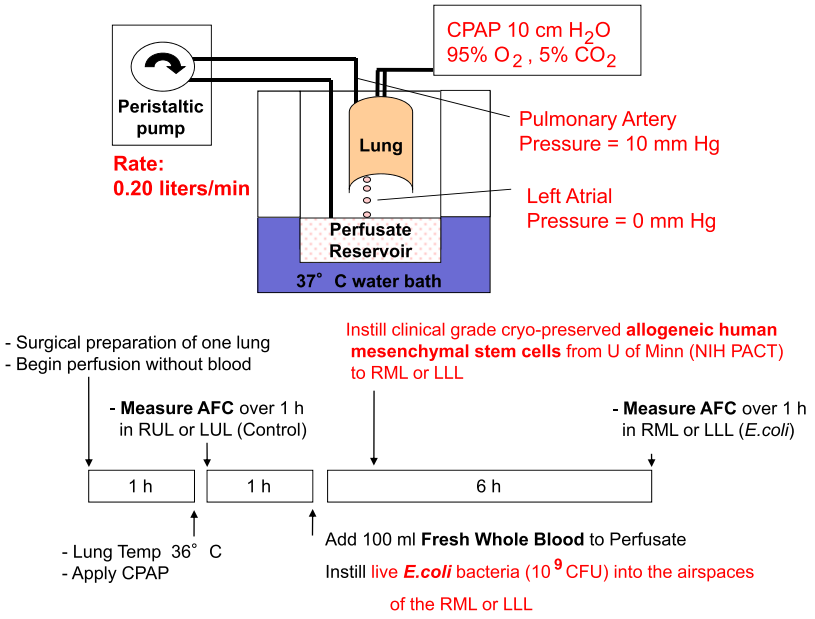

Figure 1. Schematic diagram of *Escherichia coli* pneumonia in the *ex vivo* perfused human lung. The right or left human lung declined for transplantation by the Northern California Transplant Donor Network is selected for perfusion if the total ischemic time is less than 48 hours and if the selection criteria as described in the METHODS section are met. The lung is gently rewarmed and perfused with a crystalloid solution (Dulbecco's Modified Eagle's [DME] H-21 Medium with 5% albumin) over 1 hour and oxygenated with 10 cm H_2O continuous positive airway pressure (CPAP) (FiO_2 0.95). The perfusion rate or cardiac output is set at 0.2 L/min and the left atrial pressure at 0 mm Hg to prevent hydrostatic pulmonary edema. If the alveolar fluid clearance (AFC) rate is greater than or equal to 10% per hour in the control right upper lobe (RUL) or left upper lobe (LUL), then 10^9 CFU of *E. coli* bacteria (K1 strain) is instilled into the right middle lobe (RML) or left lower lobe (LLL) and 100 ml of fresh whole blood is added to the perfusate. For mesenchymal stem cells treatment groups, human mesenchymal stem cells are instilled intrabronchially into the RML or LLL or into the perfusate (intravenously) 1 hour after the initiation of the injury.

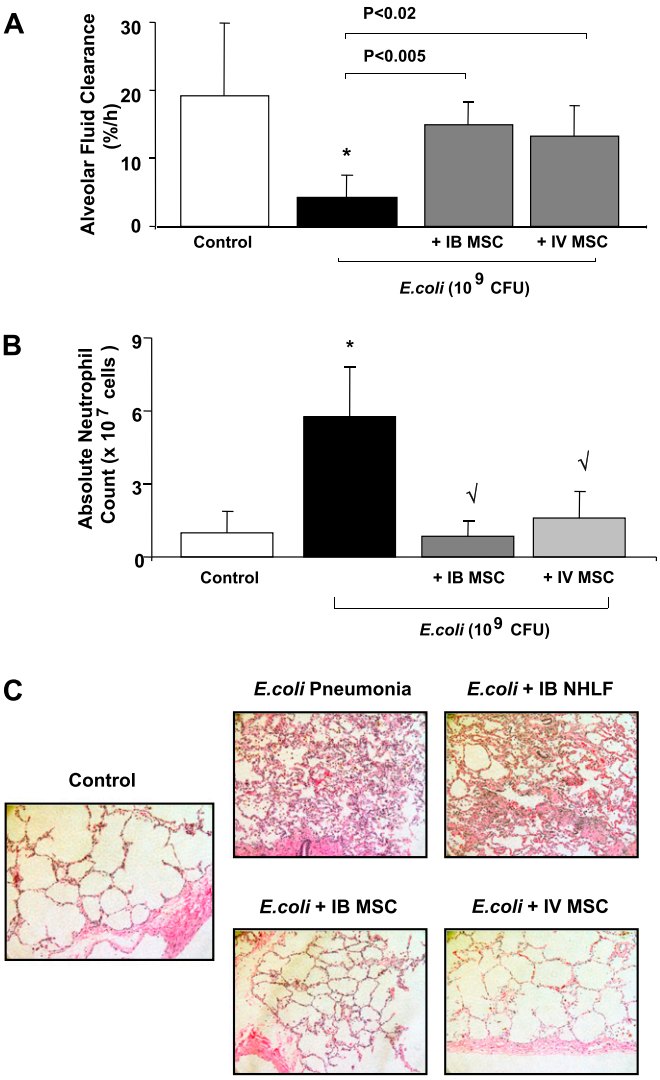

Figure 2. Effect of clinical-grade, cryopreserved allogeneic human mesenchymal stem cells (MSC) on alveolar fluid clearance and on inflammatory cell infiltration into the injured lung lobe and histology after *Escherichia coli* pneumonia. (*A*) Instillation of clinical-grade, cryopreserved human allogeneic MSC intrabronchially (IB) or intravenously (IV) restored the decrease in alveolar fluid clearance (AFC) in the lung lobe injured by *E. coli* pneumonia at 6 hours. AFC was measured by the change in protein concentration of a 5% albumin instillate in the lung lobe over 1 hour and expressed as mean AFC (% per h, per 150 ml of bronchoalveolar lavage) ± SD for each condition. n = 20 for control lobe, n = 4 for *E. coli*–injured lung lobe, n = 3 for *E. coli*–injured lung lobe treated with MSC IB or IV or normal human lung fibroblasts (NHLF), $*P < 0.008$ compared with control lobe by analysis of variance (ANOVA; Bonferroni). (*B*) Instillation of clinical-grade MSC IB or IV decreased the influx of inflammatory cells, specifically neutrophils, into the lung lobe injured by *E. coli* pneumonia at 6 hours. Absolute neutrophil counts are expressed as mean total neutrophil counts ($\times 10^7$ cells) ± SD for each condition. n = 20 for control lobe, n = 4 for *E. coli*–injured lung lobe, n = 3 for *E. coli*–injured lung lobe treated with MSC IB or IV or NHLF, $*P < 0.0001$ compared with control lobes by ANOVA (Bonferroni), $\surd P < 0.0002$ versus *E. coli* pneumonia by ANOVA (Bonferroni). (*C*) Human lungs exposed to *E. coli* bacteria with and without MSC were fixed in 10% formalin at 6 hours. Sections were stained with hematoxylin and eosin. The administration of human MSC 1 hour after *E. coli* pneumonia injury reduced the level of hemorrhage, edema, and cellularity in the injured lung lobe at 6 hours. Although not statistically significant by ANOVA, administration of human MSC IV after *E. coli* pneumonia also reduced the levels of inflammatory cytokines in a dose-dependent manner; doubling the dose of MSC to 10×10^6 cells reduced IL-1β level by 63% and tumor necrosis factor-α by 87% in the alveolar fluid compared with *E. coli*–injured lung lobes (IL-1β: 1,193 ± 549 for *E. coli*, 926 ± 156 treatment with MSC [×1], 437 ± 345 treatment with MSC [×2]; tumor necrosis factor-α: 3,019 ± 1,767 for *E. coli*, 1,604 ± 377 treatment with MSC [×1], 384 ± 189 treatment with MSC [×2], values are mean ± SD pg/ml, n = 3–4).

a clinical trial and given IB or into the perfusate (intravenous [IV]) 1 hour after endotoxin-induced ALI restored AFC to a normal level at 4 hours. Instillation of NHLF as cellular controls had no effect. Instillation of human MSC also significantly decreased IL-1β and IL-8 levels in the injured alveolus, indicating an antiinflammatory effect (*see* Figure E1 in the online supplement). In addition, PKH26-labeled human MSC preferentially migrated to endotoxin-injured lung tissue; PKH26-labeled MSC was observed in the alveolar space 2.5 times more often in the endotoxin-injured alveoli than in the uninjured controls (*see* Figure E2). To test the effect of these clinical-grade human MSC on an infectious cause of ALI, we developed an *E. coli* pneumonia model in the *ex vivo* perfused human lung (Figure 1).

Effect of MSC on Alveolar Fluid Clearance and Inflammatory Cell Infiltration into the Injured Lung after *E. coli* Pneumonia

Instillation of 10^9 CFU of *E. coli* markedly impaired AFC by 80% and was associated histologically with an influx of inflammatory cells, red blood cells, and thickening of the interstitium at 6 hours. Instillation of 5×10^6 human MSC IB or IV 1 hour after *E. coli*–induced lung injury restored AFC rate to a normal level (Figure 2A), reduced the absolute neutrophil count in the alveolus to baseline (Figure 2B), and restored lung morphology to nearly normal (Figure 2C). Instillation of NHLF had no beneficial effect.

Effect of MSCs on Alveolar Bacterial Load after *E. coli* Pneumonia

Instillation of human MSC IB or IV 1 hour after *E. coli* pneumonia significantly reduced the bacterial load at 6 hours in the injured alveolus (Figure 3A). Instillation of NHLF had no effect. Doubling the dose of MSC to 10×10^6 cells IV decreased the bacterial load by an additional 40% from *E. coli* pneumonia, suggesting a dose response. To determine if MSC secreted antimicrobial peptides and proteins into the injured alveolus, we removed cells and bacteria from the alveolar fluid of lungs exposed to *E. coli* with and without MSC therapy and reexposed this alveolar-conditioned medium to *E. coli in vitro*. Alveolar fluid from lungs treated with MSC had increased antimicrobial activity compared with the lungs exposed to *E. coli* alone (Figure 3B). Doubling the dose of MSC IV to *E. coli*–injured human lungs further increased the antimicrobial activity of the alveolar fluid.

Effect of MSC on Alveolar Macrophage Phagocytosis of *E. coli* Bacteria

In addition to the secretion of antimicrobial factors (33, 34), there is recent evidence that MSC can increase bacterial clearance by enhancing monocyte phagocytosis (29). Therefore, we tested the effect of MSC on alveolar macrophage phagocytosis

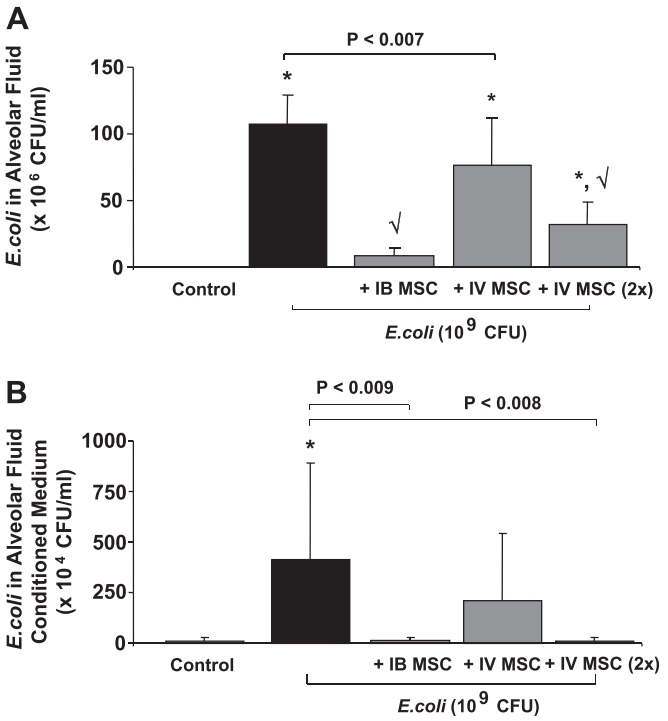

Figure 3. Effect of allogeneic human mesenchymal stem cells (MSC) on alveolar *Escherichia coli* bacterial load after *E. coli* pneumonia. (*A*) Instillation of clinical-grade, cryopreserved human allogeneic MSC intrabronchially (IB) or intravenously (IV) decreased the total bacterial load in the lung lobe injured by *E. coli* pneumonia at 6 hours. Total bacterial counts were expressed as mean ($\times 10^6$ CFU counts/ml) ± SD for each condition. n = 18 for control lobe, n = 3 for *E. coli*–injured lung lobe with or without administration of MSC IB or IV or normal human lung fibroblasts, $^*P < 0.0005$ versus control lobe CFU counts per milliliter by analysis of variance (ANOVA; Bonferroni), $^{\surd}P < 0.0001$ versus *E. coli* pneumonia CFU counts per milliliter by ANOVA (Bonferroni). (*B*) The alveolar fluid conditioned medium of lung lobes treated with human MSC IB or IV had increased antimicrobial activity against *E. coli* bacteria reexposed *in vitro*. Total bacterial counts were expressed as mean ($\times 10^4$ CFU counts/ml) ± SD for each condition. n = 19 for control lobe, n = 4 for *E. coli*–injured lung lobe, n = 3 for *E. coli*–injured lung lobe treated with MSC IB or IV or normal human lung fibroblasts, $^*P < 0.0005$ versus control CFU counts per milliliter by ANOVA (Bonferroni).

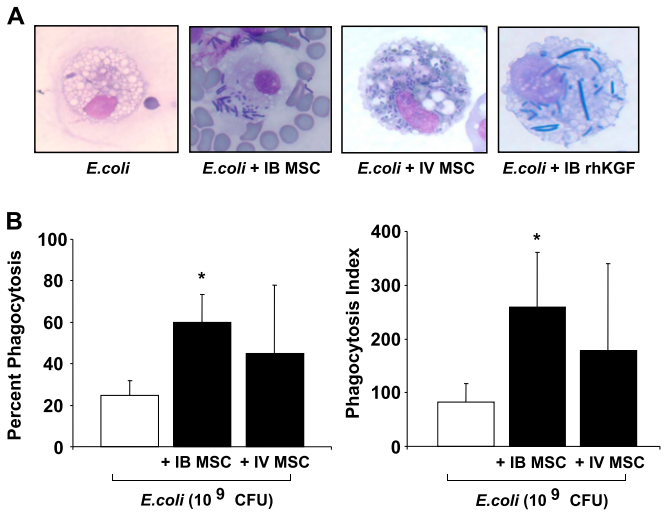

Figure 4. Effect of allogeneic human mesenchymal stem cells (MSC) on alveolar macrophage phagocytosis of *Escherichia coli* bacteria. (*A*) A representative alveolar macrophage found from cytospin slides of bronchoalveolar lavage fluid from the injured alveolus under different treatment conditions: intrabronchial (IB) MSC, intravenous (IV) MSC, and IB keratinocyte growth factor. (*B*) The instillation of human MSC IB 1 hour after *E. coli* pneumonia significantly increased the percent phagocytosis and phagocytosis index of alveolar macrophages against *E. coli* bacteria at 6 hours. Although not statistically significant, the instillation of human MSC IV 1 hour after *E. coli* pneumonia increased the percent phagocytosis and phagocytosis index of alveolar macrophages against *E. coli* bacteria by 80% and 110%, respectively, at 6 hours. The percent phagocytosis and phagocytosis index were calculated by quantifying the number of alveolar macrophages containing bacteria out of 100 macrophages from the bronchoalveolar lavage fluid of three different lung preparations per condition and quantifying the average number of bacteria per macrophage in 100 macrophages containing the bacteria from three different lung preparations per condition, respectively. The values are expressed as mean ± SD for each condition. n = 3. $^*P < 0.05$ for % phagocytosis and $^*P < 0.03$ for phagocytosis index.

in the human lung injured with *E. coli*. Although there was no significant difference in total macrophage counts, instillation of human MSC IB 1 hour after *E. coli* pneumonia significantly increased alveolar macrophage phagocytosis for *E. coli* bacteria at 6 hours. Although not statistically significant, instillation of human MSC IV 1 hour after *E. coli* pneumonia also increased alveolar macrophage phagocytosis for *E. coli* bacteria by more than 80% at 6 hours (Figure 4).

Effect of MSC with and without Antibiotics on Human Lungs Injured with *E. coli* Pneumonia for 10 Hours

To determine if the therapeutic effect of MSC would persist in human lungs injured with *E. coli* bacteria for a longer time period, we extended the pneumonia model to 10 hours, increased the bacterial load to 10^{10} CFU of *E. coli* to induce bacteremia, and administered MSC IB 2 hours after the induction of injury. MSC administration restored AFC and reduced the inflammatory cell infiltration into the injured alveolus at 10 hours. In addition, MSC eliminated the bacteremia after the more severe pneumonia (Figures 5A–5D). To determine the effect of antibiotics alone, we administered 0.2 g of ampicillin into the perfusate at 1 hour. The antibiotic treatment eliminated the bacteremia (Figure 5D), but did not significantly alter the number of bacteria (Figure 5C) or the number of neutrophils in the alveolar fluid (Figure 5B). Most importantly, antibiotics alone had no effect on AFC (Figure 5A). However, the addition of IB MSC at 2 hours after IV ampicillin at 1 hour significantly restored AFC (Figure 5A) and had an additive effect in reducing the number of neutrophils in the alveolar fluid (Figure 5B) and the total bacterial load in the injured alveolus (Figure 5C).

Effect of KGF on *E. coli* Pneumonia

Although not statistically significant, KGF levels in the alveolar fluid after administration of human MSC IB or IV for *E. coli* pneumonia were numerically higher by 17% and 38%, respectively, compared with *E. coli*–injured lung lobes (29 ± 18 for *E. coli*, 34 ± 30 treatment with MSC IB, 40 ± 17 treatment with MSC IV [×1]; values are mean ± SD pg/ml; n = 3–4). Based on our prior publication, which identified KGF as a key secreted protein by MSC that enhanced AFC after ALI (30), and a recent study that reported an antimicrobial effect of KGF for gram-positive and -negative pneumonia (31), we tested the hypothesis that recombinant human KGF would replicate some of the

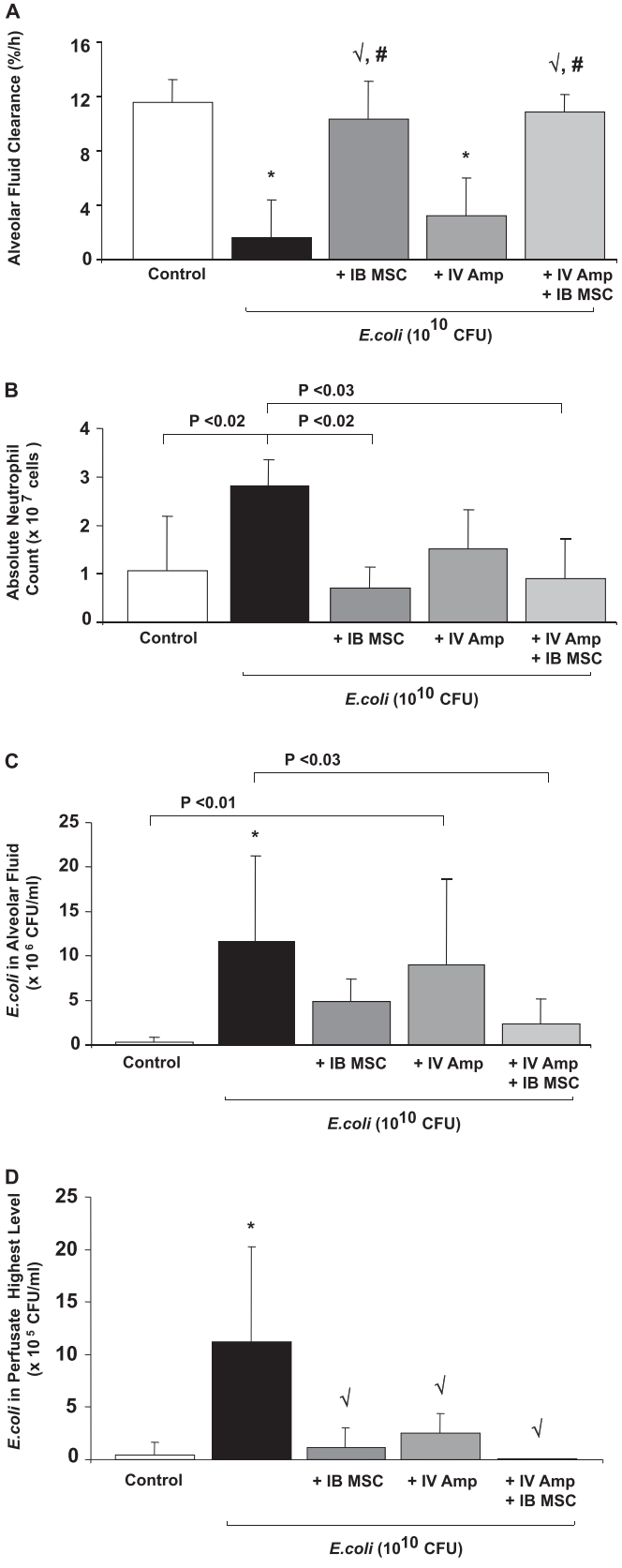

Figure 5. Effect of antibiotics or human mesenchymal stem cells (MSC) on *Escherichia coli* pneumonia at 10 hours. We extended the *ex vivo* perfused human lung model injured with *E. coli* pneumonia to 10 hours to determine if MSC treatment would be therapeutic if given at a later time-point. We also increased the *E. coli* intrabronchial (IB) dose to 10^{10} CFU from 10^9 CFU to cause a transient bacteremia in the perfusate. Similar to the previous set of experiments at 6 hours, IB MSC restored all the parameters of lung injury when given 2 hours after the induction of *E. coli* pneumonia. (*A*) Loss of alveolar fluid clearance (AFC) is expressed as mean AFC (% per h, per 150 ml of bronchoalveolar lavage ± SD for each condition). n = 12 for control lobe, n = 3 for *E. coli*–injured lung lobe treated with or without administration of MSC or ampicillin IV ± MSC, $^*P < 0.0001$ versus control lobe, $^{\surd}P < 0.0.0001$ versus *E. coli* and $^{\#}P < 0.0005$ versus *E. coli* + IV Amp AFC by analysis of variance (ANOVA; Bonferroni). (*B*) Influx of neutrophils into the injured alveolus. Absolute neutrophil counts are expressed as mean total neutrophil counts ($\times 10^7$ cells) ± SD for each condition. (*C*) Increase in the total bacterial CFU counts in the injured alveolus. Total bacterial counts were expressed as mean ($\times 10^6$ CFU counts/ml) ± SD for each condition. $^*P < 0.002$ versus control CFU counts per milliliter by ANOVA (Bonferroni). (*D*) Bacteremia present in the perfusate. The highest bacterial count found in the perfusate per hour was expressed as mean ($\times 10^5$ CFU counts/ml) ± SD for each condition. $^*P < 0.0001$ versus control lobe, $^{\surd}P < 004$ versus *E. coli* CFU counts per milliliter by ANOVA (Bonferroni). Treatment of *E. coli* pneumonia with ampicillin (0.2 g) IV at 1 hour restored most of the parameters of acute lung injury similar to MSC except for the restoration of AFC (*A–D*). The addition of IB MSC at 2 hours given after IV Amp at 1 hour after *E. coli* pneumonia had an additive effect in restoring AFC rate in the injured alveolus and decreasing the total bacterial load in the alveolus and perfusate (*C* and *D*).

effects of MSC. KGF (100 ng) IB 1 hour after *E. coli* pneumonia was as effective as human MSC in treating several parameters of ALI: the loss of AFC, increase in the influx of neutrophils, the increase in alveolar *E. coli* bacterial load, and a decrease in alveolar macrophage phagocytosis (Figures 6A–6D).

Effect of KGF on Blood Monocytes

To determine if KGF had a direct effect on macrophage-monocyte activity against bacteria, we exposed blood monocytes to KGF. The addition of KGF to human blood monocytes increased *E. coli* bacteria killing by 20% (Figure 7A). This was a modest effect compared with the bacterial killing in Figure 3A but statistically significant. By reverse-transcriptase polymerase chain reaction and Western blot analyses, human blood monocytes expressed both the mRNA and protein for FGFR2, the receptor for KGF (Figure 7B). The addition of KGF decreased lactate dehydrogenase (LDH) release after 24 hours, an effect that was associated with an increase in intracellular AKT phosphorylation at 1 hour (Figures 7C and 7D), indicating that KGF increased the survival of monocytes, one possible additional mechanism for increased bacterial clearance. Although not statistically significant, STAT5 phosphorylation was elevated by 20% with KGF treatment. In separate experiments, we administered several doses of KGF (10 ng/ml to 1 μg/ml) to monocytes *in vitro* and found that there was a dose response for both LDH release and bacteria killing (*see* Figure E3).

Effect of MSC on Monocytes

Similar to the effect of KGF, the addition of human MSC, mixed together or separated by a Transwell plate, with human blood monocytes increased *E. coli* bacterial killing by 25% and 50%, respectively (Figure 8A). The level of tumor necrosis factor (TNF)-α was also decreased and the level of IL-10 was increased, suggesting an antiinflammatory effect (Figure 8B). To determine if MSC reprogrammed the monocytes to an anti-inflammatory cell with an increase in phagocytosis, gene markers of M2 phenotype were measured by reverse-transcriptase polymerase chain reaction. However, MSC did not increase the expression of human MRC1 and arginase on the monocytes (*see*

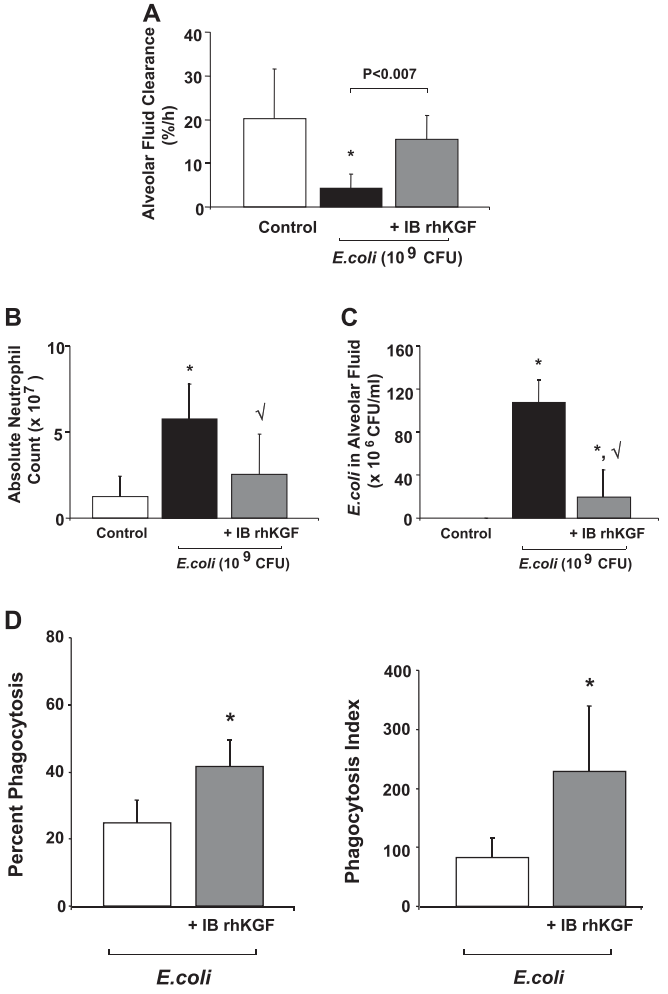

Figure 6. Effect of recombinant human keratinocyte growth factor (KGF) on *Escherichia coli* pneumonia. KGF instillation (100 ng) intrabronchially (IB) 1 hour after *E. coli* pneumonia restored many of the parameters of acute lung injury similar to mesenchymal stem cells (MSC). (*A*) Instillation of KGF IB restored the decrease in alveolar fluid clearance (AFC) in the lung lobe injured by *E. coli* pneumonia at 6 hours. AFC is expressed as mean AFC (% per h, per 150 ml of bronchoalveolar lavage) ± SD for each condition). n = 20 for control lobe, n = 3–4 for *E. coli*–injured lung lobe treated with or without KGF, $*P < 0.009$ versus control lobe AFC by analysis of variance (ANOVA; Bonferroni). (*B*) Instillation of KGF IB decreased the influx of neutrophils into the injured lung lobe at 6 hours. Absolute neutrophil counts are expressed as mean total neutrophil counts ($\times 10^7$ cells) ± SD for each condition. n = 20 for control lobe, n = 4 for *E. coli*–injured lung lobe treated with or without KGF, $*P < 0.0001$ versus control lobe by ANOVA (Bonferroni), $\surd P < 0.006$ versus *E. coli* pneumonia by ANOVA (Bonferroni). (*C*) Instillation of KGF IB decreased the total bacterial load in the injured lung lobe at 6 hours. Total bacterial counts were expressed as mean ($\times 10^6$ CFU counts/ml) ± SD for each condition. n = 18 for control lobe, n = 3 for *E. coli*–injured lung lobe with or without KGF, $*P < 0.007$ versus control CFU counts per milliliter by ANOVA (Bonferroni), $\surd P < 0.0001$ versus *E. coli* pneumonia CFU counts per milliliter by ANOVA (Bonferroni). (*D*) Instillation of KGF IB 1 hour after *E. coli* pneumonia significantly increased the percent phagocytosis and phagocytosis index of alveolar macrophages against *E. coli* bacteria at 6 hours. The values are expressed as mean ± SD for each condition. n = 3. $*P < 0.03$ for % phagocytosis and $*P < 0.05$ for phagocytosis index.

Figure E4). Human MSC, whether mixed together or separated with human blood monocytes, also increased GM-CSF secretion by 17 times and 6 times from baseline (Figure 8C); in the perfused human lung at 6 hours, MSC administration IB or IV at 1 hour increased GM-CSF levels by 200% and 80%, respectively, in the injured alveolus. The addition of KGF or human MSC also had a similar effect on human blood monocytes, cultured for 7 days to acquire the phenotype of alveolar macrophages, in terms of bacterial killing (Figure 8D). Finally, inhibition of KGF by a neutralizing antibody abrogated the antimicrobial effect of human MSC in the *ex vivo* perfused human lung and cultured blood monocytes *in vitro*, demonstrating the importance of KGF secretion in bacterial killing (Figures 9A and 9B). Inhibition of GM-CSF by a neutralizing antibody also abrogated the antimicrobial effect of MSC *in vitro*, indicating an additional paracrine factor with antimicrobial properties (Figure 9C).

DISCUSSION

The primary findings of these studies can be summarized as follows: (*1*) Clinical-grade human MSC were as effective as cultured MSC in restoring AFC after *E. coli* endotoxin–induced ALI whether these MSC were given IB or IV (*see* Figure E1). (*2*) Clinical-grade human MSC were also effective in restoring normal AFC after *E. coli* pneumonia–induced ALI whether given IB or IV (Figure 2A). (*3*) The restoration of AFC by human MSC after *E. coli* pneumonia was associated with increased bacterial killing, explained in part by an increase in alveolar macrophage phagocytosis and secretion of antimicrobial soluble factors (Figures 3 and 4). (*4*) The therapeutic effects of human MSC on *E. coli* pneumonia was duplicated in separate experiments with the IB instillation of KGF, a paracrine factor secreted by MSC and previously found to be important for net fluid transport (Figure 6) (30). (*5*) Human MSC and KGF in coculture experiments increased the antimicrobial effect of human blood monocytes, which expressed the KGF receptor by reverse-transcriptase polymerase chain reaction and Western blot analyses. To our knowledge, this is the first demonstration that KGF activates monocytes directly, not through epithelial cells, to increase the antimicrobial activity of monocytes (Figures 7 and 8). (*6*) Inhibition of KGF by a neutralizing antibody abrogated the antimicrobial effect of MSC on alveolar macrophages or blood monocytes against *E. coli* (Figure 9). (*7*) KGF increased AKT phosphorylation and decreased LDH release by blood monocytes in a dose-dependent manner, suggesting an antiapoptotic effect as an additional explanation for the increase in bacterial clearance (Figures 7C and 7D; *see* Figure E3). (*8*) In coculture experiments, human MSC secreted higher levels of GM-CSF, suggesting another paracrine mechanism for the increase in bacterial phagocytosis of monocytes (Figure 8C). (*9*) MSC *in vitro* decreased TNF-α and increased IL-10 secretion by monocytes injured by LPS, indicating an immunomodulatory effect (Figure 8B). (*10*) In experiments in which the duration and severity of *E. coli* pneumonia was increased to 10 hours with one log higher number of bacteria, MSC had an additive effect on injured lungs that were treated with IV antibiotics in restoring AFC and further reducing the bacterial load in the injured alveolus and perfusate (Figure 5).

Recent studies have demonstrated the beneficial effects of MSC in the treatment of bacterial-induced sepsis and pneumonia. In the mouse model of sepsis, Nemeth and coworkers (17) found that syngeneic MSC reduced mortality, improved organ function, and decreased total bacterial counts in the blood and peritoneal fluid, in part by the secretion of prostaglandin E_2. Gonzales-Rey and coworkers (35) reported the protective effect

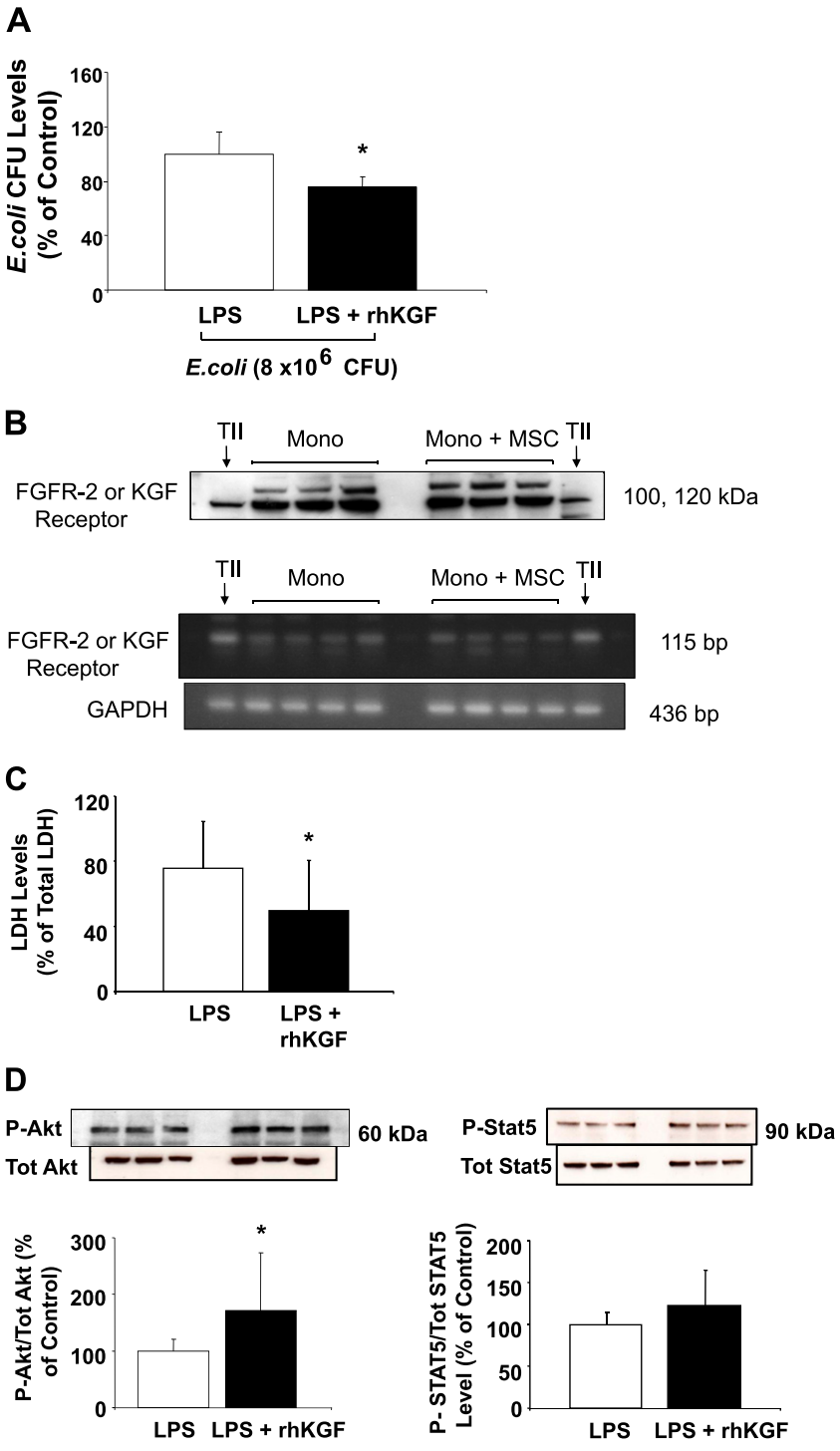

Figure 7. Effect of recombinant keratinocyte growth factor (KGF) on peripheral blood monocytes. (*A*) The simultaneous addition of KGF (100 ng/ml) to isolated human blood monocytes increased *Escherichia coli* bacteria killing by 20%. Total bacterial counts were expressed as mean (% of control) ± SD for each condition. n = 14–16, $*P < 0.0001$ versus control CFU count. (*B*) By reverse-transcriptase polymerase chain reaction and Western blot analyses, human blood monocytes were found to express the mRNA and protein for FGFR2, the receptor for KGF. Primary cultures of human alveolar epithelial type II cells (TII) were used as a positive control. (*C* and *D*) The addition of KGF decreased lactate dehydrogenase (LDH) release after 24 hours, which was associated with an increase in intracellular AKT phosphorylation at 1 hour. LDH released was expressed as mean (% of total LDH) ± SD for each condition. n = 20–24, $*P < 0.0008$ versus control LDH release; the ratio of phosphorylated AKT/total AKT was expressed as mean (% of control) ± SD for each condition. n = 10, $*P < 0.03$ versus control P-AKT/Tot AKT ratio. The ratio of phosphorylated STAT5/total STAT5 was expressed as mean (% of control) ± SD for each condition. n = 10, $*P > 0.05$ versus control P-STAT5/Tot STAT5 ratio. GAPDH = glyceraldehyde phosphate dehydrogenase.

of adipose tissue–derived human and mouse MSC in mouse experimental colitis and sepsis, which was associated with improved bacterial clearance. However, the actual mechanisms underlying enhanced bacterial clearance were not clearly identified.

Macrophages and monocytes play an important role in the production of inflammatory mediators during sepsis and seem to be a major cell target in the protective effect of MSC. Mei and coworkers (29) reported that the improvement in bacterial clearance in syngeneic MSC-treated septic mice could be in part explained by enhanced phagocytic activity of splenic monocytes. Kim and Hematti (36) reported that human MSC improved phagocytic activity of monocyte-derived macrophages when cocultured *in vitro*. They reported that coculture of human MSC and macrophages induced an alternative state (M2) of macrophage activation, which is characterized by antiinflammatory properties and more potent phagocytic activity. In a mouse model of peritoneal sepsis from gram-negative bacteria, we found that blood monocytes isolated from mice treated with MSC showed increased phagocytic activity compared with monocytes from control groups (37). We also demonstrated that MSC can inhibit bacterial growth directly in part through the secretion of antimicrobial peptides and proteins, such as LL-37 and lipocalin-2 (33, 34). Recently, several investigators found

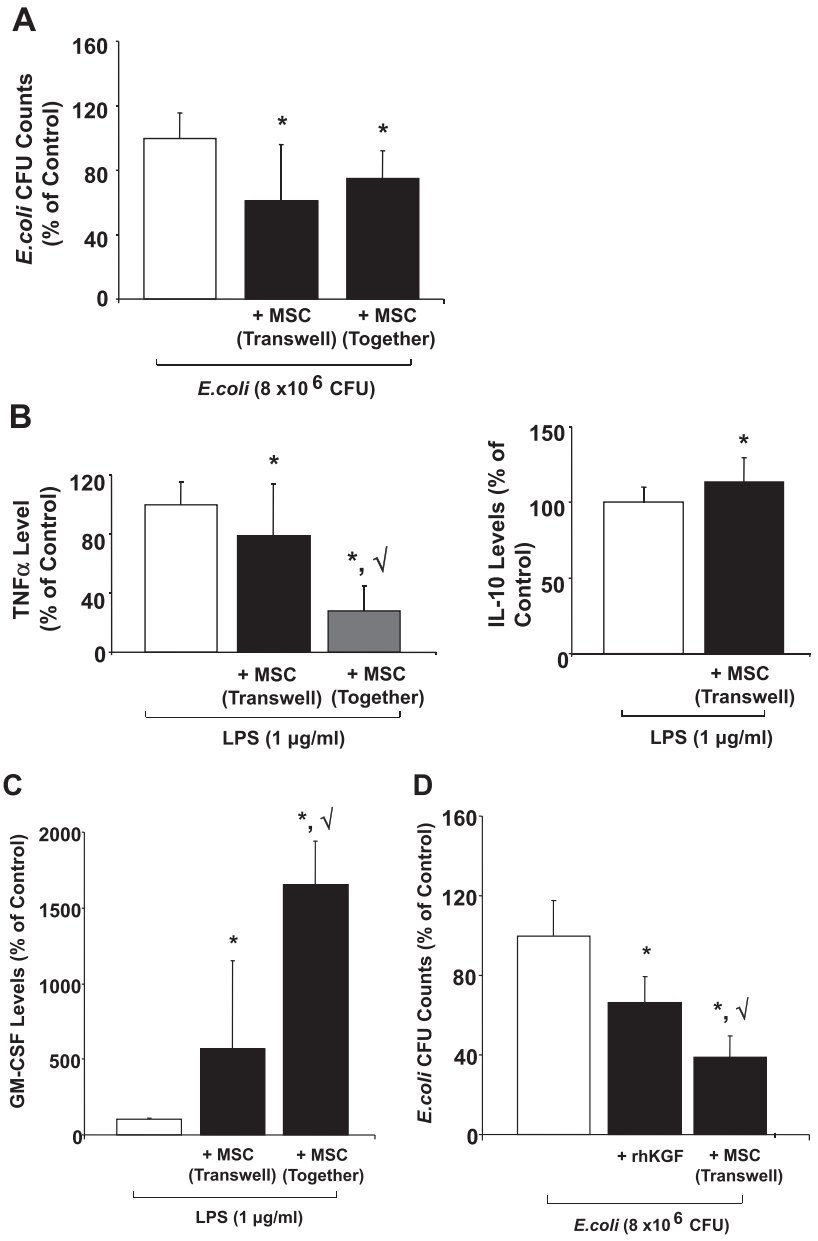

Figure 8. Effect of human mesenchymal stem cells (MSC) on peripheral blood monocytes. (*A*) The simultaneous addition of human MSC, mixed together or separated by a Transwell plate, with isolated human blood monocyte increased *Escherichia coli* bacteria killing. Total bacterial counts were expressed as mean (% of control) ± SD for each condition. n = 8–15, $*P < 0.0002$ versus control CFU counts. (*B*) In addition, the level of tumor necrosis factor (TNF)-α was decreased and the level of IL-10 was increased, suggesting an antiinflammatory effect. The TNF-α level was expressed as mean (% of control) ± SD for each condition. n = 8–11, $*P < 0.02$ versus control and $\surd P < 0.0001$ versus MSC (Transwell) for TNF-α level. The IL-10 level was expressed as mean (% of control) ± SD for each condition. n = 8–11, $*P < 0.03$ versus control for IL-10 level. (*C*) Human MSC, whether mixed together or separated by a Transwell plate, with human blood monocytes increased granulocyte-macrophage colony–stimulating factor (GM-CSF) secretion by ×17 and ×6 from baseline. The GM-CSF level was expressed as mean (% of control) ± SD for each condition. n = 8–15, $*P < 0.003$ versus control and $\surd P < 0.0001$ versus MSC (Transwell) for GM-CSF level. (*D*) The addition of keratinocyte growth factor (KGF) or human MSC had the same antimicrobial effect on monocytes cultured for 7 days to take on the phenotype of alveolar macrophages. Total bacterial counts were expressed as mean (% of control) ± SD for each condition. n = 4, $*P < 0.05$ versus control CFU counts per milliliter and $\surd P < 0.05$ versus KGF CFU counts per milliliter.

that MSC have Toll-like and formyl peptide–like receptors and become activated in response to different bacterial products, suggesting that MSC may be directly involved in innate immune response (13, 38).

In this study, we identified several mechanisms underlying the antimicrobial effect of MSC, primarily dependent on the secretion of KGF. KGF is a potent epithelial-specific mitogen and differentiation factor that plays a central role in development and repair of injured epithelial tissues (39). KGF is produced exclusively by mesenchymal cells and acts on epithelial cells through the alternatively spliced FGF-2 tyrosine kinase receptor, FGFR2-IIIb. Recombinant KGF pretreatment reduced mortality after intratracheal instillation of hydrochloric acid (40, 41), bleomycin (42, 43), hyperoxia (44, 45), and *Pseudomonas aeruginosa* (46). The protective effects of KGF have been linked to stimulation of type II cell proliferation and differentiation, DNA repair, up-regulated alveolar epithelial vectorial ion and fluid transport, and enhanced surfactant lipid and protein production. Wu and coworkers (31) also found that KGF enhanced the clearance of *E. coli* and *P. aeruginosa* ALI in mice in part through the augmentation of recruitment, phagocytic activity, and oxidant responses of alveolar macrophages. However, KGF treatment was only effective if given before or simultaneous with the injury.

In our studies, MSC or KGF given as therapy decreased the bacterial load in the perfused human lung injured by *E. coli* pneumonia (Figures 3A and 6C). MSC and KGF also increased the phagocytosis and killing of human alveolar macrophages and monocytes against *E. coli* bacteria, in part through an antiapoptotic effect (Figures 7 and 8), and possibly through the release of GM-CSF or other antimicrobial peptides and proteins by alveolar epithelial cells or by MSC itself (Figure 8C). These beneficial effects of MSC supplemented the benefit of antibiotics in human lung injured to 10 hours (Figure 5). The importance of KGF secretion was confirmed in these experiments when administration of an antihuman KGF antibody abolished the antimicrobial effect of MSC (Figure 9). Although MSC decreased TNF-α and increased IL-10 secretion by monocytes after LPS stimulation *in vitro*, suggesting an immunomodulatory phenotype (Figure 8B), we did not find an increase in

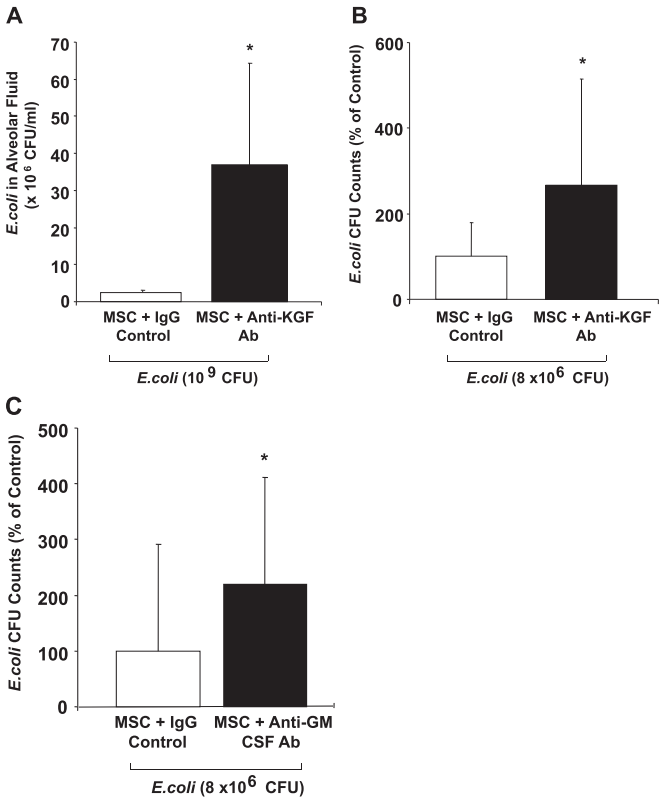

Figure 9. Effect of antihuman keratinocyte growth factor (KGF) or anti–granulocyte-macrophage colony–stimulating factor (GM-CSF) neutralizing antibody on the effect of human mesenchymal stem cells (MSC) on alveolar macrophages and peripheral blood monocytes. The inhibition of KGF by a neutralizing antibody abrogated the antimicrobial effect of human MSC on (*A*) alveolar macrophages in the *ex vivo* perfused human lung and (*B*) blood monocytes cultured *in vitro*. (*C*) Similar to anti-KGF Ab, the inhibition of GM-CSF by a neutralizing antibody abrogated the antimicrobial effect of human MSC *in vitro*. Total bacterial counts in the alveolar fluid for the perfused human lung were expressed as mean ($\times 10^6$ CFU counts/ml) $\pm$ SD for each condition. n = 3–5, $^*P < 0.05$ versus MSC CFU counts per milliliter treated with goat control IgG. Total bacterial counts for monocytes grown *in vitro* were expressed as mean (% of control) $\pm$ SD for each condition. n = 8–9. For anti-KGF Ab, $^*P < 0.05$ versus MSC treated with goat control IgG. n = 14–16. For anti–GM-CSF Ab, $^*P < 0.04$ versus MSC treated with goat control IgG.

MRC1 or arginase expression in monocytes, markers of the M2 phenotype (*see* Figure E4).

There are some limitations to the current studies. It is unclear if the antimicrobial properties of MSC are dependent on cell-cell contact, the inflammatory milieu, or both. For instance, MSC had a more significant effect on suppressing the release of TNF-α and increasing GM-CSF secretion by 300% *in vitro* if the cells were mixed together with the monocytes after LPS stimulation compared with separation by a Transwell plate (Figures 8B and 8C). In addition, Islam and coworkers (47) recently reported that MSC formed connexin 43 gap junctional channels with LPS-injured alveolar epithelium in mice, releasing mitochondria-containing microvesicles that the epithelium engulfed, which restored alveolar ATP levels and reduced lung injury. The contribution of cell-mediated effects of MSC on the alveolar epithelium and macrophages needs to be studied further to determine if MSC are more potent than the individual secreted peptides and proteins, such as KGF.

In conclusion, clinical-grade, cryopreserved allogeneic human MSC are therapeutic in *E. coli* pneumonia in the *ex vivo* perfused human lung. The effects are mediated by the capacity of MSC to suppress inflammatory responses, restore AFC to a normal level, and enhance bacterial clearance, including the ability to prevent translocation of bacteria into the bloodstream. The antimicrobial effects of MSC could be largely duplicated by KGF, a major paracrine product of MSC. In *ex vivo* and *in vitro* studies, MSC or KGF increased alveolar macrophage or blood monocyte phagocytosis and killing of *E. coli* bacteria, explained in part by a decrease in monocyte apoptosis through AKT phosphorylation. In addition, MSC secreted GM-CSF, a cytokine previously found to have significant antimicrobial activity (31). These antimicrobial properties of MSC may reduce concern for clinical testing of cell-based therapy with significant immunesuppressive effects after sepsis- or pneumonia-induced ALI.

Author disclosures are available with the text of this article at www.atsjournals.org.

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