Therapeutic Effects of Human Mesenchymal Stem Cell–derived Microvesicles in Severe Pneumonia in Mice

Antoine Monsel^{1,2,3}, Ying-gang Zhu³, Stephane Gennai³, Qi Hao³, Shuling Hu³, Jean-Jacques Rouby¹, Michelle Rosenzweig², Michael A. Matthay³, and Jae W. Lee³

¹Multidisciplinary Intensive Care Unit, Department of Anesthesiology and Critical Care, La Pitié-Salpêtrière Hospital, Assistance Publique-Hôpitaux de Paris, University Pierre and Maris Curie (UPMC) Univ Paris 06, Paris, France; ²Department of Immunology-Immunopathology-Biotherapy UPMC Univ Paris 06, Sorbonne Universités, INSERM UMRs 959, Paris, France; and ³Department of Anesthesiology & Medicine, University of California San Francisco, San Francisco, California

Abstract

Rationale: Microvesicles (MVs) are anuclear fragments of cells released from the endosomal compartment or shed from surface membranes. We and other investigators demonstrated that MVs released by mesenchymal stem cells (MSCs) were as effective as the cells themselves in inflammatory injuries, such as after endotoxin-induced acute lung injury. However, the therapeutic effects of MVs in an infectious model of acute lung injury remain unknown.

Objectives: We investigated the effects of human MSC MVs on lung inflammation, protein permeability, bacterial clearance, and survival after severe bacterial pneumonia.

Methods: We tested the effects of MVs derived from human MSCs on *Escherichia coli* pneumonia in mice. We also studied the interactions between MVs and human monocytes and human alveolar epithelial type 2 cells.

Measurements and Main Results: Administration of MVs derived from human MSCs improved survival in part through keratinocyte growth factor secretion and decreased the influx of inflammatory cells, cytokines, protein, and bacteria in mice injured with bacterial pneumonia. In primary cultures of human monocytes or alveolar type 2 cells, the uptake of MVs was mediated by CD44 receptors, which were essential for the therapeutic effects. MVs enhanced monocyte phagocytosis of bacteria while decreasing inflammatory cytokine secretion and increased intracellular ATP levels in injured alveolar epithelial type 2 cells. Prestimulation of MSCs with a toll-like receptor 3 agonist further enhanced the therapeutic effects of the released MVs.

Conclusions: MVs derived from human MSCs were as effective as the parent stem cells in severe bacterial pneumonia.

Keywords: acute respiratory distress syndrome; bacterial pneumonia; mesenchymal stem cells; microvesicles

Bacterial pneumonia is among the primary causes of respiratory failure in critically ill patients and is the leading etiology of acute respiratory distress syndrome. Despite improvements in supportive care and appropriate antibiotic use, morbidity

and mortality from acute respiratory distress syndrome remain high (1, 2). We and others reported that mesenchymal stem (stromal) cells (MSCs) were effective in preclinical models of acute lung injury (ALI) from pneumonia or sepsis due to

their ability to secrete paracrine factors such as growth factors, antiinflammatory cytokines, and antimicrobial peptides (3–11). Moreover, in an *ex vivo* perfused human lung injured with *Escherichia coli* pneumonia, we demonstrated that the

(Received in original form October 1, 2014; accepted in final form March 31, 2015)

Supported by an International Research Grant from the Société Française d'Anesthésie-Réanimation (Paris, France) (A.M.); by the Medical Research Grant from the group Pasteur-Mutualité (Paris, France) (A.M.); by National Heart, Lung, and Blood Institute grants R37 HL-51856 (M.A.M.) and HL-113022 (J.W.L.); and by Hamilton Endowment Funds (UCSF Department of Anesthesiology, San Francisco, CA) (J.W.L.).

Author Contributions: A.M. contributed to overall study design, performance of the experiments, data analysis and interpretation, and writing of the manuscript. Y.-g.Z. contributed to the study design, performance of the experiments, and data analysis and interpretation. S.G., Q.H., and S.H. contributed to the performance of the experiments and data analysis. J.-J.R. and M.R. contributed to the data analysis and writing of the manuscript. M.A.M. contributed to the study design, financial support, data analysis and interpretation, and editing the manuscript. J.W.L. contributed to overall study design, financial support, performance of the experiments, data analysis and interpretation, writing of the manuscript, and final approval.

Correspondence and requests for reprints should be addressed to Jae W. Lee, M.D., Department of Anesthesiology, University of California San Francisco, 505 Parnassus Avenue, Box 0648, San Francisco, CA 94143. E-mail: leejw@anesthesia.ucsf.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 192, Iss 3, pp 324–336, Aug 1, 2015

Copyright © 2015 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201410-1765OC on June 11, 2015

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: We and other investigators have demonstrated that microvesicles derived from human mesenchymal stem (stromal) cells (MSC MVs) reduced the severity of sterile inflammatory injury to the kidneys or lungs in part through the transfer of mRNAs for growth factors and antiinflammatory cytokines. However, the effect of MSC MVs on lung injury after an infectious etiology, such as severe bacterial pneumonia, is currently unknown.

What This Study Adds to the

Field: Using an *in vivo* model of *Escherichia coli* pneumonia in mice, administration of human MSC MVs was as effective as their parent stem cells in improving survival and mitigating lung inflammation, protein permeability, and bacterial growth. The antimicrobial effect of MSC MVs was in part through enhancement of monocyte phagocytosis of bacteria, which could be further increased by prestimulation of MSCs with a toll-like receptor 3 agonist before the release of MVs. The uptake of MSC MVs through the CD44 receptor into injured human monocytes and alveolar epithelial cells was critical for their therapeutic effects. Administration of a keratinocyte growth factor neutralizing antibody abrogated the survival advantage mediated by MSC MVs, suggesting a possible mechanism for the therapeutic effect.

addition of MSCs with antibiotics had a more potent antimicrobial effect than antibiotics alone in reducing total bacterial load (8). This was in addition to the ability of the MSCs to treat the major abnormalities that underlie lung injury, including impaired alveolar fluid clearance, altered lung permeability, and dysregulated inflammation (8). However, concerns remain regarding whether MSCs can become tumorigenic or have other side effects (12–14).

Recently, MSCs have been found to release microvesicles (MVs) that were as

biologically active as the cells themselves. MVs are anuclear plasma membrane-bound fragments, 50 to 200 nm in size, constitutively released from multiple cell types from the endosomal compartment as exosomes or shed from the plasma membrane. Similar to stem cells, MVs derived from MSCs home to the inflammatory site and transfer proteins/peptides, mRNA, microRNA, lipids, and/or organelles with reparative and antiinflammatory properties to the injured tissue (15, 16). Camussi and colleagues demonstrated that the protective effects of MSC MVs in acute kidney injury in mice were through the transfer of mRNA and microRNA to the injured renal epithelium, leading to a decrease in apoptosis (16–20). We recently reported that MVs released by human MSCs reduced the severity of endotoxin-induced ALI in mice through the transfer of mRNA for keratinocyte growth factor (KGF) to the injured alveolus (21).

Currently, the effect of MSC MVs in ALI from bacterial pneumonia is unknown. We hypothesized that administration of MSC MVs was as effective as the MSCs in reducing the severity of *E. coli* pneumonia in part through increased bacterial clearance. Some of the results of these studies have been previously reported in the form of an abstract (22).

Methods

A detailed description of the methods is provided in the online supplement.

MSCs

Human bone marrow-derived MSCs were obtained from a National Institutes of Health repository from Texas A&M Health Science Center. Normal human lung fibroblasts (NHLFs) were used as cellular controls.

Isolation of MSC-derived MVs

MVs were obtained from the supernatants of MSCs and NHLFs using ultracentrifugation as previously described (18). MSCs or NHLF MVs were resuspended according to the final cell count after 48 hours (10 μ l per 1×10^6 cells). Electron microscopy, protein and mRNA levels, and Western blot analysis for CD44 expression were performed to characterize MSC MVs. In additional

experiments, MSC MVs were fluorescent labeled (23) or blocked with CD44 or KGF neutralizing antibody to study MV uptake and function. Polyinosinic-polycytidylic acid [Poly (I:C)], a TLR3 agonist (24), was used to stimulate MSCs before the isolation of the released MVs [Poly (I:C) MVs].

E. coli Pneumonia-induced ALI in Mice

Male C57BL/6 mice (10–12 wk, 20–25 g) were injured with intratracheal instillation of *E. coli* K1 strain (2 or 3×10^6 colony-forming units [cfu]) using protocols approved by the Institutional Animal Care and Use Committee at University of California San Francisco (25). Four hours later, phosphate-buffered saline (PBS) as vehicle, 800,000 MSCs as a positive cellular control, NHLF MVs as a negative control, or MSC MVs were instilled intratracheally (30 or 60 μ l) or injected intravenously (90 μ l). In separate experiments, CD44 or KGF neutralizing antibody was given intravenously with MSC MVs. Mice were killed at 18, 24, or 72 hours, and bronchoalveolar lavage (BAL) samples or lungs were collected for assessment of neutrophil counts, cytokine levels, bacterial load, protein levels, and histology.

Statistics

Results are expressed as mean $\pm$ SD. Comparisons between two groups were made using unpaired *t* test or the Mann–Whitney *U* test. Comparisons between more than two groups were made using ANOVA or Kruskal–Wallis test using the Bonferroni's correction for multiple-comparison testing. The log-rank test was used for comparing survival data at 72 hours. A *P* value <0.05 was considered statistically significant. All statistical analysis was performed using GraphPad Prism software (La Jolla, CA). In the figure legends, *n* refers to the number of samples or mice, not the number of replicate experiments of the same sample or mouse.

Results

Isolation and Characterization of MSC MVs

The therapeutic dose chosen, 90 μ l, of MSC MVs was the MVs released by 9×10^6 MSCs over 48 hours. The viability of MSCs and NHLFs before MV isolation was $>95\%$ (trypan blue exclusion). Scanning electron

microscopy showed that the isolation technique yielded spheroid phospholipid bilayer-bound structures measuring approximately 200 nm (Figure 1A). Protein and total RNA quantities of 90 μ l MSC MVs were, respectively, 90 ± 48 μ g (n = 24) and 97 ± 90 ng (n = 7) (Figure 1B). This protein concentration falls into a range of doses used in previous studies after lung (26) or kidney injury (18). Western blot analyses showed that MSC MVs expressed CD44 (Figure 1C), a key receptor involved in MSC trafficking (16).

MSC MVs Improved Survival in *E. coli*-induced ALI in Part through KGF Secretion

Intratracheal instillation of 3×10^6 cfu of *E. coli* resulted in significant ALI, leading to

40% survival at 72 hours. Intravenous administration of 90 μ l of MSC MVs 4 hours after injury increased survival to 88% at 72 hours compared with mice treated with PBS or NHLF MVs as negative controls (Figure 2A). MSCs increased the survival rate (67% at 72 h) compared with both negative control groups. No difference was found between the MSC MV and MSC groups. NHLF MVs at the same dose had no therapeutic effect (Figure 2A). MSC MVs contained the mRNA for KGF (Figure 2B). Instillation of MSC MVs or MSCs increased human KGF protein levels in the injured alveolus compared with controls (Figure 2C). Administration of MSC MVs with an anti-KGF neutralizing antibody abrogated the beneficial effect on survival (Figure 2D).

Figure 1. Characterization of microvesicles (MV) released from human mesenchymal stem cells (MSCs) (MSC MVs). (A) After serum starvation, human MSCs constitutively release MVs (arrows), small-membrane enclosed bodies from the plasma membrane (scale bar = 1 μ m). Insert shows purified MSC MVs as a homogeneous population of spheroid particles (scale bar = 2 μ m). (B) The protein concentration of the therapeutic dose, 90 μ l of MSC MVs, used for the small animal studies was similar to the dose of MSC MVs previously used in the literature. MSC MVs contained a significant amount of total RNA. MSC MV protein and RNA contents are expressed as mean $\pm$ SD total protein (μ g/90 μ l) and total RNA (ng/90 μ l), respectively (n = 24 for protein; n = 7 for RNA content). (C) Western blot analyses of MSC MVs demonstrated significant levels of CD44, a receptor previously involved in MSC trafficking to inflammatory sites. Similar to MSCs, we hypothesized that MSC MVs homed to injured tissues using the CD44 receptor. MW = molecular weight.

MSC MVs Decreased Lung Inflammation, Protein Permeability, and Histological Severity in *E. coli* Pneumonia

Intratracheal instillation of approximately 2×10^6 cfu *E. coli* produced nonlethal but severe lung injury over 24 hours, characterized by an influx of neutrophils, high levels of monocyte inflammatory protein-2 (MIP-2), increased lung protein permeability, and development of histological evidence of ALI in the injured alveolus (Figures 3 and 4). Intravenous administration of 90 μ l of MSC MVs 4 hours after the injury reduced the influx of white blood cells by 40% and neutrophils by 53% and decreased the total protein concentration by 22% in the BAL fluid at 24 hours compared with PBS-treated mice (Figures 3). BAL fluid MIP-2 levels and histological severity score were also decreased compared with mice given either PBS or NHLF MVs at 18 hours after injury (Figures 3B and 4). No therapeutic effect was observed with the intravenous administration of the same dose of NHLF MVs (Figures 3 and 4).

In preliminary experiments, 60 μ l of MSC MVs given intratracheally 4 hours after injury had similar beneficial effects, reducing the influx of neutrophils by 50% and the protein levels by 25% in the BAL fluid as compared with mice given PBS (Figure 3C). However, there was no significant effect of intratracheal MSC MVs on MIP-2 levels. All further studies were done with intravenous administration of MSC MVs to correlate with ongoing clinical trials of cell-based therapy for ALI.

Dose-Response Effect of MSC MVs on *E. coli*-induced ALI

An initial dose-response effect of intravenous MSC MVs on *E. coli*-induced ALI was generated to arrive at the optimal intravenous dose of 90 μ l (see Figure E1 in the online supplement).

Effect of MSC MVs on *E. coli* Total Bacterial Load

Intratracheal instillation of *E. coli* resulted in a significant bacterial load (median, 1.2×10^5 cfu; 25th–75th percentile, 8.5×10^3 to 9.3×10^5) in the injured alveoli at 18 hours. Intravenous administration of MSC MVs significantly decreased the bacterial load in BAL fluid and lung homogenate by 97 and 50%, respectively, as compared with mice treated with PBS (Figures 5A and 5B).

Figure 2. Keratinocyte growth factor (KGF)-mediated effect of mesenchymal stem cell (MSC) microvesicles (MVs) on survival after severe *Escherichia coli* pneumonia in mice. Intravenous administration of MVs released from MSCs (MSC MVs) improved survival in mice with severe *E. coli* pneumonia. Administration of a neutralizing KGF antibody with MSC MVs abrogated this therapeutic effect. (A) Administration of MSCs or MSC MVs significantly increased survival over 72 hours. * $P < 0.01$ versus phosphate-buffered saline (PBS), # $P < 0.05$ versus normal human lung fibroblast (NHLF) MV-treated group, and $^{\dagger}P < 0.05$ versus PBS by log-rank test. (B) Reverse transcriptase polymerase chain reaction demonstrated that MSC MVs expressed KGF messenger RNA. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control to normalize loading of the RNA samples. The PCR products were 210 bp in size for KGF. (C) Intravenous administration of MSC MVs increased human KGF protein levels in the bronchoalveolar lavage fluid (BAL) of *E. coli*-injured mice at 18 hours, which was not accounted for by MV intracellular KGF protein levels; lysates of 90 μ l of MVs yielded only 16.5 ± 5.4 pg total of KGF protein. Data are shown as mean $\pm$ SD for each condition ($n = 29$ for PBS, $n = 12$ for MSCs, $n = 22$ for MVs released from standard mesenchymal stem cells, and $n = 10$ for polyinosine-polycytidylic acid [Poly (I:C)] MVs). *** $P < 0.01$ versus the Poly (I:C) MV group by ANOVA (Bonferroni). (D) Administration of an anti-KGF neutralizing antibody with the MSC MVs significantly decreased survival over 72 hours compared with the MSC MV + control IgG-treated group. * $P < 0.01$ versus PBS; $\checkmark P < 0.01$ versus MSC MV + anti-KGF antibody-treated group by log-rank test. MV + IgG = MVs released from MSCs combined with control IgG antibody; MV + anti-KGF Ab = MVs released from MSCs combined with an anti-KGF neutralizing antibody; NHLF MV = MVs released from normal human lung fibroblasts.

MSC MV treatment also eliminated the bacteremia seen (Figure 5C). Administration of NHLF MVs had no therapeutic effect.

Intratracheal treatment with MSC MVs decreased the incidence of bacteremia from 100% in the PBS group to 11% in the treated mice (Figure 5E). Although there was a reduction in the BAL fluid bacterial cfu counts by 40% with MSC MV treatment, this change was not significant (Figure 5D).

CD44 Receptor-Dependent Uptake of MSC MVs into Primary Cultures of Human Monocytes and Human Alveolar Epithelial Type 2 Cells

The uptake of fluorescently-labeled MSC MVs was increased by 100% in human monocytes and by 150% in human alveolar epithelial type 2 cells with injury (LPS and/or cytomix) (Figure 6). The impact of inflammation on the uptake of MVs was confirmed using MVs isolated from murine green fluorescent protein-transfected MSCs on

human monocytes with or without LPS exposure, allowing us to rule out any "contamination" by an excess of the fluorescent dye. Exposure to the anti-CD44 neutralizing antibody suppressed this inflammatory uptake of MSC MVs, suggesting a CD44-dependent mechanism (Figure 6). A loss of the therapeutic effect of MSC MVs on survival was also seen when MSC MVs preincubated with anti-CD44 blocking antibody were administered to mice with severe *E. coli* pneumonia compared with IgG control (Figure 6D). The expression of the CD44 ligands L-selectin and osteopontin was increased in human monocytes and alveolar epithelial type 2 cells after inflammatory injury (Figure E2).

Effect of MSC MVs on Human Monocytes and Alveolar Epithelial Type 2 Cells

MSC MV treatment reduced *E. coli* bacterial cfu counts in primary cultures of human monocytes by 25% compared with PBS control (Figure 7A). This antimicrobial effect was in part due to an increase in percentage phagocytosis and phagocytic index of human monocytes against *E. coli* bacteria. MSC MV administration also decreased tumor necrosis factor- α (TNF- α) secretion by 30% (Figure 7B), which was associated with a numerical increase in prostaglandin E2 (PGE2) levels by 40% by monocytes ($P = \text{NS}$). Although the antimicrobial effect was similar between MSC MV and MSC treatment, TNF- α secretion was reduced further in the MSC group (by 65%) compared with the MSC MV group (by 30%) (Figure 7B). MSC MV treatment restored intracellular ATP levels in injured human alveolar epithelial type 2 cells to control levels (Figure 7C).

Human monocytes exposed with MSC MVs exhibited lower levels of mRNA for human inducible nitric oxide synthase, a type 1 (M1, proinflammatory) marker, and higher levels of mRNA for transglutaminase 2, a type 2 (M2, antiinflammatory) marker. However, there were no changes on other M2 markers (CD163 or CD206) by PCR or flow cytometry (Figure E3).

Prestimulation of MSCs with the Toll-Like Receptor 3 Agonist Poly (I:C) Increased the Expression of Cyclooxygenase 2 and IL-10 mRNA in MSCs and Human Monocytes
Human MSCs expressed mRNA for TLR-3 by reverse transcriptase polymerase chain

Figure 3. Effect of microvesicles (MVs) released from mesenchymal stem cells (MSCs) (MSC MVs) on the influx of inflammatory cells and on inflammation after *Escherichia coli*-induced acute lung injury in mice. Intravenous and intratracheal administration of human MSC MVs reduced the influx of inflammatory cells and lung protein permeability in the injured alveoli after *E. coli* pneumonia. (A) Intravenous administration of MSC MVs decreased total white blood cell (WBC) and neutrophil counts in the bronchoalveolar lavage (BAL) fluid of *E. coli*-injured mice at 24 hours. Data are shown as mean $\pm$ SD for each condition (n = 21 for sham, n = 20 for phosphate-buffered saline (PBS), n = 6 for MSCs, n = 22 for MSC MVs, and n = 5 for normal human lung fibroblast [NHLF] MVs). * $P < 0.05$ versus PBS by ANOVA (Bonferroni) for neutrophil count, ** $P < 0.01$ versus PBS; $\checkmark P < 0.05$ versus NHLF MV group by ANOVA (Bonferroni) for total WBCs. (B) Intravenous MSC MVs reduced lung protein permeability and MIP-2 levels in BAL fluid of mice injured with *E. coli* pneumonia at 24 and 18 hours, respectively (n = 17–21 for sham, n = 19–22 for PBS, n = 6–9 for MSCs, n = 14–20 for MSC MVs, and n = 5–14 for NHLF MVs). * $P < 0.05$ versus MSCs for protein concentration by ANOVA (Bonferroni), *** $P < 0.01$ versus PBS, and $\checkmark P < 0.05$ versus NHLF MVs. (C) Intratracheal MSC MV administration decreased total WBCs, neutrophil counts, and the protein concentration in the BAL fluid of *E. coli*-injured mice at 24 hours. Data are shown as mean $\pm$ SD for each condition (n = 2–3 for sham, n = 5–20 for PBS, n = 9–14 for MSC MVs, and n = 5 for NHLF MVs). MIP-2 = macrophage inflammatory protein-2.

reaction (Figure 8A). MSC pretreatment with Poly (I:C) increased mRNA expression for cyclooxygenase 2 (COX2) and IL-10 in MSCs (Figures 8B and 8C) and increased the

expression of COX2 in the released MVs compared with standard MSC MVs (Figure 8D). No detectable level of mRNA for IL-10 was found in MSC MVs with and

without pretreatment with Poly (I:C). More importantly, primary cultures of human monocytes exposed to Poly (I:C)-pretreated MVs exhibited higher level of mRNA for

Figure 4. Effect of the administration of microvesicles (MVs) released from mesenchymal stem cells (MSCs) (MSC MVs) on lung injury after severe *Escherichia coli* pneumonia in mice. (A) Intravenous MSC MVs or MSCs significantly improved lung injury as assessed by histology. Hematoxylin and eosin-stained lung sections at 18 hours exhibited a reduction in neutrophil influx, edema, wall thickening, and airspace congestion. (B) Lung injury as assessed by semiquantitative scoring was reduced by MSC or MSC MV treatment. Data are shown as mean $\pm$ SD for each condition ($n = 4-5$). * $P < 0.01$ versus phosphate-buffered saline (PBS), $\checkmark P < 0.01$ versus the normal human lung fibroblast (NHLF)-treated group, and $\dagger P < 0.01$ versus MSCs by Kruskal-Wallis test (Dunn).

COX2 and IL-10 compared with standard MSC MVs (Figures 8E and 8F).

MVs Released from Poly (I:C)-pretreated MSCs Enhanced Human Monocytes' Phagocytic Capacity against Bacteria and Further Decreased TNF- α and Increased IL-10 Secretion

Treatment of human monocytes with Poly (I:C)-pretreated MVs further reduced bacterial cfu counts in the culture medium by 15% compared with standard MSC MV treatment (Figures 9A and 9D). The percentage phagocytosis remained similar in both groups. However, Poly (I:C)-treated MVs increased the phagocytic index of monocytes by 2.8-fold compared with standard MSC MVs (Figures 9E and 9F). In addition, monocytes treated with Poly (I:C)-treated MVs had reduced TNF- α (by 14%) and increased IL-10 levels (by 22%) compared with standard MSC MVs (Figures 9G and 9H).

In Vivo Therapeutic Effect of MVs Released from Poly (I:C)-pretreated MSCs

Compared with standard MSC MVs, intravenous administration of

Poly (I:C)-treated MVs further increased KGF secretion in BAL fluid (Figure 2C) and further reduced the BAL fluid bacterial cfu count by 74% after *E. coli* pneumonia but had no effect on other lung inflammatory parameters (Figures 9 and E4).

Discussion

The main findings of this work are summarized as follows. (1) Intravenous administration of MSC MVs as therapy improved survival through a KGF-mediated effect (Figure 2) and reduced the total bacterial load, inflammation, and lung protein permeability in the injured alveolus in mice with *E. coli* pneumonia (Figures 2-4); (2) the therapeutic effects of MSC MVs were equivalent to MSCs (Figures 2-4); (3) the antimicrobial effect of MSC MVs was mediated in part through an increase in monocytes phagocytosis, which was further increased by Poly (I:C) prestimulation of MSCs (Figures 5A and 9); (4) MSC MVs decreased TNF- α secretion by LPS-primed human monocytes and restored intracellular ATP levels in injured human alveolar epithelial type 2 cells,

suggesting immunomodulatory and metabolomic effects of MVs (Figure 7); (5) TLR3 prestimulation further increased mRNA expression for COX2 and IL-10 in MSCs and human monocytes exposed to Poly (I:C)-treated MVs (Figure 8), and treatment with Poly (I:C)-MVVs further decreased TNF- α secretion and increased IL-10 secretion by monocytes (Figures 9G and 9H); (6) treatment with Poly (I:C)-treated MVs further decreased bacterial cfu counts *in vitro* and *in vivo* compared with standard MSC MVs (Figures 9D and 9I); and (7) CD44 neutralizing antibody abrogated MSC MV uptake in injured human monocytes and alveolar epithelial type 2 cells (Figure 6) and suppressed the therapeutic effects of MSC MVs on mice survival in *E. coli* pneumonia (Figure 6D), demonstrating the critical role of CD44 in the uptake of the MVs into the injured cell for its therapeutic effect.

This is the first study demonstrating that MSC MVs are as effective as MSCs in improving survival in a model of *E. coli* pneumonia. We previously reported that MSC MVs reduced the severity of endotoxin-induced ALI in mice (21). However, the model was not lethal, and

Figure 5. Effect of microvesicles (MVs) released from mesenchymal stem cells (MSCs) (MSC MVs) on total bacterial load. Intravenous administration of human MSC MVs reduced the total bacterial load in the alveolar space (A), lung tissue (B), and bloodstream (C) after *Escherichia coli* pneumonia. Intratracheal administration of human MSC MVs numerically reduced the total alveolar bacterial load (D) and significantly reduced the incidence of bacteremia (E). (A) Intravenous MSC MVs decreased the total alveolar bacterial load in mice injured by *E. coli* pneumonia at 18 hours. Total bacterial counts were expressed as mean (colony-forming unit [cfu] counts/ml) $\pm$ SD for each condition (n = 15 for phosphate-buffered saline [PBS], n = 9 for MSCs, n = 11 for MSC MVs, and n = 7 for normal human lung fibroblast [NHLF] MVs). * P < 0.05 versus PBS; ✓ P < 0.05 versus NHLF MV group by Kruskal–Wallis test (Dunn). (B) Intravenous MSC MVs decreased the total bacterial load in the lung homogenate of mice injured by *E. coli* pneumonia at 18 hours. Total bacterial counts were expressed as mean (cfu counts/ml) $\pm$ SD for each condition (n = 9 for PBS; n = 8 for MSC MVs). * P < 0.05 versus PBS group by Mann–Whitney test. (C) Compared with PBS, the MSC MV–treated group was without bacteremia at 18 hours. Total bacterial counts were expressed as individual plotted value (cfu counts/ml) (the bar represents the median for each condition; n = 10 for both groups). (D) Intratracheal MSC MVs numerically decreased total bacterial load in the injured alveolus and decreased the bacteria load in the blood of mice injured by *E. coli* pneumonia at 24 hours. (E) Total bacterial counts were expressed as individual plotted value (cfu counts/ml) (the bar represents the median for each condition; n = 5 for PBS, n = 9 for MSC MVs, and n = 5 for MVs released from NHLFs [NHLF MVs]). BAL = bronchoalveolar lavage.

MSC MVs and endotoxin were given intratracheally simultaneously (27). Here, we found that intravenous MSC MVs given 4 hours after bacterial inoculation were as effective as MSCs in improving survival and attenuating *E. coli* bacteria–induced ALI. Other researchers have demonstrated that exosomes released by mouse MSCs

mitigated proinflammatory and proliferative responses in a hypoxia–induced pulmonary hypertension model in mice (26). However, the pathophysiology of pulmonary hypertension is primarily sterile inflammation.

Our data suggested several potential mechanisms underlying the beneficial effects

of MSC MVs: (1) an increase in human KGF protein levels in the injured alveolus in part through the transfer of KGF mRNA from MSC MVs; (2) an increase in alveolar and total lung tissue bacterial clearance through an increase in monocyte bacterial phagocytosis; (3) an immunomodulatory effect on monocytes and alveolar

Figure 6. Role of CD44 in the uptake of microvesicles (MVs) released from mesenchymal stem cells (MSCs) (MSC MVs) into primary cultures of human monocytes and human alveolar epithelial type 2 cells. In both human monocytes and human alveolar epithelial type 2 cells, MSC MV uptake was dependent on CD44, the cell surface receptor for hyaluronic acid, after injury. (A) LPS stimulation increased the uptake of fluorescent-labeled MSC MVs into monocytes, which was dependent on the CD44 receptor on the MVs. Fluorescence intensity was expressed as mean (arbitrary units) $\pm$ SD for each condition ($n = 352$ – 477 cells for all groups). $^*P < 0.01$ versus LPS–, $^{\dagger}P < 0.01$ versus CD44-preincubated MSC MVs, and $^{\S}P < 0.01$ versus IgG preincubated MSC MVs by ANOVA (Bonferroni). Photomicrographs display the pattern of fluorescence levels observed in each experimental condition. Scale bar = $20\ \mu\text{m}$. (B) Using MVs released by green fluorescent protein–transfected MSCs, we confirmed that LPS stimulated the uptake of MSC MVs

Figure 7. Functional effects of microvesicles (MVs) released by mesenchymal stem cells (MSCs) (MSC MVs) on primary cultures of human monocytes and human alveolar epithelial type 2 (ATII) cells. (A) Treatment of human blood monocytes with MSC MVs for 24 hours increased *Escherichia coli* bacterial clearance by 24%. Total bacterial counts are expressed as mean (% of phosphate-buffered saline [PBS]) $\pm$ SD for each condition ($n = 46$ for PBS, $n = 12$ –13 for MVs released by normal human lung fibroblasts [NHLFs] [NHLF MVs] or MSCs, and $n = 26$ for MSC MVs). $^*P < 0.05$ versus PBS, $^{**}P < 0.01$ versus PBS, and $^{\checkmark}P < 0.01$ versus NHLF MVs by ANOVA (Bonferroni). (B) MSC MVs decreased monocyte tumor necrosis factor α (TNF- α) secretion by 30%. TNF- α secretion levels are expressed as mean (% of PBS) $\pm$ SD for each condition ($n = 21$ for PBS, $n = 10$ for NHLF MVs or MSCs, and $n = 15$ for MSC MVs). $^{***}P < 0.01$ versus PBS, $^{****}P < 0.01$ versus PBS, and $^{\checkmark}P < 0.01$ versus MSCs by ANOVA (Bonferroni). (C) MSC MVs restored intracellular ATP levels in human ATII cells injured with an inflammatory insult (cytomix) at 48 hours ($n = 11$ –14 per condition).

macrophages, suppressing cytokine-induced lung injury and lung protein permeability; and (4) a beneficial effect on alveolar epithelial type 2 cell metabolism.

In our *E. coli* pneumonia model, MSC MV administration increased KGF protein levels in the injured alveolus in part through the expression of MV KGF mRNA,

as we previously demonstrated (21) (Figure 2C). Inhibition of KGF eliminated the therapeutic benefits of MSC MVs, leading to higher mortality (Figure 2D). These results were consistent with previous studies demonstrating the important role of KGF in the paracrine-mediated effects of MSCs, such as on restoration of alveolar fluid clearance, lung permeability, and inflammation and inhibition of bacterial growth (8).

Human monocytes exposed to MSC MVs exhibited lower levels of mRNA for inducible nitric oxide synthase and higher levels of mRNA for transglutaminase 2. Although we found no differences in CD163 mRNA expression or CD206 by flow cytometry (for additional M2 markers, see Figure E3), the results suggested that MSC MVs partially changed monocytes to a more antiinflammatory phenotype (8, 28–31), which we found *in vitro* and *in vivo* with MSC MV treatment (Figures 3, 4, 7, and 9). In our previous work, we also did not find that MSC treatment increased CD206 expression in monocytes (8).

In animal models of sepsis (9, 28, 32) and pneumonia (6, 8, 33), MSCs have been shown to repolarize monocytes-macrophages from the M1 to the M2 phenotype characterized by high levels of IL-10, low levels of TNF- α production, and increased phagocytosis. Németh and colleagues found that PGE2 secreted by MSCs was essential for reprogramming monocytes-macrophages toward a M2 phenotype (9). Because MSC MVs expressed mRNA for COX2 (Figure 8D), the key enzyme in PGE2 synthesis, we speculate that the transfer of COX2 mRNA from MSC MVs to monocytes, with a resultant increase in PGE2 secretion, might be involved in shifting monocytes toward an antiinflammatory state (34–36).

Similar to MSCs, MSC MVs enhanced bacterial clearance primarily by increasing monocyte phagocytosis. However, because MSCs can secrete antimicrobial-soluble factors (6–8), further studies investigating other mechanisms underlying the

Figure 6. (Continued). ($n = 269$ –343). $^*P < 0.01$ versus LPS– by Student's *t* test. Photomicrographs display the pattern of fluorescence levels (green fluorescent protein) observed in both experimental conditions. Scale bar = 20 μ m. (C) Stimulation by LPS with an inflammatory injury (cytomix + LPS) increased the uptake of fluorescent-labeled MSC MVs into alveolar epithelial type 2 cells, which was dependent on the CD44 receptor on the MVs. Fluorescence intensity was expressed as mean (arbitrary units [A.U.]) $\pm$ SD for each condition ($n = 179$ –239 cells for all groups). $^*P < 0.01$ versus cytomix–LPS–, $^{\dagger}P < 0.01$ versus CD44-preincubated MSC MVs, and $^{\S}P < 0.01$ versus IgG preincubated MSC MVs by ANOVA (Bonferroni). Photomicrographs display the pattern of fluorescence levels observed in each experimental condition. Scale bar = 20 μ m. (D) Blocking the CD44 receptor on MSC MVs decreased survival in mice injured with *Escherichia coli* pneumonia treated with the MVs as compared with blocking with an IgG control antibody ($n = 21$ for MV + IgG, $n = 33$ for MV + CD44ab). $^*P < 0.01$ versus MV + IgG by log-rank test. Ab = antibody.

Figure 8. Effect of mesenchymal stem cell (MSC) pretreatment with polyinosine-polycytidylic acid [Poly (I:C)], a toll-like receptor 3 (TLR3) agonist, on messenger RNA (mRNA) expression for cyclooxygenase 2 (COX2) and IL-10 in MSCs and its released microvesicles (MVs) and in monocytes exposed to MVs released by Poly (I:C)-pretreated MSCs (MSC MVs). In these experiments, total RNA was extracted from human MSCs (B, C), MSC MVs (D), or human monocytes (E, F), and semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed. *Top:* Representative agarose gels of semiquantitative RT-PCR products from COX2 (B, D, E) and IL-10 (C, F) mRNA amplification. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control to normalize loading of the RNA samples. The PCR products were 295 bp in size for COX2 and 500 bp for IL-10. *Bottom:* Densitometry readings derived from the PCR gels. The band density relative to that of the GAPDH is expressed as mean $\pm$ SD. (A) Human MSCs expressed TLR3. (B, C) Human MSCs were cultured with or without Poly (I:C) for 1 hour and serum starved for 48 hours. TLR3 stimulation further increased the expression of COX2 and IL-10 mRNA in MSCs [$n = 8-22$ for standard control (STD MSCs) and Poly (I:C)-stimulated MSCs]. $P < 0.01$ by Student's *t* test. (D) TLR3 stimulation also further increased the mRNA expression for COX2 in the released MVs, Poly (I:C) MVs ($n = 10$ for MVs released from standard MSCs [STD MVs] and MVs released from MSCs prestimulated with Poly (I:C) MVs [Poly (I:C) MVs]). $P < 0.01$ by Mann-Whitney test. (E, F) mRNA expression for COX2 and IL-10 was increased in human monocytes exposed to Poly (I:C) MVs ($n = 5-8$). $P < 0.01$ by Student's *t* test.

bactericidal effects of MSC MVs are needed.

MSC MVs restored ATP levels in injured alveolar epithelial type 2 cells,

suggesting a metabolic benefit. This result is in line with transcriptome and proteome profiling of MSC MVs showing that MVs carry key enzymes involved in metabolism,

such as glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase, which may be transferred to the injured tissue (36-38). Another mechanism might be

Figure 9. Monocytes exposed to microvesicles (MVs) derived from polyinosine-polycytidylic acid [Poly (I:C)]-stimulated mesenchymal stem cells (MSCs) had increased bacterial phagocytosis index and decreased inflammatory cytokine secretion. (A–C) Representative phagocytosis of *Escherichia coli* bacteria by human monocytes exposed to phosphate-buffered saline (PBS) (A, control), MVs released from standard MSCs (STD MVs) (B), or MVs released from MSCs prestimulated with Poly (I:C) [Poly (I:C) MVs] (C). Scale bar = 5 μ m. (D–F) Human monocytes exposed to Poly (I:C) MVs exhibited a higher *E. coli* bacterial clearance capacity compared with STD MV treatment (D). Although the phagocytosis rate was similar between groups (E), treatment with Poly (I:C) MVs further increased the phagocytosis index for bacteria compared with treatment with STD MVs (F). Data are expressed as mean $\pm$ SD [n = 5–13 for PBS, n = 6–22 for STD MVs, and n = 6–19 for Poly (I:C) MVs]. *** $P < 0.01$ versus PBS; ✓ $P < 0.01$ versus STD MVs by ANOVA (Bonferroni). (G, H) Poly (I:C) MVs further decreased tumor necrosis factor α (TNF- α) and increased IL-10 levels secretion by monocytes [n = 11–13 for PBS, n = 11–12 for STD MVs, and n = 11–20 for Poly (I:C) MV]. ** $P < 0.01$ versus PBS, *** $P < 0.01$ versus PBS, and ✓ $P < 0.01$ versus STD MVs by ANOVA (Bonferroni). (I) In mice injured with *E. coli* pneumonia, intravenous administration of Poly (I:C) MVs further decreased the alveolar bacterial load as compared to STD MSC MVs. Total bacterial counts are expressed as mean (% of STD MSC MVs) $\pm$ SD for each condition [n = 14 for STD MSC MVs; n = 8 for Poly (I:C) MVs]. BAL = bronchoalveolar lavage.

through the transfer of mitochondria (39) or mRNA for key mitochondrial genes (21, 37). Although ATP restoration may be critical for preserving key functions of alveolar epithelial type 2 cells, such as fluid clearance and surfactant production, it is unknown whether MSC MVs affect the pathway involved in ATP production, such as the rate of ATP production and/or utilization. Additional studies are warranted.

Similar to MSCs (40, 41), we found that CD44 was not only crucial for incorporation of MSC MVs into injured human monocytes and alveolar epithelial type 2 cells but also for their therapeutic effect in mice with pneumonia (Figure 6D). These results confirmed previous studies highlighting endocytosis and direct membrane fusion as two major ways of transferring MV cargo to recipient cells (42). Furthermore, we found that the CD44 ligands L-selectin and osteopontin were significantly overexpressed upon inflammation (Figure E2).

Recent studies indicated that TLR3 activation with Poly (I:C) can boost the immunomodulatory potential of MSCs, while TLR4 activation could reduce it (24, 43–45). Watterman and colleagues showed that pretreated MSCs secreted higher levels of PGE2 (24). In the present study, Poly (I:C) pretreatment further increased

expression of COX2 mRNA in not only MSCs and its released MVs but also in monocytes exposed to Poly (I:C) MVs. Moreover, these monocytes displayed a more obvious M2 phenotype as seen in the decrease in TNF- α level and the increase in IL-10 level and bacteria phagocytosis (Figures 8 and 9); it is conceivable that the transfer of COX2 mRNA from Poly (I:C) MVs to monocytes caused the phenotype switch (9). More importantly, we found a 7-fold higher increase in alveolar KGF protein levels (Figure 2C) and a 74% reduction of total alveolar bacterial load (Figure 9I) in mice treated with Poly (I:C) MVs compared with standard MSC MVs. We previously found that MSC KGF secretion increased monocytes-macrophages phagocytic activity (8). Our study is the first suggesting that the immunoregulatory and antimicrobial properties of MSC MVs might be enhanced by MSC pretreatment.

The current study has some limitations. (1) Ultracentrifugation may isolate a heterogeneous population of MVs, including smaller exosomes. Further studies are needed to determine the contribution of each in the overall effect. (2) To compare our findings with studies using MSCs in ALI, we dosed MVs by total cell count, not by protein concentration. However,

we found that the MV protein content was within the range used in previous studies. (3) In addition to mRNA, MV microRNA and lipid content may play a significant therapeutic role. (4) MSC MVs may interact with other cells, such as regulatory T cells, which are involved in ALI resolution. The impact of MVs on other immune cells and on the resolution phase of ALI will need to be studied further.

In conclusion, MVs released from human bone marrow-derived MSCs improved survival from *E. coli* pneumonia in mice. This was associated with enhanced phagocytosis of bacteria by human monocytes with a reduction in inflammation and increased ATP levels in alveolar epithelial type 2 cells. TLR3 agonist pretreatment of MSCs further increased the effects of MSC MVs on monocyte's immunoregulatory and phagocytosis properties. More importantly, MSC MVs were as effective as MSCs as a therapeutic in ALI from bacterial pneumonia, suggesting a possible alternative to using cells given the potential limitations of any stem cell-based therapy. ■

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

Acknowledgment: The authors thank Jia Liu and Tristan Mirault.

References

- Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–1349.
- Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD. Incidence and outcomes of acute lung injury. *N Engl J Med* 2005;353:1685–1693.
- Gupta N, Su X, Popov B, Lee J-W, Serikov V, Matthay MA. Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol* 2007;179:1855–1863.
- Xu J, Qu J, Cao L, Sai Y, Chen C, He L, Yu L. Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *J Pathol* 2008;214:472–481.
- Mei SHJ, McCarter SD, Deng Y, Parker CH, Liles WC, Stewart DJ. Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. *PLoS Med* 2007;4:e269.
- Gupta N, Krasnodembskaya A, Kapetanaki M, Mouded M, Tan X, Serikov V, Matthay MA. Mesenchymal stem cells enhance survival and bacterial clearance in murine *Escherichia coli* pneumonia. *Thorax* 2012;67:533–539.
- Krasnodembskaya A, Song Y, Fang X, Gupta N, Serikov V, Lee J-W, Matthay MA. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 2010;28:2229–2238.
- Lee JW, Krasnodembskaya A, McKenna DH, Song Y, Abbott J, Matthay MA. Therapeutic effects of human mesenchymal stem cells in ex vivo human lungs injured with live bacteria. *Am J Respir Crit Care Med* 2013;187:751–760.
- Németh K, Leelahavanichkul A, Yuen PST, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;15:42–49.
- Gonzalez-Rey E, Anderson P, González MA, Rico L, Büscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009;58:929–939.
- Mei SHJ, Haitsma JJ, Dos Santos CC, Deng Y, Lai PFH, Slutsky AS, Liles WC, Stewart DJ. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 2010;182:1047–1057.
- Izadpanah R, Kaushal D, Kriedt C, Tsien F, Patel B, Dufour J, Bunnell BA. Long-term *in vitro* expansion alters the biology of adult mesenchymal stem cells. *Cancer Res* 2008;68:4229–4238.
- Røsland GV, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwiec J, Tonn JC, Goldbrunner R, Lønning PE, et al. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. *Cancer Res* 2009;69:5331–5339.
- Prockop DJ, Brenner M, Fibbe WE, Horwitz E, Le Blanc K, Phinney DG, Simmons PJ, Sensebe L, Keating A. Defining the risks of mesenchymal stromal cell therapy. *Cytotherapy* 2010;12:576–578.
- Ratajczak MZ. The emerging role of microvesicles in cellular therapies for organ/tissue regeneration. *Nephrol Dial Transplant* 2011;26:1453–1456.
- Bruno S, Grange C, Derigibus MC, Calogero RA, Saviozzi S, Collino F, Morando L, Busca A, Falda M, Bussolati B, et al. Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J Am Soc Nephrol* 2009;20:1053–1067.

17. Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C, Camussi G. Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. *Nephrol Dial Transplant* 2011;26:1474–1483.
18. Bruno S, Grange C, Collino F, Deregibus MC, Cantaluppi V, Biancone L, Tetta C, Camussi G. Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury. *PLoS One* 2012;7:e33115.
19. Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B, Camussi G. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood* 2007;110:2440–2448.
20. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, Ratajczak MZ. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 2006;20:847–856.
21. Zhu YG, Feng XM, Abbott J, Fang XH, Hao Q, Monsel A, Qu JM, Matthay MA, Lee JW. Human mesenchymal stem cell microvesicles for treatment of *Escherichia coli* endotoxin-induced acute lung injury in mice. *Stem Cells* 2014;32:116–125.
22. Monsel A, Zhu Y-G, Hao Q, Liu J, Gennai S, Matthay MA, Lee JW. Microvesicles derived from human bone marrow mesenchymal stem cells improve survival in *E. coli* pneumonia-induced acute lung injury in mice and enhance monocyte phagocytosis of bacteria. *Am J Respir Crit Care Med* 2014;189:A5307.
23. El-Andaloussi S, Lee Y, Lakhali-Littleton S, Li J, Seow Y, Gardiner C, Alvarez-Erviti L, Sargent IL, Wood MJ. Exosome-mediated delivery of siRNA *in vitro* and *in vivo*. *Nat Protoc* 2012;7:2112–2126.
24. Waterman RS, Tomchuck SL, Henkle SL, Betancourt AM. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS One* 2010;5:e10088.
25. Su X, Looney M, Robriquet L, Fang X, Matthay MA. Direct visual instillation as a method for efficient delivery of fluid into the distal airspaces of anesthetized mice. *Exp Lung Res* 2004;30:479–493.
26. Lee C, Mitsialis SA, Aslam M, Vitali SH, Vergadi E, Konstantinou G, Sdrimas K, Fernandez-Gonzalez A, Kourembanas S. Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* 2012;126:2601–2611.
27. Rittirsch D, Hoesel LM, Ward PA. The disconnect between animal models of sepsis and human sepsis. *J Leukoc Biol* 2007;81:137–143.
28. Krasnodembskaya A, Samarani G, Song Y, Zhuo H, Su X, Lee J-W, Gupta N, Petrini M, Matthay MA. Human mesenchymal stem cells reduce mortality and bacteremia in gram-negative sepsis in mice in part by enhancing the phagocytic activity of blood monocytes. *Am J Physiol Lung Cell Mol Physiol* 2012;302:L1003–L1013.
29. Kim J, Hematti P. Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. *Exp Hematol* 2009;37:1445–1453.
30. Maggini J, Mirkin G, Bognanni I, Holmberg J, Piazzón IM, Nepomnaschy I, Costa H, Cañones C, Raiden S, Vermeulen M, et al. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS One* 2010;5:e9252.
31. Zhang Q-Z, Su W-R, Shi S-H, Wilder-Smith P, Xiang AP, Wong A, Nguyen AL, Kwon CW, Le AD. Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing. *Stem Cells* 2010;28:1856–1868.
32. Ionescu L, Byrne RN, van Haaften T, Vadivel A, Alphonse RS, Rey-Parra GJ, Weissmann G, Hall A, Eaton F, Thébaud B. Stem cell conditioned medium improves acute lung injury in mice: *in vivo* evidence for stem cell paracrine action. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L967–L977.
33. Kim ES, Chang YS, Choi SJ, Kim JK, Yoo HS, Ahn SY, Sung DK, Kim SY, Park YR, Park WS. Intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells attenuates *Escherichia coli*-induced acute lung injury in mice. *Respir Res* 2011;12:108.
34. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* 2014;14:195–208.
35. Mokarizadeh A, Delirez N, Morshedi A, Mosayebi G, Farshid AA, Mardani K. Microvesicles derived from mesenchymal stem cells: potent organelles for induction of tolerogenic signaling. *Immunol Lett* 2012;147:47–54.
36. Arslan F, Lai RC, Smeets MB, Akeroyd L, Choo A, Aguer ENE, Timmers L, van Rijen HV, Doevendans PA, Pasterkamp G, et al. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res (Amst)* 2013;10:301–312.
37. Lai RC, Yeo RWY, Tan KH, Lim SK. Mesenchymal stem cell exosome ameliorates reperfusion injury through proteomic complementation. *Regen Med* 2013;8:197–209.
38. Kim HS, Choi DY, Yun SJ, Choi SM, Kang JW, Jung JW, Hwang D, Kim KP, Kim DW. Proteomic analysis of microvesicles derived from human mesenchymal stem cells. *J Proteome Res* 2012;11:839–849.
39. Islam MN, Das SR, Emin MT, Wei M, Sun L, Westphalen K, Rowlands DJ, Quadri SK, Bhattacharya S, Bhattacharya J. Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nat Med* 2012;18:759–765.
40. Tögel F, Isaac J, Hu Z, Weiss K, Westenfelder C. Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury. *Kidney Int* 2005;67:1772–1784.
41. Zhu H, Mitsuhashi N, Klein A, Barsky LW, Weinberg K, Barr ML, Demetriou A, Wu GD. The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. *Stem Cells* 2006;24:928–935.
42. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013;200:373–383.
43. Cassatella MA, Mosna F, Micheletti A, Lisi V, Tamassia N, Cont C, Calzetti F, Pelletier M, Pizzolo G, Krampera M. Toll-like receptor-3-activated human mesenchymal stromal cells significantly prolong the survival and function of neutrophils. *Stem Cells* 2011;29:1001–1011.
44. Le Blanc K, Mougiakakos D. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol* 2012;12:383–396.
45. van den Akker F, de Jager SC, Sluijter JP. Mesenchymal stem cell therapy for cardiac inflammation: immunomodulatory properties and the influence of toll-like receptors. *Mediators Inflamm* 2013;2013:181020.