Stem cell conditioned medium improves acute lung injury in mice: in vivo evidence for stem cell paracrine action

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Bone-marrow derived mesenchymal stem cells (MSCs) differentiate into cartilage, fat, and bone, but also into muscle, liver, kidney, heart, and brain cells (58, 59, 69). These properties have been harnessed for organ regeneration (69). In the lung, bone marrow–derived cells, including MSCs, engraft and adopt a lung epithelial cell phenotype (3, 21, 33, 34, 54, 62, 71). MSCs can also suppress local immune responses and have the capacity to avoid immune rejection in allotransplantation (29, 35, 38, 47, 59). These immunomodulatory properties might be of therapeutic benefit in ALI/ARDS and other lung diseases characterized by inflammation. Both intratracheal and systemic MSC administrations improve lipopolysaccharide (LPS)-induced ALI in mice (21, 44). These findings are congruent with the therapeutic benefits of MSCs described in other lung injury models (3, 5, 25, 37, 48, 53, 54, 62, 71). In all these studies, the engraftment rates of MSCs were low (32, 33, 62, 71). Together with the rapid therapeutic benefits seen within 48 to 72 h of MSC delivery in the ALI models (20, 44), these findings suggest that beyond cell replacement, MSCs may be releasing factors responsible for the beneficial effects of cell therapy. In addition, since the use of MSCs as whole-cell therapy may hold some risks to the patient (2, 38), we investigated the effects of MSC-derived conditioned medium (CdM) in LPS-induced ALI in mice.

MATERIALS AND METHODS

All procedures were approved by the Animal Welfare Committee of the University of Alberta.

MSCs and lung fibroblast isolation, culture, and characterization. Bone marrow was harvested from adult (8–10 wk) C57BL/6 mice (Charles River), and MSCs were cultured and characterized as previously described (25). The femur and tibia were excised and the extracted marrow was dissociated and plated in a tissue culture flask. After 24 h, the medium was aspirated, and adherent cells were rinsed three times with PBS and replenished with fresh media. Cells were grown to confluence, trypsinized, and reseeded at a density of 7,000 cells/cm². Differentiation of MSCs was performed over 21 days on passage 2–3 cells (25). MSCs were evaluated for expression of a panel of surface markers according to established criteria (13, 19, 44, 46, 68). Antibodies against the following markers were obtained from Becton Dickinson (BD, Mississauga, ON, Canada): stem cell antigen-1 [Sca-1, fluorescein isothiocyanate (FITC)], CD31 [phycocyanin (PE)], CD11b (FITC), CD45 (FITC), CD44 (FITC), CD73 (PE), CD14 (FITC), CD34 (FITC), c-kit (FITC), Flk-1 (PE), CD106 [vascular cell adhesion molecule-1 (VCAM1)], CD29 (PE), CD105 (PE) was obtained from BioLegend (San Diego, CA). MSCs between

DESPITE IMPROVEMENTS IN MANAGEMENT, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) remain major causes of morbidity and mortality in critically ill patients of all ages (63). The incidence of ALI/ARDS in the United States is 138,000 persons per year and is anticipated to double in the next 25 years (63). Treatment of ALI/ARDS remains primarily supportive (41).
passages 7–11 were detached from culture surfaces, counted, and divided into aliquots of ~0.5–1 × 10⁶ cells/sample in 12 × 75 mm polystyrene round-bottom tubes (BD Falcon). Cells were washed twice with flow buffer (0.05% sodium azide, 0.1% bovine serum albumin in PBS), incubated with the respective antibodies at 4°C with gentle shaking for 30 min, washed twice, resuspended in flow buffer, and analyzed by flow cytometry (FACSCalibur, BD). Cellquest (BD) and FlowJo (version 5.7.2) software were used for analyses.

Lung fibroblasts were isolated from adult (8–10 wk) C57BL/6 mice (67). Fibroblast (Fib) identity was confirmed by immunofluorescence staining for the intermediate filament protein vimentin. Fibroblasts (Fib) were cultured with serum-free DMEM (25) rinsed three times with PBS. Cells were cultured with serum-free DMEM for 3 days and then washed twice with flow buffer (0.05% sodium azide, 0.1% bovine serum albumin in PBS), incubated with the respective antibodies at 4°C with gentle shaking for 30 min, washed twice, resuspended in flow buffer, and analyzed by flow cytometry (FACSCalibur, BD). Cellquest (BD) and FlowJo (version 5.7.2) software were used for analyses.

CdM preparation. Passage 2–8 MSCs and fibroblasts were grown to ≥80% confluency. Medium (DMEM) was aspirated and cells were rinsed three times with PBS. Cells were cultured with serum-free DMEM (+ PSF) for 24 h. CdM was collected and filtered through a 0.2-μm filter to remove cellular debris. Adherent cells were trypsinized, stained with trypan blue, and counted. The medium from 5 × 10⁶ cells yielded 15 ml of primary CdM that was further desalted and concentrated ~25-fold, yielding 600 μl CdM, using ultrafiltration units with a 3-kDa molecular weight cutoff (Amicon Ultra-PL 3, Millipore, Billerica, MA). Similar to work by others (24), serum-free CdM was prepared by stirring the medium to obtain IGF-I-neutralized CdM (Neut-CdM).

For IGF-I studies, IGF-I was quantified in DMEM, Fib-CdM, and MSC-CdM by using a commercially available ELISA kit (R&D Systems) according to manufacturer’s instructions. Neutralizing antibody to IGF-I (R&D Systems, 100 ng/ml) was added to the serum-free medium to obtain IGF-I-neutralized CdM (Neut-CdM).

Murine LPS-induced ALI. Eight- to 10-wk-old male C57BL/6 mice were anaesthetized with 5% isoflurane and injected intratracheally (i.t.) with 4 mg/kg LPS (Escherichia coli 055:B5, Sigma-Aldrich, Oakville, ON, Canada). Four hours post-LPS, mice were reanaesthetized and received a 30-μl i.t. instillation of MSCs, Fib, MSC-CdM, CdM, or DMEM. We ensured equivalence between cell-based and CdM-based treatment by administering the same number of cells (250,000 cells/30 μl DMEM) that produced 30 μl concentrated CdM. For IGF-I studies, recombinant mouse IGF-I (rIGF-I, R&D Systems, 100 μg/kg) was administered i.t. in a total volume of 30 μl saline solution.

Mice (n ≥ 5 per group per endpoint) were euthanized via an intraperitoneal injection of pentobarbital at 48 h post-LPS for either bronchoalveolar lavage fluid (BALF) or lung histological analysis. BALF analysis and AM isolation. Lungs were lavaged with 2.5 ml ice-cold phosphate-buffered saline (PBS) injected at 0.5-ml increments via a 20-gauge catheter inserted in the trachea. BALF was centrifuged for 10 min at 400 g and BALF cells were enumerated by

![Fig. 1](https://example.com/figure1.png)  
**Fig. 1.** Characterization of mesenchymal stem cells (MSCs) isolated from C57BL/6 mice. **A:** MSCs differentiated along adipogenic, osteogenic, and chondrogenic lineages. **Top:** differentiated MSCs. **Bottom:** control MSCs. **Left to right:** respectively: oil red O staining (adipocytes), alizarin red (osteocytes), safranin O (chondrocytes). Size bar: 60 μm. **B:** representative flow-cytometry histograms. **C:** quantification of MSC surface marker expression. Values are expressed as means ± SE.
use of the Scepter automated cell counter (Millipore). Differential cell counts were performed on cytospin preparations (Thermo Shandon, Pittsburgh, PA) stained with Hema 3 Manual Staining System (Fisher Scientific, Nepean, ON, Canada) by counting 300 cells per cell smear and multiplying by total cell number per milliliter.

For alveolar macrophage (AM) isolation, an established protocol was followed (73). Briefly, BALF was centrifuged at 300 g for 10 min and the cellular pellet was washed with PBS, resuspended in red blood cell lysis buffer (8.3 g NH4Cl, 1 g KHCO3, 1.8 ml of 5% EDTA in 1 liter of distilled water) for 5 min at room temperature, and centrifuged again at 300 g for 10 min. The pellet was resuspended in RPMI-1640 medium and plated at a density of 600,000 cells/ml in a 24-well tissue culture plate. After 2 h, medium was removed and adherent cells were washed three times with PBS. AMs were stained with Hema 3 Manual Staining System for morphological assessment. AMs were evaluated for the expression of characteristic surface markers (22) with use of antibodies against CD11b (FITC) and CD11c [allophycocyanin (APC)] (BD Biosciences) by flow cytometry according to the same protocol described for MSC characterization.

Assessment of lung permeability. Lung edema due to LPS-induced increase in lung permeability was measured by using the wet-to-dry weight ratio of lung lobes as previously described (21). Briefly, lungs were weighed upon excision (wet weights), homogenized in 1 ml of water, and placed in a drying oven at 55°C for 24 h; dry weights were recorded; and wet-to-dry ratio was calculated.

Lung histological analysis. Lungs were inflated and fixed with 4% formaldehyde solution through a tracheal catheter at a constant pressure of 20 cmH2O (25). Lungs were processed and paraffin embedded, and 4-μm-thick serial sections were stained with hematoxylin and eosin. Images were captured (Openlab, Improvision, version 5.0.2.) with Leica CTRMIC microscope, and 40 high-powered fields per lung were examined by a blinded investigator to quantify the histopathology score. Parameters assessed were alveolar septal congestion, alveolar hemorrhage, intra-alveolar fibrin, and intra-alveolar infiltrates. A score from 0 to 3 was given for each criterion and a total score was established, all according to a previously published protocol (43, 44). Briefly, lung injury score = [(alveolar hemorrhage points/no. of fields) + (2 × (alveolar infiltrate points/no. of fields)] × (fibrin points/no. of fields)].

AM LPS exposure and AM phenotype characterization. Freshly isolated AMs were cultured with RPMI-1640 media containing 10% FBS in a 24-well plate. After overnight adherence, RPMI-1640 medium was replaced with either DMEM alone, DMEM+LPS (1 μg/ml), or MSC-CdM+LPS (1 μg/ml). After 48 h in culture, medium was aspirated and cells were rinsed 3× with PBS. The monolayer of cells was scraped, cells were spun down, and pellets were frozen. Arginase activity in AM was measured by determining the amount of urea generated by the enzyme (11, 65). Briefly, the frozen AM pellets were thawed and homogenized in 100 μl per sample of lysis buffer [0.1% Triton X-100 with protease inhibitors: phenyl-methyl-sulfonyl fluoride (PMSF) (1 mM), leupeptin (0.5 μg/ml), aprotinin (5 μg/ml), EDTA (2 mM)]. The samples were sonicated twice for 3–5 s and stored at −80°C. Samples were thawed and 50 μl of the homogenate was added to 50 μl Tris-HCl (25 mM, pH 7.5) containing MnCl2 (5 mM) and incubated at 37°C for 10 min to activate the enzyme. Then 50 μl of this heat-activated supernatant mixture was incubated with 50 μl l-arginine (0.5 mol/l) at 37°C for 60 min. The reaction was stopped by adding 400 μl of an acid mixture (1 H2SO4:3 H3PO4:7 H2O), and 25 μl of α-nitositosopropiophenone (9% dissolved in 100% ethanol) was added to the above mixture and incubated at 100°C for 45 min for color development. The mixture was cooled at room temperature in the dark for 10 min. A standard curve for urea (0–30 μg) was prepared. Urea concentration in the homogenate was measured by using a colorimeter at 550/540 nm with 200 μl of the aliquot. Arginase activity was expressed as units per milligram protein per hour.

For further macrophage phenotype characterization by immunodetection (Western blot) and multicolor flow cytometry, AMs were isolated from experimental animals and allowed to adhere to plastic as described above. The following antibodies were used: CD11b (Pacific Blue), CD11c (PE, both from Biolegend), rabbit anti-mouse inducible nitric oxide synthase (iNOS; Abcam) with donkey anti-rabbit secondary conjugated with FITC (Biodena, Foster City, CA) or streptavidin (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse Ym1 (R&D Systems) and donkey anti-goat secondary [Cy5 (Biodena) or streptavidin (Santa Cruz)].

Immunodetection was performed as previously described (70). For flow cytometry, the staining was performed as described for MSC characterization, with the addition of a 30-min incubation of the cells at 4°C in Cytofix/Cytoperm Buffer (BD), followed by resuspension in permeabilization buffer (BD) before incubation with primary antibodies. Analysis was performed using FACSCanto with FACS Diva software (BD).

**Fig. 2.** MSC-conditioned medium (CdM) decreased bronchoalveolar lavage fluid (BALF) total cell and neutrophil number in LPS-induced lung inflammation. A: LPS-DMEM mice (n = 10) had significantly more inflammatory cells than uninjured controls (n = 5) and control-CdM (n = 6) mice. Treatment with MSCs (n = 5) or MSC-CdM (n = 10), but not fibroblast (Fib; n = 5) or Fib-CdM (n = 5), significantly attenuated lung cells influx. BALF of mice treated with DMEM had 2 times more polymorphonuclear leukocytes (PMNs) than those treated with MSC-CdM. There were no differences in mononuclear cells (MN) number. *P < 0.05 control, control-CdM vs. LPS-MSC-CdM; †P < 0.01 control, control-CdM vs. all other LPS groups (LPS-DMEM, LPS-Fib, LPS-Fib CdM, LPS-MSC); ‡P < 0.01 LPS-MSC vs. LPS-DMEM, LPS-Fib, LPS-Fib CdM. B: LPS-DMEM (n = 5) lungs had increased wet-to-dry weight ratios compared with control (n = 5) and control-CdM (n = 5) lungs. LPS-MSCs (n = 4) and LPS-MSC-CdM (n = 5), but not LPS-Fib (n = 4) or LPS-Fib-CdM (n = 5), had significantly decreased wet-to-dry weight ratios compared with LPS-DMEM. *P < 0.05 LPS-DMEM vs. LPS-MSC; †P < 0.05 LPS-Fib and LPS-Fib CdM vs. LPS-MSC CdM; #P < 0.01 control, control-CdM vs. LPS-DMEM, LPS-Fib, LPS-Fib CdM, LPS-MSC CdM; §P < 0.01 LPS-MSC vs. LPS-Fib and LPS-Fib CdM.
Antibody array of MSC-CdM. MSC-CdM and Fib-CdM were prepared, concentrated, and desalted as described above. Samples were analyzed for a panel of 96 factors by use of an antibody array (RayBio Mouse Antibody Array G Series 1000, RayBiotech, Norcross, GA). Relative intensity of each factor in MSC-CdM and Fib-CdM is reported compared with a positive and negative control, as well as fold change of specific factors in MSC-CdM vs. Fib-CdM.

Statistical analysis. Data are expressed as means ± SE. Group comparisons were analyzed by one-way ANOVA with a Fisher’s least significant difference post hoc test. An unpaired Student’s t-test was used when appropriate. Data was assessed with statistical software Statview (version 5.0.1, SAS Institute, Cary, NC). Values were considered significant with \( P < 0.05 \).

RESULTS

MSC display functional characteristics and surface marker phenotype of murine MSCs. Bone marrow-derived MSCs differentiated into adipogenic, osteogenic, and chondrogenic mesenchymal lineages (Fig. 1A). Cell surface antigen phenotype was assessed by flow cytometry. MSCs expressed high levels of Sca-1 (92.93% of cells) and CD29 (89.99%) and moderate levels of CD105 (33.92%), CD106 (14.36%), CD11b (12.10%), and CD45 (11.62%) and were considered negative for CD14 (0.30%), c-kit (0.53%), CD34 (1.13%), CD73 (1.49%), Flk-1 (3.01%), and CD31 (4.08% of cells) (Fig. 1B and C).

MSC-CdM decreased lung inflammation and lung vascular permeability in LPS-induced lung injury. LPS significantly increased the total cell and neutrophil count in the BALF compared with uninjured controls (Fig. 2A). This inflammatory influx was attenuated by MSCs and MSC-CdM treatment, but not by treatment with DMEM, the vehicle control, Fib, or Fib-CdM (Fig. 2A). MSCs and MSC-CdM, but not DMEM, Fib, or Fib-CdM, prevented the LPS-induced increase in lung vascular permeability, as assessed by lung wet-to-dry ratio (Fig. 2B).

MSC-CdM failed to prevent LPS-induced body weight loss. All mice given LPS were lethargic and had reduced activity and decreased body weight over 48 h compared with control mice (Fig. 3A).

Fig. 3. MSC-CdM reduced LPS-induced lung injury but failed to prevent body weight loss. A: no effect of treatment on LPS-induced body weight loss. Control \((n = 13)\), control-CdM \((n = 6)\), LPS-DMEM \((n = 36)\), LPS-Fib \((n = 9)\), LPS-Fib-CdM \((n = 10)\), LPS-MSC \((n = 8)\), LPS-MSC CdM \((n = 35)\). **\( P < 0.01 \) control groups vs. each LPS group. B: LPS-MSC \((n = 5)\) and LPS-MSC-CdM \((n = 8)\) lungs had improved lung injury score compared with LPS-DMEM \((n = 8)\), LPS-Fib \((n = 5)\), and LPS-Fib CdM \((n = 8)\). **\( P < 0.01 \) control groups vs. each LPS group; §\( P < 0.01 \) LPS-MSC and LPS-MSC CdM vs. LPS-DMEM, LPS-Fib, and LPS-Fib CdM. C: representative images of lungs from experimental animals. Size bar: 130 \( \mu \)m.
MSC-CdM improved LPS-induced lung injury. Histological assessment of lung injury by using a semiquantitative histopathology score revealed that mice treated with LPS had increased septal thickening, alveolar hemorrhage, alveolar inﬁbrates, and ﬁbrin strands compared with controls, whereas treatment with MSCs or CdM signiﬁcantly attenuated these features (Fig. 3, B and C).

MSC-CdM determined alternative activation of AMs following LPS exposure in vitro and in vivo. Isolated AMs were macroscopically (Fig. 4A) and phenotypically (CD11c+CD11b−) (Fig. 4B) consistent with AMs. In vitro, AMs were exposed to LPS to test the role of MSC-CdM on the induction and maintenance of the M2 phenotype in AMs. LPS increased arginase activity in AMs compared with non-LPS-exposed cells, and MSC-CdM further elevated these levels compared with LPS-exposed cells cultured with DMEM (Fig. 4C). MSC-CdM also increased Ym1 protein expression compared with LPS-DMEM and LPS-Fib-CdM (Fig. 4D).

To further investigate the effects of MSC-CdM in vivo, AMs were isolated from LPS-exposed animals that had received rIGF-I or Fib-CdM by ELISA (Fig. 7A). Administration of rIGF-I signiﬁcantly attenuated the LPS-induced BALF inﬂammatory cell inﬂux (Fig. 7B), increase in lung vascular permeability (Fig. 7C), and lung injury (Fig. 7D–H).

To further investigate the effects of IGF-I in vivo, AMs were isolated from LPS-exposed animals that had received rIGF-I or MSC-CdM with IGF-I neutralizing antibody. rIGF-I induced a iNOS−Ym1+ phenotype in AMs in LPS-treated animals, whereas neutralization of IGF-I in MSC-CdM attenuated the induction of a M2 phenotype (Fig. 8).

DISCUSSION

We provide evidence that the therapeutic beneﬁts of MSCs are attributable to a paracrine mechanism. In vivo, administration of MSC-CdM alone attenuated lung neutrophil inﬂux and improved lung histology. This effect was comparable with administration of an equivalent number of MSC and absent in mice treated with control cells (lung ﬁbroblasts) or control cell CdM. MSC-CdM induced an M2 phenotype in AMs exposed to LPS in vitro and in vivo in LPS-exposed animals. We also suggest that MSC-CdM
contains soluble factors capable of attenuating lung injury. Identification of these soluble factors secreted by MSCs may yield new therapeutic options for ALI/ARDS.

Recent studies have shown that MSCs modulate immune cell function (28, 39, 46) and have cell-protective effects through the release of cytokines and growth factors (17, 18, 24, 37). MSC-CdM decreased hypoxia-induced cell death and improved tube formation of human aortic endothelial cells (24). CdM derived from MSCs engineered to overexpress the prosurvival gene Akt protected cardiomyocytes from hypoxia-induced cell death and limited myocardial infarct size in vivo (17, 18). MSC-CdM improved healing in an excisional wound splinting model in mice (9), oxygen-induced AT2 and pulmonary microvascular endothelial cell injury in vitro (71), neonatal oxygen-induced lung injury in vivo (3), and reversed hepatocyte death and increased survival in Gal-N-induced fulminant hepatic failure (55). Moreover, human MSC-CdM attenuated endotoxin-induced lung injury in an ex vivo perfused human lung model (37) and mouse MSC-CdM prevented the development of murine asthma (25).

In the present study, the therapeutic benefit seen with CdM mirrored the protective effects described with whole cell therapy. Similar to previous findings with whole cell therapy after LPS injury (21, 44), we observed that a single intratracheal injection of MSC-CdM or MSCs 4 h after LPS administration decreased lung neutrophil influx and lung permeability and improved the lung histopathology score compared with control media, lung fibroblasts, or their CdM. These findings open new therapeutic options by identifying potential healing molecules contained in MSC-derived CdM and understanding their mechanism of action.

Our data suggest that MSC-CdM promote a M2 “healer” macrophage phenotype. In the development of ALI/ARDS, neutrophils and macrophages are activated to eliminate pathogens but also contribute to tissue injury through the release of antimicrobial compounds (72). Macrophages are phenotypically heterogeneous because they respond to stimuli in their

Fig. 5. MSC-CdM promotes the M2 AM phenotype in vivo. A: representative scatterplots of 4-color-stained AMs from experimental lungs. a.u., Arbitrary units. B: AMs from LPS-DMEM, LPS-Fib, LPS-Fib CdM lungs displayed an iNOS+/Ym1- (gate P3; M1) phenotype. AMs from LPS-MSC and LPS-MSC CdM showed an iNOS-Ym1+ (gate P2; M2) phenotype (n = 5/group). Q1-1, Q1-2, Q1-3, Q1-4: quadrants resulted from quadrant gating. For M1 macrophages: no significant differences in control vs. control-CdM and LPS-MSC, control-CdM vs. LPS-MSC CdM, or LPS-DMEM vs. LPS-Fib CdM. P < 0.05 LPS-MSC vs. control-CdM and LPS-MSC CdM. P < 0.01 for all other group pairings. For M2 macrophages: no significant differences in control vs. LPS-Fib; LPS-DMEM vs. LPS-Fib CdM. P < 0.01 for all other group pairings.
Microenvironment and they also differ genetically (20). Classically activated macrophages (M1) kill invading microorganisms and tumors and promote type I immunity by secreting high levels of proinflammatory cytokines and low levels of anti-inflammatory cytokines (40). In contrast, alternatively activated macrophages (M2) secrete lower levels of proinflammatory cytokines and higher levels of anti-inflammatory cytokines. M2 macrophages promote type II immunity and are thought to dampen the immune response and to promote wound healing, angiogenesis, and debris scavenging (12). M1 and M2 macrophages can be characterized by receptor expression, effector function, cytokine and chemokine production (26, 40), or a set of marker genes: M1 macrophages generate nitric oxide by upregulation of iNOS, whereas M2 macrophages express Ym1 [also known as T-lymphocyte-derived eosinophil chemotactic factor (ECF-L) and chitinase 3-like 3 (CHI3L3)], FIZZ1 [found in inflammatory zone-1, also known as resistin-like molecule alpha (RELM-)], and arginase-1 (Arg-1) (20, 50). The upregulation of arginase-1 expression and activity is crucial in the metabolic switch from M1 to M2 phenotype in mice (60). In our LPS-induced inflammation model, we screened for the effect of MSC-CdM on AM phenotype in vitro. AMs isolated from healthy mice and subsequently exposed to LPS followed by MSC-CdM had higher Arg-1 activity and expressed higher levels of Ym1, findings characteristic of M2 macrophages. To more thoroughly test for the occurrence of alternative macrophage activation in vivo, we isolated AMs from experimental animals and found that LPS, as well as Fib and FibCdM, increased the numbers of macrophages expressing iNOS in the absence of Ym1 (a pattern corresponding with the M1 phenotype), whereas MSCs and MSC-CdM upregulated Ym1 macrophages lacking iNOS expression (M2). These data support the immunomodulatory capacity of MSC-CdM shifting the immune environment from pro- to anti-inflammatory via the induction of a “healer” M2 AM phenotype from a “killer” M1 macrophage. Our results are in line with several recent reports indicating that MSCs exert anti-inflammatory properties via macrophage reprogramming (31, 39, 49, 52). However, the murine MSC populations differ amongst these reports, mainly.
because of the current lack of consensus in establishing the universal murine MSC phenotype (7, 56). Comparison is also limited by differences in experimental protocols and variations in CdM preparation methods.

The paracrine mechanism of action of MSCs opens new therapeutic perspectives. Indeed, several paracrine mediators that can mediate restorative effects of MSCs have been identified, including interleukin-10, IL-1 receptor antagonist (IL-1ra), and keratino-IGF-I (pg/mL)

![](IGF-I ELISA.png)

**Fig. 7.** IGF-I decreases LPS-induced lung injury. 

A: ELISA measurement showing that IGF-I is present at higher levels in MSC-CdM compared with Fib-CdM. *P < 0.05 control; #P < 0.01. B–D: treatment with recombinant mouse IGF-I (rIGF-I; n = 5) significantly attenuated BALF cells influx (b), lung permeability (c), and lung injury score (d) compared with LPS-DMEM (n = 5). *P < 0.05 control vs. LPS groups; #P < 0.05 LPS-rIGF-I vs. LPS-DMEM. 

E: representative images of lungs from experimental animals. Size bar: 130 µm.

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![](4-color-stained AMs.png)

**Fig. 8.** IGF-I promotes the M2 AM phenotype. 

A: representative scatterplots of 4-color-stained AMs from experimental lungs. 

B: LPS-rIGF-I treatment enhanced Ym1 expression and prevented inducible nitric oxide synthase (iNOS) induction compared with LPS-DMEM. LPS-Neut-CdM (LPS-MSC CdM + nAb IGF-I) attenuated Ym1 expression in AMs and was less effective in preventing iNOS induction (n = 5/group).
cyte growth factor (KGF). IL-1ra was identified as an MSC-derived paracrine factor that reduced the severity of bleomycin-induced lung injury (53). Allogeneic human MSCs or their CdM attenuated endotoxin-induced lung injury in an ex vivo perfused human lung model partly through KGF, a well-known growth factor to reduce lung injury (37). In our study, we found that MSC-CdM contained higher levels of KGF and HGF than Fib-CdM. IL-10 was shown to be responsible for the therapeutic benefits of exogenous MSCs in murine sepsis (49). Our group has recently reported that bone marrow MSC (BMSC)-derived adiponectin contributes to the antiatherogenic effect of BMSC CdM in a murine atheroscleroma-induced asthma model (25). Human cord blood-derived MSCs, but not fibroblasts, produce high levels of angiotensin converting enzyme 2, an exopeptidase recently shown to be lung protective (61). Recent findings suggest the possibility that cell-to-cell communication mediated by transfer of exosomes/microvesicles or whole organelles contributes to the therapeutic benefit of MSCs (1, 10). Indeed, MSC-derived microvesicles protect from kidney injury (57) and MSC mitochondria transfer mediates the protective effects of MSC in ALI (27). Further identification of cellular communication mediators, including soluble factors, may lead to the development of critically missing pharmacological therapies for ALI/ARDS and other inflammatory diseases (6).

To broaden the search for candidate soluble factors, we performed a multiplex analysis screening for 96 factors present in MSC-CdM and showed that their secretory profile differed from that of lung fibroblasts. In a similar manner, Schinköthe et al. (66) screened human MSCs to attain the first large-scale description of factors they secrete and categorized these into functional groups: antiapoptotic, immunosuppressive, proproliferative, and angiogenic modulating. Among factors that may account for the alternative macrophage activation, we found that MSC-CdM contained several known M2 activators, whereas Fib-CdM contained higher amounts of the potent classical (M1) activator IL-6, whereas MSC-CdM had higher levels of factors that have recently been shown to contribute to the promotion of an M2 environment. We have previously reported that adiponectin, which may exert similar M2-activating effects (50) was found in our MSCs from both WT C57/BL6 and Balb/C mouse strains but displayed similar M2-activating effects (50) was found in our MSCs from both WT C57/BL6 and Balb/C mouse strains but was undetectable in Fib-CdM by ELISA (25).

The factors with the highest differential expression in MSC-CdM compared with Fib-CdM included platelet factor 4 (PF4) and macrophage inflammatory protein 2 (MIP-2). PF4-induced macrophages are distinct from M1 macrophages and M2 macrophages and have been proposed to have specific prothorogenic capacities (16), suggesting that PF4 does not induce a healer macrophage phenotype. With regard to the role of MIP-2 as a phenotype marker, the existing literature is unclear. MIP-2 has been presented as both an “M1 marker” (30) and a factor produced by M2 macrophages (14). Conversely, IGF-I has recently been shown to aid in creating an M2-favorable environment (8). Thus we explored the contribution of IGF-I to the therapeutic benefit by administering rIGF-I alone as in vivo treatment. Administration of rIGF-I partially reproduced the beneficial effects of MSC-CdM.

MSC-CdM also contained markedly higher levels of potential protective factors compared with Fib-CdM, suggesting that additional mechanisms could explain the therapeutic benefit of MSC-CdM in this model. SCF improves survival, proliferation, and differentiation of hematopoietic stem and progenitor cells and antiapoptotic effects reported in kidney tubular epithelial cells (7, 15). Another molecule, FcγRIIB, is increased 14-fold in MSC-CdM compared with Fib-CdM. FcγRIIB mediates the anti-inflammatory benefits of intravenous gamma globulin in a murine model of immune thrombocytopenia (64). We also found that IL-12p40, a natural antagonist of IL-12, which is a cytokine responsible for the production of IFN-γ, was increased sevenfold in MSC-CdM compared with Fib-CdM. IL-12p40 has protective effects in bacterial pneumonitis in mice (23) and selectively inhibits airway hyperresponsiveness and peribronchial fibrosis in murine asthma (52). A twofold increase in soluble TNF-α receptor II (sTNFRII), an inhibitor of TNF-α and a possible regulator of inflammatory activity, was observed in MSC-CdM compared with Fib-CdM. sTNFRII may contribute to dampening of the activity of TNF-α in LPS injury (45).

In conclusion, our study provides direct in vivo evidence that MSCs exert their therapeutic benefit through a paracrine activity. Identification of pneumoprotective soluble factors in MSC-CdM holds promise for the discovery of new pharmacological therapies for lung diseases. Although MSCs may have a unique ability to monitor the microenvironment of injured tissues and respond appropriately, therapies with the proteins or cytokines produced by activated MSCs may be more practical than cell therapies (2, 38). In vitro priming of MSCs to optimize the production of protective factors may combine the advantages of both cell and small molecule therapy.

REFERENCES


