Influenza causes prolonged disruption of the alveolar-capillary barrier in mice unresponsive to mesenchymal stem cell therapy

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Gotts JE, Abbott J, Matthay MA. Influenza causes prolonged disruption of the alveolar-capillary barrier in mice unresponsive to mesenchymal stem cell therapy. Am J Physiol Lung Cell Mol Physiol 307: L395-L406, 2014. First published July 18, 2014; doi:10.1152/ajplung.00110.2014.—Viral pneumonia is a major cause of acute respiratory distress syndrome (ARDS). Anti-inflammatory therapies for viral-induced lung injury show promise in preclinical models. Mesenchymal stem/stromal cells (MSCs) are multipotent, self-renewing cells that secrete anti-inflammatory cytokines and epithelial and endothelial growth factors. We inoculated mice intranasally with influenza A (murine-adapted Puerto Rico/8/34) or PBS, and the mice were killed at multiple time points after infection for measures of lung injury and viral load. We report that influenza induces marked, long-lasting dysfunction of the alveolar-capillary barrier peaking at 1 wk but lasting longer than 3 wk postinfection. Weight loss, commonly employed as a criterion for euthanasia (and hence “survival”), was found to be poorly predictive of the severity of lung injury at its peak; rather, persistent weight loss 11 days postinfection identified mice with impaired injury resolution. Murine and human bone marrow-derived MSCs (obtained from the National Institutes of Health repository) were then administered intravenously during the rapid phase of injury progression. Murine MSCs (mMSCs) given two times 24 h apart failed to improve weight loss, lung water, bronchoalveolar lavage inflammation, or histology. However, mMSCs prevented influenza-induced thrombocytosis and caused a modest reduction in lung viral load at day 7. Human MSCs administered intravenously showed a similar lack of efficacy. The results demonstrate that the influenza murine model bears important similarities to the slow resolution of ARDS in patients. Despite their potent therapeutic effects in many models of acute inflammation and lung injury, MSCs do not improve influenza-mediated lung injury in mice.

mesenchymal stem cells; acute lung injury; acute respiratory distress syndrome; pulmonary edema; pneumonia

ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) continues to exact a heavy toll on public health, and therapeutic options remain limited. Of the many viruses that can cause severe pneumonia/ARDS, influenza has historically been the major culprit (4, 53). During influenza pandemics caused by novel strains (as in 1918 and 2009), deaths tend to occur disproportionately among younger and healthier patients (59, 62). Influenza vaccination is reasonably effective, but pandemics emerge rapidly, allowing new viruses to cause widespread disease before an appropriate vaccine can be deployed. Neuraminidase inhibitors have some efficacy in reducing symptom duration and viral shedding, but only when given early in the course of illness. In addition, they are threatened by increasing resistance (27), and a recent analysis has questioned their true therapeutic benefits (28).

Mice have been used to study influenza for decades, most commonly the H1N1 Puerto Rico/8/34 (PR8) strain, which causes anorexia, weight loss, and inflammatory lung injury, culminating in diffuse alveolar damage by 1 wk postinfection (5, 17). Most murine studies have focused on the 1st wk postinfection and the waves of injury produced by viral cytopathic effect, the innate, and adaptive immune responses (32, 39, 57), but much less is known about the process of recovery. Importantly, many investigators kill animals for an arbitrary weight loss criterion of 20%, and “survival” data in the murine influenza literature are greatly influenced by this practice.

Uncontrolled inflammation is central to the pathogenesis of influenza. Cytokine production is better correlated than viral burden with the severity of influenza infection in mice, primates, and humans (13a, 33, 48). Studies of genetically altered mice have revealed important roles for multiple cytokines in influenza pathogenesis, generally showing that disrupting pro-inflammatory pathways improves clinical outcomes despite increasing viral loads (13). Therapeutically, modulating inflammatory pathways during influenza infection is desirable because it is unlikely to exert selective pressure on this rapidly mutating virus. Mesenchymal stem/stromal cells (MSCs) are self-renewing cells isolated from bone marrow that can differentiate into muscle, bone, fat, fibroblasts, and cartilage, have potent immunomodulatory effects, and secrete a variety of epithelial and endothelial growth factors (7, 18, 37). They have shown substantial therapeutic promise in preclinical models of bronchopulmonary dysplasia (2, 61a), abdominal sepsis (42, 45), sterile (22, 36) and bacterial lung injury (21, 34, 35), and for increasing alveolar fluid clearance in lungs rejected for transplantation (41). With the exception of one other study (12), however, little is known about their potential to mitigate viral lung injury.

Here we sought to: 1) characterize the temporal disruption and repair of the alveolar-capillary barrier following influenza-induced lung injury; 2) test the ability of weight loss to discriminate lung injury severity; 3) test the hypothesis that murine (m) or human (h) MSCs reduce the degree of lung injury when administered during the phase of rapid injury progression; and 4) determine whether MSC therapy impairs host control of the viral infection. We report that: 1) the alveolar-capillary barrier undergoes severe injury between 5 and 7 days after viral inoculation, 2) barrier function remains abnormal 3 wk postinfection; 3) weight loss, a common euthanasia (and hence “survival”) criterion, is not predictive of the severity of lung injury at its peak, but rather may predict slower injury resolution during the 2nd wk postinfection; 4) neither
**A**

![Graph showing baseline weight percentage](image)

**B**

![Bar graph showing endothelial permeability](image)

**C**

![Bar graph showing wet/dry ratio](image)

**D**

![Bar graph showing excess lung water](image)

**E**

![Bar graph showing BAL protein concentration](image)

**F**

![Image of lung tissue](image)

**G**

![Image of lung tissue](image)

**H**

![Image of lung tissue](image)
hMSC nor mMSC reduce influenza-induced lung injury; and 5) mMSC therapy modestly reduces lung viral load.

**MATERIALS AND METHODS**

**Animals.** Eight-week-old female C57BL/6 mice purchased from NCI were used for all experiments. All work was conducted under a protocol approved by the University of California, San Francisco Institutional Animal Care and Use Committee (IACUC).

**Viral infection and quantification.** Animals were anesthetized deeply with isoflurane, and 100 foci-forming units of influenza A/H1N1/PR8 dissolved in 30 μl of PBS were pipetted on their nostrils. The mice were allowed to recover and then weighed daily. Mice used for viral quantification experiments were not processed for other measures of lung injury. Infective viral particles were assayed by inoculation of either stock virus or homogenate (in 1 ml PBS) of left lung, spleen, and brain on 96-well plates of confluent MDCK cells. After 1 h, samples were decanted and replaced with serum-free media containing tosyl phenylalanine chloromethyl ketone trypsin at 1.5 μg/ml. Fifteen hours later, the cells were fixed in 100% methanol and then underwent indirect immunocytochemistry using Millipore mouse anti-influenza A (MAB 8257) at 1.25 μg/ml, followed by Vector biotinylated horse anti-mouse, and the biotin/avidin system (PK-4002) with diaminobenzidine as a chromogen. Samples were processed in triplicate over 105 dilutions, and foci were counted in wells that yielded 30–100 discrete foci.

**MSC culture and administration.** Mouse MSCs and hMSCs were obtained from the National Institutes of Health repository in Temple, TX. These cells met all the criteria for MSCs as defined by the International Society of Cellular Therapy (14). Cells were thawed and expanded in tissue culture flasks, being passage every 3–5 days by trypsinization when they reached 80% confluence. As in prior studies, all MSCs used were passage 7 or less. Before injection, cells were trypsinized and washed twice in PBS and then resuspended in 1 ml PBS. In some experiments, MSCs were preincubated with fluorescent nanocrystals (Qtracker 525 Cell Labeling Kit; Life Technologies, Grand Island, NY) before injection; mice injected with labeled MSCs were killed 3 h later by thoracotomy, exanguination, and intracardiac perfusion of 15 ml PBS.

**Lung injury endpoints.** For gravimetric measurements (wet-to-dry ratio, excess extravascular lung water, and endothelial permeability), mice were killed by bilateral thoracotomy after overdose of ketamine and intracardiac perfusion of 15 ml PBS. Samples of blood, lung homogenate, and plasma were collected in EDTA tubes. The lungs were removed and homogenized in 1 ml PBS. Samples of blood, lung homogenate, and homogenate supernatant were weighed before and after desiccation, and another fraction of homogenate was assayed for hemoglobin concentration such that the blood volume of the lung could be calculated (58). As in prior work, the wet-to-dry ratio and excess extravascular lung water were calculated. In selected experiments, 0.1 μCi of 125I-labeled albumin was injected intravenously 3 h before death, permitting measurement of lung endothelial permeability as previously described (16): counts per minute of blood-free lung divided by counts per minute of plasma multiplied by the calculated plasma volume. In separate animals, bronchoalveolar lavage (BAL) was accomplished by tracheal cannulation and lavage with 1 ml PBS. BAL cell count, hemoglobin, hematocrit, and platelet count were measured with a Hemavet 950 cell counter (Drew Scientific, Waterbury, CT). Cyto spun preparations of BAL fluid were made and stained with Hema 3 solution (Thermo Fisher Scientific, Waltham, MA), and 200 cells/mouse were analyzed at ×100 magnification and classified as neutrophils, lymphocytes, or monocyte/macrophages. BAL protein was measured with the BCA Protein Assay (Thermo Fisher Scientific). Histological analysis was performed on the lavaged mice. Lungs were fixed by intratracheal installation of 1 ml 4% paraformaldehyde followed by overnight fixation, dehydration, paraffin embedding, and staining of 4-μm sections with hematoxylin and eosin. For blinded histological analysis, four sections (2 from the right lung and 2 from the left lung) were scored at ×40 for inflammatory infiltrate and septal thickening (0 = normal, 1 = mild, 2 = moderate, 3 = severe, and 4 = very severe/necrotic). Fields were evaluated at 150-μm intervals throughout the entirety of each of the four sections, yielding >100 scored fields/mouse. Interleukin-6 (IL-6) was measured in duplicate serum samples by Quantikine Mouse IL-6 ELISA (R&D Systems, Minneapolis, MN).

**Statistical analysis.** Comparisons between two groups were done with unpaired t-test or Wilcoxon rank sum (when data were not normally distributed). P < 0.05 was considered to be statistically significant. Comparisons of more than two groups were made with ANOVA with Bonferroni corrections. Weight loss trajectories were analyzed with the generalized estimating equations (GEE) model. The relationship between weight loss and lung water at different postinfection time points was analyzed with linear regression. All statistical analyses were performed with Stata-13 (StataCorp, College Station, TX).

**RESULTS**

**Influenza induces a severe but survivable injury.** Mice underwent intranasal inoculation with 100 foci-forming units (FFU) of PR8 influenza virus or PBS control. Influenza-infected mice lost over 20% of their baseline body weight at −1 wk postinfection (Fig. 1A) and then rapidly recovered weight over the next week with no deaths observed in over 40 mice. Protein in the BAL is commonly measured as a surrogate for disruption of the alveolar-capillary barrier. However, this measure reflects the accumulated changes in the barrier function of the lung endothelium and alveolar epithelium as well as alveolar fluid clearance, making it ill-suited to characterize dynamic barrier changes (63). Although labor intensive, measuring albumin extravasation from the vascular to the extravascular lung compartment provides a more rigorous assessment of barrier function. Endothelial permeability, assessed 3 h following intravenous injection with 0.1 μCi 125I-albumin increased dramatically between 5 and 7 days postinfection (dpi; Fig. 1B). Surprisingly, endothelial permeability remained significantly elevated at 3 wk postinfection. Both the wet-to-dry ratio (Fig. 1C) and excess lung water (Fig. 1D) followed a similar temporal course, peaking at 7 dpi but remaining ele-

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**Fig. 1.** Time course of influenza-induced lung injury and its resolution. A: mice were inoculated with 100 foci-forming units (FFU) influenza Puerto Rico/8/34 (PR8) virus or PBS control. Despite substantial weight loss, influenza-infected mice recovered their baseline body weight by 12 days postinfection (dpi) with 100% survival in this cohort of over 40 infected animals. Data are means ± SE. *Significant group/day interaction by generalized estimating equations (GEE) model. B–E: lung endothelial permeability (B), wet-to-dry ratio (C), and excess lung water (D) peak at 7 dpi but remained elevated up to 3 wk postinfection. Although bronchoalveolar lavage (BAL) protein normalized by 22 dpi (E), endothelial permeability to albumin remained significantly elevated (B). Data are means ± SD, n = 5–10 mice/group at each time point. *P < 0.05 vs. PBS by one-way ANOVA (Bonferroni corrected). F: typical patchy injury seen at low power 7 dpi with 100 FFU of influenza (scale bar 200 μm). Higher-power views of PBS control (G) and influenza-infected mice (H) demonstrating mixed inflammatory infiltrate and septal thickening, scale bar 50 μm.
vated up to 2 wk after the peak. In contrast to the sustained increase in lung endothelial permeability, BAL protein normalized by 22 dpi (Fig. 1E). Influenza infection produces severe but patchy lung injury (Fig. 1F), with obvious septal thickening and mixed inflammatory infiltrate (Fig. 1H) compared with control (Fig. 1G).

**Weight loss and lung injury.** Weight loss is commonly used as both an outcome measure and criterion for euthanasia in murine influenza studies. The influenza literature is replete with studies that report changes in survival following a variety of interventions; given that many of these survival differences are driven by changes in weight loss (and meeting or not meeting a prespecified criterion for euthanasia, most commonly 20%), we tested whether weight loss is predictive of the severity of lung injury. As shown in Fig. 2A, at the peak of lung injury 7 dpi, weight loss is not predictive of excess lung water, with an $R^2 < 0.01$. However, during the period of rapid weight recovery, this relationship improved (Fig. 2, B and C), reaching statistical significance at 11 dpi, suggesting that delayed recovery of baseline weight predicts slower resolution of lung injury.

**mMSCs do not improve influenza-induced lung injury.** mMSCs were administered intravenously (by retro-orbital injection) to influenza-infected mice on 5 and 6 dpi. To assess for potential effects both on the maximum extent of injury and the speed of resolution, mice were killed at 7, 9, and 11 dpi. As shown in Fig. 3B, mMSC treatment did not alter the trajectory of weight loss and recovery. Radiolabeled albumin injected intravenously 3 h before death was used to measure lung endothelial permeability (Fig. 3C), showing no significant difference between mMSC and PBS control at 7–11 dpi. Similarly, the wet-to-dry ratio (Fig. 3D) and excess lung water (Fig. 3E) were unchanged by mMSC administration. Additional measures of lung injury were performed at 7 dpi (Fig. 4A). As shown in Fig. 4B, BAL protein was not different between mMSC and PBS-treated mice. mMSC administration did not affect the cellularity (Fig. 4C) or the composition of the inflammatory infiltrate (Fig. 4D). To adequately compare the histological extent of lung injury (given the patchy nature of the injury; Fig. 1F), over 100 fields from 4 sections (spanning two lung lobes) were scored (0–4) by a blinded observer on the extent of inflammatory infiltrate (Fig. 4E) and septal thickening (Fig. 4F), showing no significant difference following mMSC treatment.

**Intravenously delivered mMSCs reach the lung and are biologically active.** To verify that the cells administered by retro-orbital injection reached the lung, MSCs were labeled in vitro with Qtracker nanocrystals before infusion. As shown in Fig. 5A, clearly labeled cells could be found in abundance in distal lung tissue 3 h postinfusion. Influenza caused a mild thrombocytosis by 7 dpi (Fig. 5B). mMSCs administered intravenously on 5 and 6 dpi prevented influenza-induced thrombocytosis, indicating that the cells were having a clear biological effect. Because IL-6 is known to be a potent stimulator of thrombopoiesis (3), and is also known to be increased in influenza-infected mice at 7 dpi (6), we measured serum IL-6 in this model. As shown in Fig. 5D, mMSC administration on 5 and 6 dpi did not reduce serum IL-6.

**mMSCs modestly reduce lung viral load.** Although mMSC therapy failed to reduce the severity of lung injury, a related issue is whether these cells, which have potent immunomodulatory effects, might impair host control of the infection. This question takes on added significance now that MSCs are in early clinical trials in ARDS and may be administered to patients with influenza (NCT01775774, http://clinicaltrials.gov). To improve our ability to make quantitative comparisons across a range of tissues and conditions, we employed an immunocytochemical approach to viral load analysis. Homogenates of lung, brain, and spleen were incubated with confluent MDCK cells in 96-well plates, followed by fixation and stain-
ing with an antibody to influenza nucleoprotein A and then secondary immunocytochemistry and visible chromogen. This technique produced discrete, easily countable foci (Fig. 6A).

As shown in Fig. 6B, applying this technique to aliquots of influenza virus verified that the assay was highly reliable. Influenza infection produced the expected early rise in lung viral titer, falling to near the level of detection by day 10 (Fig. 6C). mMSC administration on 5 and 6 dpi modestly reduced lung viral load at 7 dpi (Fig. 6E).

hMSCs do not improve influenza-induced lung injury. Both mMSCs and hMSCs have been shown to have potent anti-inflammatory and immunomodulatory effects in murine models of lung injury and sepsis (37). However, there are several key differences between these cell populations. For example, the molecular mechanisms of T cell response inhibition appear to occur via indoleamine 2,3-dioxygenase in hMSCs but via nitric oxide signaling in MSCs (52). Also, given that hMSCs are being tested in patients, it is important to understand whether these cells might affect influenza-induced lung injury. Therefore, we administered hMSCs intravenously during the period of rapid progression of barrier dysfunction (Fig. 7A). As shown in Fig. 7B, there was no difference in weight loss, although there was a trend toward slower weight recovery in mice treated with hMSCs. Endothelial permeability (Fig. 7C), wet-to-dry ratio (Fig. 7D), and excess lung water (Fig. 7E) were not significantly impacted by hMSC treatment.

Given the lack of therapeutic effect of MSCs given 5 and 6 dpi, we also tried administering mMSCs or hMSCs earlier in the injury process (2 and 3 dpi) and similarly found no impact on the degree of lung injury (data not shown). Finally, we tried administering the cells by the intratracheal route but unexpect-

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Fig. 3. Murine mesenchymal stem/stromal cells (MSCs) do not prevent influenza-induced alveolar-capillary barrier breakdown. A: schematic showing interventions and death time points. All mice were infected with 100 FFU influenza. On 5 and 6 dpi, 100 μl of PBS alone or PBS containing 500,000 mMSCs was infused by retro-orbital injection. B: mean weight loss during the first 11 dpi was not different between mMSC and PBS-treated groups. Data are means ± SE; n = 27–33 mice/condition. C–E: mMSC treatment did not significantly impact endothelial permeability (C), the wet-to-dry ratio (D), or excess lung water (E) at 7–11 dpi. Data are means ± SD; n = 8–10 mice/group at each time point.
edly found that this greatly increased the severity of the model: even saline given by airway (under isoflurane anesthesia) led to over 50% mortality by 7 days (data not shown).

**DISCUSSION**

The main findings of these experiments can be summarized as follows: 1) influenza infection induces a reliable and severe lung injury in mice characterized by a dramatic breakdown of the alveolar-capillary barrier between 5 and 7 dpi; 2) barrier permeability remains elevated beyond 3 wk postinfection; 3) weight loss at the peak of lung injury is not predictive of injury severity; 4) mMSCs and hMSCs fail to improve influenza-induced lung injury as assessed by multiple methods, despite evidence of biological activity; and 5) mMSCs modestly reduce lung viral load.

Fig. 4. Murine MSCs do not improve BAL or histological outcomes following influenza. **A**: schematic showing interventions and death time point at 7 dpi. All mice were infected with 100 FFU influenza. **B–D**: day 7 BAL protein (**B**), cell count (**C**), and manual differential (**D**) were not significantly different after mMSC treatment. Data are means ± SD (except individual data points in B); n = 8–10 mice/group. **E and F**: histological analysis of extent of inflammation (**E**) and septal thickening (**F**) showed no significant impact by mMSC treatment. Over 100 sections/mouse at 4 locations in 2 lung lobes were scored by a blinded observer 0–4 on extent of inflammatory infiltrate and septal thickening and averaged. Data are means ± SD; n = 10–11 mice/group.
The lung pathology wrought by influenza pneumonia in patients was described after the great 1918 pandemic (40) but more thoroughly in classic work by Hers et al. in the 1950s (24), and extended to the 2009 H1N1 pandemic recently (20). Epithelial viral cytopathic change extends from the nose through the deep airways to the alveolar ducts, affecting types 1 and 2 alveolar epithelial cells as well as alveolar macrophages. In addition, there is capillary thrombosis, necrosis, hemorrhage, and the formation of hyaline membranes. These changes constitute diffuse alveolar damage, the pathological pattern of ARDS. Notably, the patchy nature of influenza-induced injury in autopsy specimens spares significant areas of lung (24).

As in patients with influenza pneumonia, mouse-adapted PR8 virus rapidly infects and replicates (12 h after inoculation) in murine alveolar epithelial cells, with evidence of epithelial sloughing at 72 h. By 7 dpi, ill-appearing mice harbor patchy areas of consolidated lung with edematous alveolar septa and a protein-rich exudate replete with neutrophils, lymphocytes, and monocytes/macrophages (39). In 2008, Wolk and colleagues (64) reported that high-dose inoculation with a different strain of influenza A, WSN/33, increased lung wet-to-dry ratio, peaking 6–8 dpi. These authors also instilled fluorescently labeled albumin in the airspaces of anesthetized, ventilated mice and found little extravasation in the serum until 4 dpi, suggesting that the alveolar-capillary barrier retains its integrity early during the infection. More recent work with PR8 in mice has shown that lung weight nearly doubles between 2 and 6 days in parallel with the progressive development of diffuse alveolar damage (17). Despite the large body of work with murine influenza, little is known about the precise timing of the breakdown and subsequent repair of the alveolar-capillary barrier so critical in preventing airspace flooding and life-threatening hypoxemia.

Here we characterize the temporal course of barrier breakdown, finding that lung endothelial permeability to protein nearly quadruples between 5 and 7 dpi. In parallel, pulmonary edema as measured by wet-to-dry ratio or excess lung water peaks at day 7, probably in part because influenza impairs alveolar fluid clearance (10, 64). Remarkably, barrier function remains abnormal for over 3 wk postinfection, although the normalization of BAL protein by this time point suggests that the ongoing capillary leak is progressively confined to the interstitial space since reepithelialization probably occurs and active vectorial fluid transport resumes. The mechanisms responsible for prolonged endothelial leak are not clear but may...
Fig. 6. mMSCs modestly reduce lung viral load. A: example of FFU immunocytochemical assay. Pictured is a plate of confluent MDCK cells processed for immunoreactivity to influenza nucleoprotein A. Three discrete labeled MDCK nuclei (foci) are visible in this field. B: the FFU assay is reliable as shown by the results from 10 viral aliquots of equal concentration. The SD of these measurements is ~12% of the mean. C: time course of viral load in the influenza model. Triangles represent results from individual animals. The left lungs of mice were flash-frozen at death and then batch processed by homogenization in 1 ml PBS. The data show the expected peak of influenza replication 3 dpi followed by near clearance at 10 days. Virus was not detected in the brains or spleens of mice at any time point. D: schematic depicting interventions and time points of death for E. E: mice treated with mMSCs (dashes) had a significant reduction in lung viral load compared with PBS-treated mice (triangles). Virus was not detected in the brains or spleens of any mice (data not shown). *P < 0.05 vs. PBS by Wilcoxon rank sum.
be related to persistent low-grade inflammation or perhaps angiogenesis. The delayed but rapidly developing and then long-lasting barrier dysfunction mirrors the clinical course of patients with influenza-induced ARDS and identifies an optimal time window in which to test potential therapeutics.

Mirroring the temporal course of barrier dysfunction, influenza-infected mice appear relatively unaffected until 5 dpi, when they begin to lose weight rapidly, become lethargic, hypothermic, and huddle together (46). Many IACUC protocols require euthanasia for an arbitrary weight loss criterion (commonly 20%), and thus survival in mouse influenza studies is often heavily influenced by the degree of weight loss, particularly when the criterion for euthanasia approximates the average weight loss in the study. The related supposition is that weight loss is a suitable surrogate for the severity of illness. In our experiments, the mean weight loss in mice not undergoing additional interventions was a little over 20% at 7–8 dpi, yet all infected mice survived. Additionally, weight loss was poorly predictive of the degree of pulmonary edema, although a failure to rapidly regain weight did correlate with its persistence. Thus, slow weight gain during the 2nd wk postinfection might be a useful marker of impaired resolution of lung injury.

MSCs have been extensively studied and found therapeutically efficacious in diverse models of acute inflammation in many different organ systems. In the lung, MSCs improve injury following bleomycin (47), endotoxin (22, 26, 43, 66), cecal ligation and puncture (42, 45), Pseudomonas abdominal sepsis (34), and Escherichia coli pneumonia (21). In these models MSCs work by various mechanisms, including secreting angiopoietin-1 (15) and keratinocyte growth factor (36, 38), shifting cytokines from pro- to anti-inflammatory (50), improving bacterial clearance (21, 34, 35), and transferring mitochondria to energetically impaired alveolar epithelial cells (26).
Uncontrolled, excessive cytokine production and immune cell activation are known to be central to the pathophysiology of influenza-induced ARDS (13). MSCs have been shown to inhibit dendritic cell maturation (56), suppress T cell (19, 51, 67) and natural killer cell (55) proliferation and cytotoxicity, and promote the generation of regulatory T cells (49). These immunomodulatory properties suggested that MSCs might be an effective therapy for influenza-induced lung injury. However, MSCs failed to produce any evidence of physiological benefit in our studies, despite giving two doses on subsequent days during the period of rapid barrier breakdown. Administering the cells earlier in the infection and changing the route of delivery failed to change this outcome. Importantly, we demonstrate here that the cells localized to the lung and were biologically active.

Similarly negative results were reported recently by Darwish and colleagues (12). These researchers studied intravenous mMSCs and hMSCs as prophylaxis (preinfection and/or 2 dpi) or therapy (2 or 5 dpi) in mice inoculated with PR8 or 2009 pandemic H1N1, finding no evidence of beneficial effect on weight loss, survival, or lung injury. Combining MSC administration with oseltamivir was also ineffective. Taken together, our results and those of Darwish et al. provide reasonably strong evidence that MSCs do not blunt the injurious cascades of influenza in mice.

Potential reasons for their lack of efficacy include 1) the route of delivery did not allow access to the injured epithelial barrier, 2) the dose/timing of MSCs was insufficient given the prolonged nature of the injury, 3) MSCs might be targets of influenza infection, and 4) MSCs might have mixed or even stimulatory effects on immune responses. As for route of delivery, intravenously administered MSCs have proven effective in a variety of models of epithelial injury, including airway administration of endotoxin (9, 65) and bleomycin (44). Also, given the prominent role that lung endothelium appears to play in influenza pathogenesis (60), there is good reason to suspect that intravenous delivery of therapeutics is a reasonable strategy. Finally, we unexpectedly discovered that airway administration of cells or even saline during the period of rapid viral replication greatly increased the severity of the model, making this an impractical approach.

As for dose, we infused 500,000 cells on subsequent days, which yields a total cell number of over 50 × 10^6 cells/kg, substantially greater than most studies reporting therapeutic efficacy of MSCs in murine models of sterile and infectious lung injury (68), and greater than that employed by Darwish et al. (12). Furthermore, we administered the cells during the preidentified 48 h of greatest decline in barrier function. Although it would be possible to administer the cells more frequently to mice, such a strategy in patients is unrealistic. Interestingly, Khatri and Saif (30) recently reported that swine bone marrow-derived MSCs express the appropriate sialic acid receptors for hemagglutinin binding and secrete proinflammatory cytokines, including TNF-α and IL-6 when infected by influenza in vitro. Whether MSCs become infected in our murine model of intravenous MSCs is not known, but these results do suggest that airway administration of MSCs might best be avoided in patients potentially infected with influenza. Although known to potently inhibit T cell and other immune effector cell functions in vitro, MSCs may function differently when introduced in the complex signaling milieu of influenza pneumonia. Discrepancies between in vitro immune suppression and in vivo immune stimulation have been reported in collagen-induced arthritis (8, 54) and allogeneic heart transplantation (25). Furthermore, much of the optimism over MSCs for therapy of graft vs. host disease has faded among conflicting results in patients (31). Notably, the immunosuppressive effects of MSCs on B cells are less consistent (11, 61), and little is known about their effects on other T cell subsets such as Th17 cells, reported to be critical in the response to influenza (1). The mild reduction in viral load we observed following MSC treatment suggests that, on balance, MSCs were mildly immunostimulatory in this model. However, we are clearly in need of a more sophisticated understanding of the effects of MSCs on the multiple cell types and signaling cascades of the complex and evolving immune response elicited by viral infection.

In conclusion, influenza reliably produces severe but survivable lung injury in mice, and alveolar-capillary barrier dysfunction is prolonged. Even though MSCs modestly reduced viral load, they failed to reduce the severity of influenza-induced injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.E.G. and M.A.M. conception and design of research; J.E.G. and J.A. performed experiments; J.E.G. analyzed data; J.E.G. and M.A.M. interpreted results of experiments; J.E.G. prepared figures; J.E.G. drafted manuscript; J.E.G. and M.A.M. edited and revised manuscript; J.E.G., J.A., and M.A.M. approved final version of manuscript.

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