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Human mesenchymal stromal cells reduce influenza A H5N1-associated acute lung injury in vitro and in vivo

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Contributed by Robert G. Webster, February 13, 2016 (sent for review August 24, 2015; reviewed by Peter J. Openshaw, Luis Ortiz, and Daniel Perez)

Influenza can cause acute lung injury. Because immune responses often play a role, antivirals may not ensure a successful outcome. To identify pathogenic mechanisms and potential adjunctive therapeutic options, we compared the extent to which avian influenza A/H5N1 virus and seasonal influenza A/H1N1 virus impair alveolar fluid clearance and protein permeability in an in vitro model of acute lung injury, defined the role of virus-induced soluble mediators in these injury effects, and demonstrated that the effects are prevented or reduced by bone marrow-derived multipotent mesenchymal stromal cells. We verified the in vivo relevance of these findings in mice experimentally infected with influenza A/H5N1. We found that, in vitro, the alveolar epithelium's protein permeability and fluid clearance were dysregulated by soluble immune mediators released upon infection with avian (A/Hong Kong/483/97, H5N1) but not seasonal (A/Hong Kong/54/98, H1N1) influenza virus. The reduced alveolar fluid transport associated with down-regulation of sodium and chloride transporters was prevented or reduced by coculture with mesenchymal stromal cells. In vivo, treatment of aged H5N1-infected mice with mesenchymal stromal cells increased their likelihood of survival. We conclude that mesenchymal stromal cells significantly reduce the impairment of alveolar fluid clearance induced by A/H5N1 infection in vitro and prevent or reduce A/H5N1-associated acute lung injury in vivo. This potential adjunctive therapy for severe influenza-induced lung disease warrants rapid clinical investigation.

influenza | avian | acute lung injury | mesenchymal stromal cells | alveolar fluid clearance

Acute lung injury is a continuum of clinical and radiographic changes, terminating at its most severe, with acute respiratory distress syndrome. Infection with highly pathogenic avian influenza (HPAI) viruses of the H5N1 and more recent H7N9 subtypes often leads to acute lung injury whereas seasonal influenza viruses and the 2009 pandemic H1N1 influenza viruses do so more rarely. The underlying mechanisms of influenza-related acute lung injury remain unclear, and effective therapies are lacking. Viruses that are highly pathogenic to humans (e.g., H5N1 viruses) may differ intrinsically from the less pathogenic (LP) (e.g., seasonal H1N1) viruses in their replication competence, cell tropism, and/or cytokine dysregulation (1, 2). Early treatment of H5N1 disease with the antiinfluenza drug oseltamivir is helpful but does not ensure a favorable outcome (3). Thus, effective adjunctive therapies that do not compromise beneficial host defenses are needed (4).

H5N1 (5) and H7N9 (6) influenza viruses target alveolar epithelial cells, which form the crucial gas exchange interface in the lung. These cells also help to maintain intraalveolar and intravascular fluid homeostasis by vectorial transport of sodium, chloride, and water from the apical to the basolateral surface of the alveolar epithelium [alveolar fluid clearance (AFC)]. Impaired AFC and increased alveolar protein permeability (APP) contribute to acute lung injury (7). Therapies that normalize alveolar fluid clearance are likely to be free of off-target effects, unlike immunomodulation, that may promote virus replication.

Human bone marrow-derived multipotent mesenchymal stromal cells (MSCs) have applications in multiple clinical disorders, including sepsis, myocardial infarction, diabetes, and acute renal failure (8). Allogeneic MSC therapy has beneficial preclinical effects on endotoxin-, bacteria-, and ventilator-induced acute lung injury (9) via MSC secretion of the soluble paracrine growth factors angiopoietin-1 (Ang1) and keratinocyte growth factor (KGF) (9, 10). MSCs can also transfer mitochondria and microvesicles that modulate immunity and epithelial response to injury (11). Current clinical trials are testing MSCs as a therapy for sepsis and acute respiratory distress syndrome (12). However, little is known about the impact of MSCs on acute respiratory viral infections, including influenza, with the exception of a study in which MSCs failed to reduce influenza-induced lung injury in mice (13). Here, we showed that influenza A/H5N1 virus infection dysregulates AFC and APP in vitro by inducing infected cells to release soluble mediators that down-regulate alveolar sodium and chloride transporters. When we cocultured alveolar

Significance

Acute lung injury, including impaired alveolar fluid clearance, is a life-threatening complication of severe respiratory virus infection, and effective treatment is lacking. Understanding the mechanism of this complication may suggest novel therapies. Here, we found that, in vitro, influenza A/H5N1 infection impaired alveolar fluid clearance more than did seasonal virus, mimicking its greater severity in patients. We demonstrated that this impairment is mediated by the release of soluble factors from infected cells, leading to down-regulation of alveolar sodium and chloride transporters. Mesenchymal stromal cells prevented or reduced this effect in vitro and in vivo in A/H5N1-infected mice. These cells provide a potentially effective treatment for acute lung injury in severe influenza.

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The authors declare no conflict of interest.

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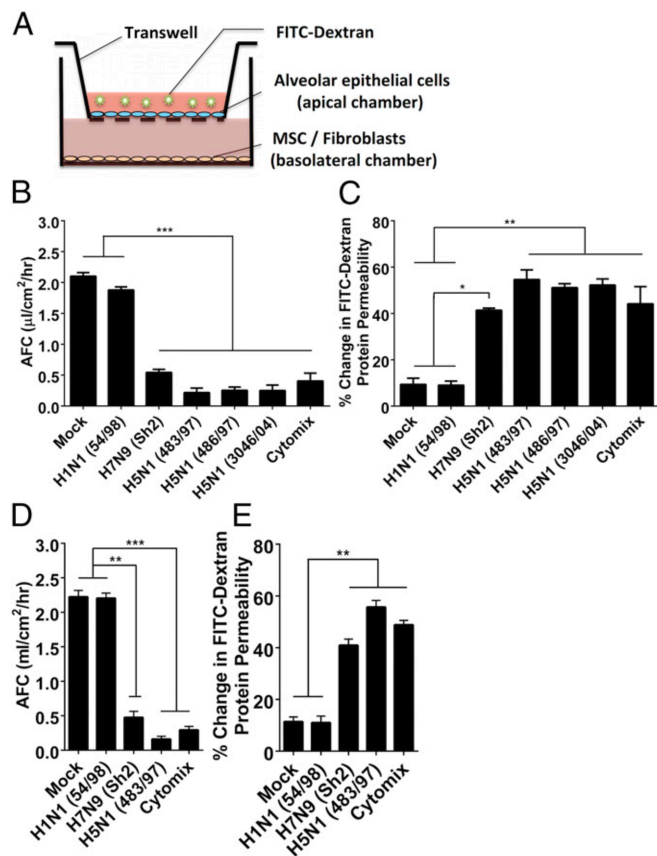

Fig. 1. Highly pathogenic influenza viruses significantly reduce human alveolar fluid clearance (AFC) and significantly increase human alveolar protein permeability (APP). (*A*) Schematic diagram of the in vitro lung injury model. FITC-dextran, fluorescein isothiocyanate-dextran; MSC, mesenchymal stromal cells. (*B* and *C*) Infection with HPAI H5N1 (A/HongKong/483/97, A/HongKong/486/97, and A/HongKong/3046 /04) and H7N9 (A/Shanghai/2/2013) viruses (MOI = 0.1) significantly (*B*) reduced the AFC and (*C*) increased the APP of human alveolar epithelial cells compared with infection with LP influenza H1N1 virus (A/HongKong/54/98). Cytomix containing proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ) was used as an injury-inducing positive control. Data are the mean ± SE from three experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. (*D* and *E*) The effect of conditioned medium (virus-free supernatant) from cells infected with H5N1 and H7N9 influenza viruses affected the AFC and APP of alveolar epithelial cells comparably with the live influenza viruses and (*D*) reduced the AFC and (*E*) increased the APP significantly more than supernatant from cells infected with H1N1 virus. Data are the mean ± SE from three experiments. $^{**}P < 0.01$; $^{***}P < 0.005$.

epithelium with MSCs, these injury mechanisms were prevented or reduced. We then treated mice infected with influenza A/H5N1 with MSCs and demonstrated a clinically significant reduction in lung pathology and increased survival in association with a modulation of these pathogenic mechanisms in vivo.

Results

HPAI H5N1 Virus Infection Reduces AFC and Enhances APP in Human Alveolar Epithelial Cells. Monolayers of primary human alveolar epithelial cells demonstrating tight junctions were grown on apical chambers of transwell culture inserts in which the basolateral chamber can be used for coculture with MSCs, or with fibroblasts or PBS as controls (Fig. 1*A*). Influenza H5N1, H7N9, and human seasonal H1N1 influenza viruses replicated comparably in these cells (Fig. S1). Under the experimental conditions used, ZO-1 tight junction staining of the alveolar epithelial was not affected in virus-infected cells, and the transepithelial resistance (>1,000 ohms) was maintained for up to 48 h postinfection. Although H1N1 54/98 virus minimally affected AFC and APP, the H5N1 viruses (483/97, 486/97, and 3046/04) and H7N9 virus (Sh2) (complete virus names are listed in *Materials and Methods*) caused much greater reduction of AFC (all $P < 0.0001$) (Fig. 1*B*) and greater increase in APP ($P = 0.008$, 0.007, 0.007, and 0.032, respectively) (Fig. 1*C*) than did mock infection at 24 h postinfection (p.i.). A mixture of proinflammatory cytokines [Cytomix (IL-1β, TNF-α, and IFN-γ)] served as positive control. The other human seasonal influenza viruses, H1N1 (OK/447), H3N2 (1174/99), and H3N2 (OK/370), affected AFC and APP similarly to H1N1 54/98 (Fig. S2). Virus-free conditioned medium from virus-infected alveolar epithelial cells (after virus filtration) similarly affected AFC (Fig. 1*D*) and APP (Fig. 1*E*). Therefore, the observed effects were caused by soluble mediators released from H5N1 or H7N9 virus-infected, but not H1N1 virus-infected, alveolar epithelial cells, rather than direct viral cytopathic effects.

Coculture with MSCs Reduces the Adverse Effect of H5N1 Infection on AFC, APP, and Proinflammatory Cytokine Responses in Human Alveolar Epithelial Cells. When H5N1 483/97-infected alveolar epithelial cells were cocultured (100,000 cells per well) with MSCs, the viral effects on AFC and APP were significantly moderated (Fig. 2), showing levels similar to those levels in mock-infected cells. Coculture with human lung fibroblasts or PBS had no effect (Fig. 2). MSCs have a dose-dependent effect on H5N1-impaired AFC and APP. However, even with 10,000 cells, MSCs still mediate a significant decrease in the impairment of AFC and reduction of the increased APP, respectively (Fig. S3).

The mRNA expression of the major proinflammatory cytokines and chemokines IFN-β, interleukin (IL)-1β, RANTES, IP10, IL6, and IL8 was significantly greater in alveolar epithelial cells infected with H5N1 483/97 virus than in the cells infected with human influenza H1N1 54/98 ($P = 0.041$, 0.035, 0.006, 0.003, 0.031, and 0.038, respectively) or mock-infected ($P = 0.033$, 0.042, 0.004, 0.003, 0.024, and 0.034, respectively). Coculture of H5N1 virus-infected alveolar epithelial cells with MSCs significantly reduced IL-1β ($P = 0.035$), RANTES ($P =$

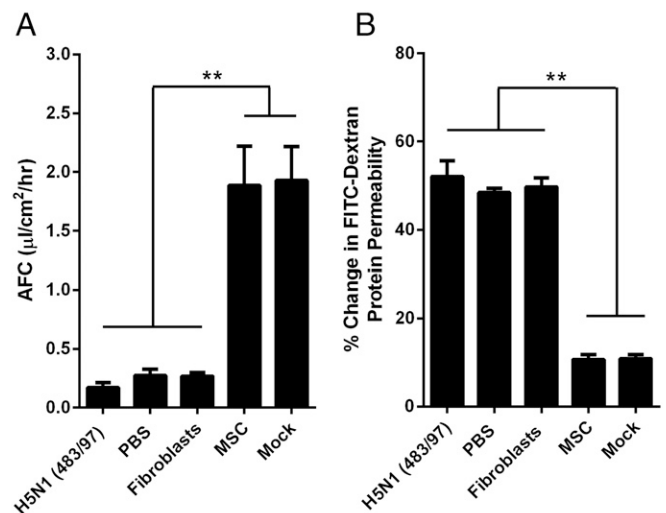

Fig. 2. MSCs prevent or reduce H5N1 virus impairment of alveolar epithelial AFC and APP. Alveolar epithelial cell monolayers on the apical chamber of transwell culture inserts were infected with H5N1 (A/HongKong/483/97) influenza virus (MOI = 0.1). Cells cocultured with MSCs in the basolateral chamber showed a significantly greater increase in net AFC (*A*) and decrease in APP (*B*) across the transwell inserts than did untreated infected cells, mock-cocultured cells, or cells cocultured with fibroblasts. Data represent the mean ± SE from three experiments. $^{**}P < 0.01$.

Fig. 3. H5N1 influenza virus infection of alveolar epithelium induces greater cytokine and chemokine expression and lower sodium and chloride transporter expression than does H1N1 virus infection whereas coculture with MSCs prevents or reduces the H5N1 effects. See Fig. 2 for experimental details. (*A*) Alveolar epithelial cells infected with HPAI H5N1 virus showed greater gene expression of proinflammatory cytokines (IL1-β; IL-6; IL-8) and chemokines (RANTES; IP-10) than did cells infected with H1N1 virus whereas coculture with MSCs reduced expression of cytokines and chemokines. (*B*) H5N1 virus infection down-regulated expression of the major sodium and chloride transporters (with the exception of α2-Na,K-ATPase and β1-Na,K-ATPase) to a greater extent than infection with H1N1 virus. Data are percent expression compared with that in mock-infected cells. (*C*) Western blot analysis showed reduced expression of CFTR and α1-Na,K-ATPase protein in H5N1 virus-infected alveolar epithelial cells at 24 h p.i. and further reduction of α1-Na,K-ATPase at 48 h p.i. With MSC coculture, expression of CFTR and α1-Na,K-ATPase was increased at 24 h p.i. and increased more substantially at 48 h p.i. Shown is mean ± SE protein expression relative to β-actin expression in three experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

0.024), IL6 ($P = 0.022$), IL8 ($P = 0.031$), and IP10 ($P = 0.006$) mRNA levels compared with the cells without MSC coculture (Fig. 3*A*).

Coculture with MSCs Prevents Down-Regulation of Sodium and Chloride Transporter Proteins in H5N1 Virus-Infected Alveolar Epithelial Cells. Major sodium and chloride transporter mRNA levels were decreased by H1N1 54/98 infection and decreased even more by H5N1 483/97 infection (Fig. 3*B*). These effects were confirmed in the protein expression of cystic fibrosis transmembrane conductance regulator (CFTR) (3.2 times less protein expression at both 24 h and 48 h p.i with H5N1, both $P = 0.006$) and α1Na, K-ATPase (3.1 times and 7.1 times less protein expression at 24 h and 48 h p.i. with H5N1, $P = 0.008$ and 0.006, respectively) (Fig. 3*C*). These H5N1-induced effects were prevented by coculture with MSCs at 48 h p.i, and expression of the transporter proteins CFTR and α1Na,K-ATPase was significantly enhanced ($P <$ 0.009 and 0.007, respectively) (Fig. 3*C*).

MSCs Cocultured with H5N1-Infected Alveolar Epithelial Cells Secrete Ang1 and KGF. Ang1 and KGF are paracrine soluble factors associated with the beneficial effects of MSCs in preclinical models (10). After coculture of MSCs with alveolar epithelial cells infected with H5N1 virus, Ang1 in MSC supernatants increased by a factor of 500 at 24 h p.i. and a factor of 1,015 at 48 h p.i., compared with levels in mock cocultures (both $P = 0.006$) (Fig. 4*A*). Similarly, KGF secretion by MSCs cocultured with H5N1 virus-infected alveolar epithelial cells was greater than that in mock cocultures by a factor of 253 ($P = 0.005$) at 48 h p.i. (Fig. 4*B*). These effects were not observed in MSCs cocultured with H1N1 virus-infected alveolar epithelial cells.

To assess the effect of Ang1 and KGF on the AFC and APP of H5N1 virus-infected alveolar epithelial cells, we selectively knocked down Ang1 and/or KGF protein in MSCs by using Ang1 and KGF siRNAs, separately or together. We cocultured H5N1 483/97-infected human alveolar epithelial cells on the transwell membrane with MSCs transfected with the siRNA or with scrambled siRNA as control on the transwell basolateral chamber. Pretreatment with Ang1 siRNA or KGF siRNA reduced the MSC-mediated increase of AFC by 35% and 30%, respectively, compared with that of mock or MSCs with scrambled siRNA-transfected ($P = 0.036$ and 0.042, respectively) (Fig. S4*A*). Similarly, MSC pretreatment with Ang1 siRNA or KGF siRNA reduced the MSC-mediated decrease of APP by 44% and 39%, respectively, compared with that of mock or MSC with scrambled siRNA-transfected ($P = 0.008$ and 0.007, respectively) (Fig. S4*B*). Further, siRNA knock-down of both Ang1 and KGF reduced the ability of MSCs to restore AFC and APP by 58% and 46%, respectively ($P = 0.027$ and 0.007, respectively) (Fig. S4). Conversely, addition of 100 ng/mL recombinant human (rh) Ang1 and KGF alone to H5N1 virus-infected alveolar epithelial cells in the absence of MSCs reduced the virus-mediated decrease of AFC by ~60% (Fig. S4*A*) and the increase of APP by ~56% (Fig. S4*B*). Therefore, MSC mediated the reduction of AFC and APP impairment in part via secreted Ang1 and KGF. However, these two factors together did not reduce H5N1-mediated lung injury to the extent achieved by MSC coculture, suggesting that other soluble factors or other mechanisms play a synergistic role.

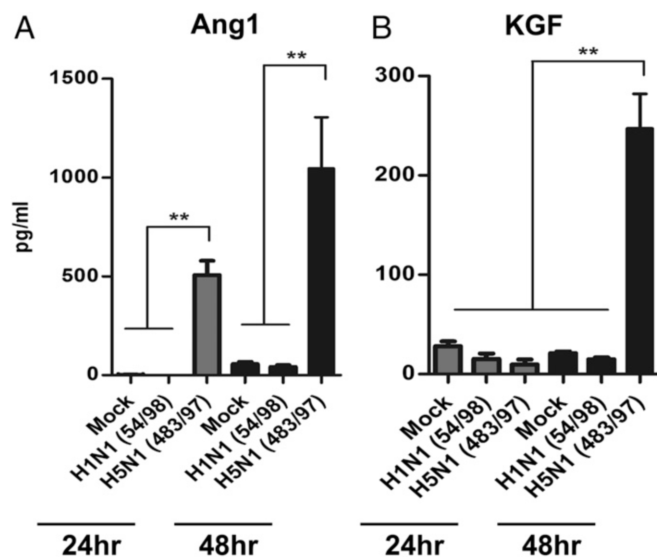

Fig. 4. Growth factor secretion by mesenchymal stromal cells is enhanced by coculture with H5N1 influenza virus-infected alveolar epithelial cells and contributes to reduction of AFC and APP impairment. Secretion of (*A*) Ang1 and (*B*) KGF by MSCs was increased significantly more by coculture with alveolar epithelium infected with H5N1 (A/HongKong/483/97) influenza virus vs. H1N1 (A/HongKong/54/98) virus, as measured by ELISA. Mock, mock infection. Data are the mean ± SE from three experiments. $^{**}P < 0.01$.

Effect of MSC Therapy in H5N1 Virus-Infected Mice. We assessed the effect of MSC treatment on the survival, body weight, lung alveolar protein permeability, alveolar fluid clearance, lung cellular infiltration, and lung pathology of H5N1 virus-infected mice. In young mice (6–8 wk of age) challenged with H5N1 486/97 virus and treated at day 5 p.i. with i.v. MSCs (5×10^5 cells in 100 μL), survival and weight loss were comparable with that with mouse fibroblast treatment (Fig. S5). However, survival ($P = 0.032$) (Fig. 5*A*) and body weight ($P < 0.05$) (Fig. 5*B*) were significantly greater in infected, MSC-treated aged mice (8–12 mo of age) than in aged controls, despite similar lung virus titers at days 7 and 10 p.i. (i.e., days 2 and 5 post-MSC treatment) (Fig. S6*A*).

Comparison of the wet-to-dry lung weight indicated the net effect of alveolar fluid clearance whereas extravasation of Evans blue dye was an indicator of fluid leakage into the alveolar spaces. MSC-treated mice had a significantly lower wet-to-dry lung weight than control mice at day 7 p.i. ($P = 0.023$) (Fig. 5*C*). A significantly lower concentration of Evans blue dye in the lungs of MSC-treated vs. control mice at day 10 p.i. ($P = 0.015$) (Fig. 5*D*) suggested that MSC treatment reduces vascular leakage of plasma protein. The total protein concentration in the bronchoalveolar lavage (BAL) fluid was significantly less in MSC-treated than in control mice at day 7 p.i. ($P = 0.004$) (Fig. 5*E*).

MSC treatment did not affect the total number of cells in the BAL fluid (Fig. S6*B*). However, the BAL fluid at day 7 p.i. showed significantly fewer $CD4^+$ T cells ($P = 0.008$) and NK cells ($P = 0.039$) and significantly more macrophages/monocytes ($P = 0.028$) after MSC treatment than after fibroblast treatment (Fig. 5*F*). After MSC treatment, the macrophage/monocyte population in the BAL fluid was predominantly alveolar macrophages with an M2 phenotype ($CD11c^+$ $Ly6C^{int}$ $F4/80^+$) (14) ($P = 0.023$) (Fig. 5*G*).

The concentrations of most cytokine and chemokine proteins tested (IP10, MCP-1, MCP-3, M1P-1 alpha, RANTES, IL-4, IL-17, and TNF-alpha) were significantly lower in the BAL fluid of MSC-treated mice than in that of fibroblast-treated mice at day 7 p.i. (Fig. S6*C*). CFTR protein expression was significantly higher in the lung lysates of MSC-treated mice than in the fibroblast-treated mice at day 10 p.i., as shown by Western blot analysis ($P = 0.007$, Fig. 5*H*).

Mice infected with the H5N1 influenza viruses with or without MSC treatment showed comparable lung pathology (Fig. 6*A*) and histopathology scores (Fig. 6*C*) at day 10 p.i., but MSC-treated mice had significantly less lung pathology at day 18 p.i. (Fig. 6*B*). The peribronchial, perivascular, and overall histopathology scores of the MSC-treated mice were significantly lower than the fibroblast-treated mice at day 18 p.i. ($P = 0.018$, 0.023, and 0.015, respectively) (Fig. 6*C*). MSC-treated and control mice did not seem to differ in alveolar epithelial or endothelial cell proliferation.

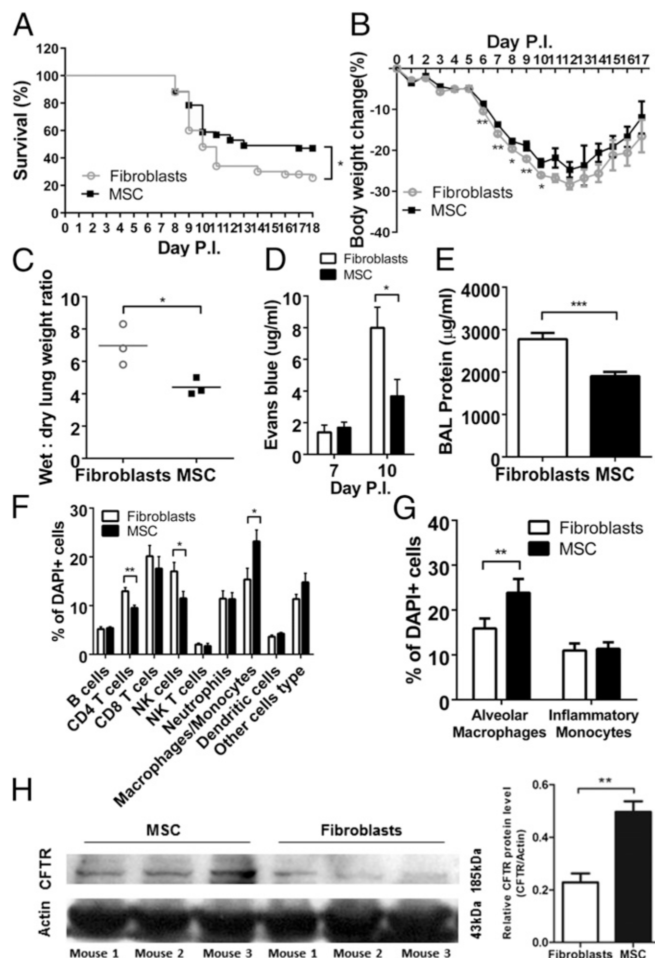

Fig. 5. Effects of treatment with MSC in an aged animal model of highly pathogenic H5N1 influenza virus infection. All experiments were carried out in aged (8–12 mo) mice inoculated with A/Hong Kong/486/1997(H5N1) virus and injected i.v. with 5×10^5 MSC/100 μL or mouse fibroblasts (control) on day 5 p.i. (*A*) Survival of mice injected with MSC ($n = 51$) or mouse fibroblasts ($n = 50$). Data are the means from three experiments. (*B*) Percent body weight change of mice injected with MSCs ($n = 79$) or mouse fibroblasts ($n = 83$). Data are the mean from three experiments. (*C*) Wet-to-dry lung weight ratio of mice treated with MSCs ($n = 3$) or mouse fibroblasts ($n = 3$) on day 7 p.i. (*D*) Lung tissue concentration of Evans blue dye at days 7 and 10 p.i. in mice treated with MSCs ($n = 4$) or mouse fibroblasts ($n = 4$). (*E*) Total protein concentration (indicating lung injury) at day 7 p.i. in BAL fluid from mice treated with MSCs ($n = 4$) or mouse fibroblasts ($n = 4$). (*F*) Immune cell profile of BAL fluid on day 7 p.i. from mice treated with MSCs ($n = 5$) or mouse fibroblasts ($n = 5$). (*G*) Mean (± SD) percentage of alveolar macrophages and inflammatory monocytes at day 7 p.i. in BAL fluid from mice treated with MSCs ($n = 5$) or mouse fibroblasts ($n = 5$). Counts were repeated at least twice. (*H*) CFTR transporter protein at day 10 p.i. in lung lysates of virus-infected mice injected with MSCs ($n = 3$) or mouse fibroblasts ($n = 3$). In all comparisons, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

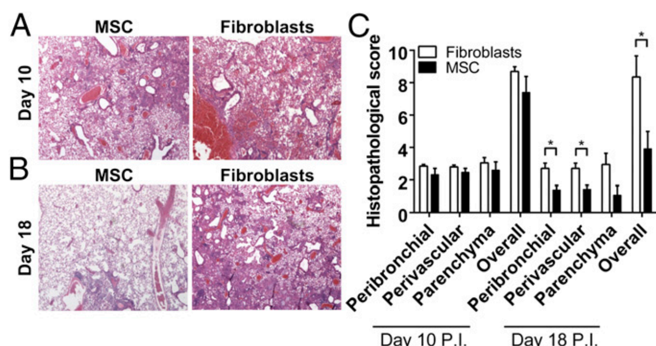

Fig. 6. Improved lung pathology and histopathology scores of aged mice treated with MSCs after inoculation with highly pathogenic H5N1 virus. (*A* and *B*) Lung tissue at (*A*) day 10 p.i. and (*B*) day 18 p.i. from H5N1 virus-infected mice after treatment with MSCs ($n = 4$) or mouse fibroblasts ($n = 4$) on day 5 p.i. Hematoxylin/eosin stain. (Magnification: 100×.) (*C*) Histopathology scores of peribronchiolar, perivascular, and parenchymal lung tissues at days 10 and 18 p.i. from influenza virus-infected mice treated with MSCs or mouse fibroblasts. $*P < 0.05$.

Discussion

Here, we demonstrated that soluble mediators released from influenza A H5N1 virus-infected alveolar epithelial cells impair alveolar fluid clearance and protein permeability by down-regulating alveolar sodium and chloride transporter proteins. This pathology was prevented or reduced by coculture with MSCs in vitro and by MSC treatment in vivo in aged mice.

Highly pathogenic H5N1 and H7N9 influenza viruses caused markedly greater impairment of AFC and APP in cultured human alveolar epithelial cells than did the seasonal H1N1 and H3N2 influenza viruses. This effect was caused largely by soluble mediators and growth factors released by H5N1 or H7N9 virus-infected, but not H1N1-infected, alveolar epithelial cells rather than by direct viral cytopathic effects or viral replication competence alone. Infection with H5N1, compared with seasonal H1N1, influenza virus also led to greater down-regulation of the Na,K-ATPase and CFTR transporter proteins in alveolar epithelium. Influenza virus infection was previously shown to impair alveolar fluid transport by inhibiting epithelial sodium channel activity through interaction of viral hemagglutinin (15) and matrix (16) proteins with the epithelial sodium channel. To our knowledge, ours is the first demonstration of impaired alveolar fluid clearance and protein permeability due to soluble mediators released by infected alveolar epithelium.

Alveolar fluid clearance was previously found to be impaired in patients with sepsis-induced acute injury; a similar in vitro experimental model of *Escherichia coli* endotoxin-induced acute lung injury showed that alveolar fluid clearance was impaired via proinflammatory cytokines and chemokines (10), demonstrating the physiological relevance of the model we used. In our study, this model comprised alveolar epithelium infected with the highly pathogenic H5N1 viruses that induce higher levels of proinflammatory cytokines and chemokines than does seasonal H1N1 virus. Activation of proinflammatory pathways was previously shown to result in down-regulation of sodium and chloride transporters responsible for vectorial fluid transport across the alveolar epithelium (17), increasing paracellular protein permeability. To our knowledge, ours is the first demonstration of this phenomenon in an experimental model of virus-infected alveolar epithelium.

We found that when MSC expression of the growth factors Ang1 and/or KGF was knocked down by siRNA, the MSCs were less able to attenuate the effects of H5N1 virus infection on alveolar AFC and APP. However, recombinant Ang1 and KGF only partially restored the attenuation of pathology. Therefore, secreted Ang1 and KGF account only partially for the therapeutic effect of MSCs on alveolar epithelial AFC and APP.

When mice were infected with H5N1 virus, MSC therapy increased survival and reduced weight loss only among the aged mice. At day 18 p.i., these mice had fewer lung lesions and a greater number of bronchoalveolar antiinflammatory M2 macrophages, which also enhance tissue repair (18), and proinflammatory cytokine and chemokine levels were low. However, their lung virus titers remained comparable with those in untreated mice; therefore, the observed phenotype did not reflect a direct antiviral effect of MSCs.

MSCs, alone or as an adjunct to antiviral therapy, were recently reported not to improve the survival of 7- to 10-wk-old C57BL/6 mice inoculated with A/PuertoRico/8/34 (mouse-adapted H1N1) or A/Mexico/4108/2009 (pandemic H1N1) influenza virus (13, 19). Our findings are similar; MSCs failed to improve survival (Fig. S5) or histopathology in young (6–8 wk of age) mice inoculated with H5N1 influenza viruses. MSC treatment was beneficial only in aged mice (8–12 mo of age). Age affects various MSC functions, including expression and secretion of soluble factors important in recovery from lung injury (20). Aged animals show lower expression of genes involved in cell activation and migration and of cytokine receptors (e.g., TNFR1 and TNFR2) and chemokine receptors (CCR7, CX3CR1, and CXCR5) involved in MSC migration and chemotaxis (21). Thus, younger animals have more potent endogenous MSC responses (22) whereas exogenous MSC therapy is more likely to exert an apparent benefit in older animals.

Age-related loss of lung repair capacity may also explain the progressive age-related increase in the clinical severity of severe acute respiratory syndrome (SARS) (23, 24) and pandemic H1N1 virus infection (25) and the higher mortality reported in older patients with acute respiratory distress syndrome (26). Age also determines the severity of SARS in both mouse and macaque experimental models (27, 28), and aged mice were more susceptible to exogenous endotoxin (LPS) in an animal model of sepsis-induced kidney injury (29). Although age-related changes may account for some of these findings, there may be other factors as well. We hypothesize that, in the context of severe influenza, aged and young mice differ in the mechanisms of both lung pathology and repair. As we previously reported, the pathogenic mechanisms of H5N1 viruses differ in important ways from the seasonal or pandemic H1N1 influenza viruses (30, 31). Likewise, the pathogenic mechanisms and the potential benefits of MSC therapy very likely depend on factors relating to both the virus and the host.

We did not investigate the effect of combining MSC therapy with antiviral therapy, as would be done clinically. Our primary interest at this stage was the impact of MSC therapy on the pathogenesis of severe influenza, which was best shown by using MSC therapy alone. Future studies should investigate the synergy of MSC with antivirals and the mechanisms underlying the interaction of age with the effects of MSC therapy.

In summary, infection with highly pathogenic H5N1 and H7N9 influenza viruses dysregulated alveolar fluid clearance and protein permeability in vitro, as observed in humans; these effects were mediated by infected cells' release of soluble factors that down-regulate the sodium and chloride transporters. The ability of highly pathogenic influenza virus to modulate AFC and APP may provide useful parameters for assessing virulence. In vivo, MSC therapy diminished lung injury and increased survival in aged H5N1-infected mice. These preclinical data suggest that MSCs may provide a therapeutic benefit to humans with severe pulmonary illness caused by influenza viruses such as H5N1 and H7N9, especially older patients, who are at higher risk. The identification of alveolar epithelial sodium and chloride transporters as key pathogenic targets and the modulation of these pathogenic mechanisms by factors released by MSCs suggest additional

therapeutic possibilities for the clinical management of viral acute lung injury.

Materials and Methods

Detailed descriptions of experiments are provided in *SI Materials and Methods*.

Influenza A Viruses. The H5N1 influenza A viruses used were A/HongKong/483/97 (483/97), A/HongKong/486/97 (486/97), and A/Vietnam/3046/04 (3046/04); all were highly pathogenic viruses. The H7N9 influenza A virus was A/Shanghai/2/2013 (Sh2). The seasonal influenza A viruses were H1N1 [A/HongKong/54/98 (54/98) and A/Oklahoma/447/08 (OK447/08)] and H3N2 [A/HongKong/1174/99 (1174/99) and A/Oklahoma/370/05 (OK370/05)]. All viruses were human isolates. They were grown in Madin–Darby canine kidney (MDCK) cells and then aliquotted and stored at −80 °C. Viruses were titrated in MDCK cells [50% tissue culture infective dose ($TCID_{50}$)].

Acute Lung Injury Coculture Model. A previously described in vitro model (Fig. 1) (7) was adapted for use in a biosafety level-3 (BSL-3) laboratory and used to measure net AFC and APP during influenza virus infection. Alveolar epithelial cells were isolated from resected, nonmalignant lung tissues at Queen Mary Hospital, Hong Kong. The study was approved by the Institutional Review Board of the University of Hong Kong (IRB reference no. UW 09-394) and Hospital Authority, Hong Kong West Cluster. The cells were plated on 24-well transwell insets to establish an air–liquid interface. Net AFC was measured by adding FITC-labeled dextran (Sigma) to the cells 24 h after virus infection [multiplicity of infection (MOI) = 0.1] or after incubation with virus-free conditioned medium for 1 h. Cytomix (mixed proinflammatory cytokines in buffer; R&D Systems) served as positive control.

Bone marrow-derived human MSCs obtained from the Texas A&M Health Science Center were added to the basolateral chamber of the transwell to assess their effect on AFC and APP; human lung fibroblasts were used as controls. To assess the roles of the MSC-secreted soluble growth mediators Ang1 and KGF, MSCs were reverse-transfected with 300 nM Ang1 (121280; Ambion) and/or 100 nM KGF (10818; Ambion) silencing RNA (siRNA) duplex or with scrambled siRNA (negative control). The recombinant human proteins, 100 ng/mL rhAng-1 and 100 ng/mL rhKGF (R&D Systems), were used to compare the effect of defined soluble proteins on H5N1 virus-impaired AFC and APP.

Mouse Studies. Female Balb/C mice aged 6–8 wk (young) or 8–12 mo ("aged") were inoculated intranasally with 10^6 $TCID_{50}$ of 486/97 influenza virus in 25 μL. At day 5 p.i., mice were injected i.v. with 5×10^5 human bone marrow-derived MSCs in 100 μL. NIH 3T3 mouse embryo fibroblasts (5×10^5) in 100 μL (ATCC) served as control. MSCs do not express major histocompatibility antigens and do not elicit a rejection response, and human MSCs have been used in previous studies on endotoxin-induced acute lung injury models (10). Survival and body weight were monitored for 18 d. At day 7 p.i., virus was titrated in three mouse lungs per group (MSC-treated, fibroblast-treated groups). BAL fluid was collected on day 7 p.i. for cytokine assay and immune cell profiling (*SI Materials and Methods*). At day 10 p.i., three mice per group were killed for measurement of pulmonary microvascular permeability and CFTR protein expression. On days 10 and 18 p.i., four mouse lungs per group were fixed for histopathology. The animal infection experiments were approved by the University of Hong Kong Animal Ethics Committee (CULATR 3445-14).

Measurement of Cytokines, Sodium and Chloride Transporter Proteins, and MSC-Secreted Soluble Proteins. Total RNA and total cellular protein were extracted from virus-infected alveolar epithelial cells or mouse lungs. Cytokines, major transporter proteins, and Ang1 and KGF soluble proteins secreted by MSCs were measured as described in *SI Materials and Methods*.

Statistical Analysis. Mean values from three independent experiments were compared by unpaired two-tailed *t* tests. One-way analysis of variance with a post hoc Bonferroni test was used as appropriate. Differences were considered significant at $P < 0.05$. All statistical analyses used GraphPad Prism version 6.0 software.

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