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Therapeutic potential of porcine bronchoalveolar fluid-derived mesenchymal stromal cells in a pig model of LPS-induced ALI

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Abstract

In this study, we isolated mesenchymal stromal (stem) cells (MSCs) from broncho-alveolar lavage fluid (BAL) of 2-6 week-old commercial pigs. BAL-MSCs displayed fibroblastic morphology and possessed self-renewal properties. Similar to bone-marrow MSCs, BAL-MSCs expressed mesenchymal markers and both cell types lacked the expression of hematopoietic markers. BAL-MSCs, when cultured in differentiation induction media, differentiated into adipocytes, osteocytes and chondrocytes. Next, we examined if BAL-MSCs have the ability to treat lipopolysaccharide (LPS)-induced acute lung injury (ALI) in a pig model. Five-week-old commercial pigs were inoculated intra-tracheally with *E. coli* LPS [(1 mg/kg body weight (b.wt.)). Twelve hours after the LPS inoculation, groups of pigs were inoculated intra-tracheally with BM-MSCs or BAL-MSCs (2×10^6 cells/kg b.wt.). Forty eight hours after the cells administration pigs were euthanized and neutrophils in BAL, lung lesions, and cytokines in lung lysates, and engraftment of MSCs in lungs were examined. Engraftment of BAL-MSCs in injured lungs was significantly higher than the BM-MSCs, however, both cell types were equally effective in attenuating LPS-induced ALI as evidenced by decreased inflammation, lung lesions and proinflammatory cytokines in the lungs of pigs treated with BAL-or BM-MSCs. These data in a preclinical large animal model suggest that BAL-MSCs may be used in clinical settings to treat ALI in humans.

Keywords

Lung mesenchymal stem cells; acute lung injury; Stem cell therapy; pig model

1 INTRODUCTION

More than 400,000 people in the US die each year due to respiratory diseases. In 2009, the cost of providing healthcare related to all respiratory conditions, excluding lung cancer, was \$113 billion (Schraufnagel, 2010). Acute lung injury (ALI), and its more severe form the acute respiratory distress syndrome (ARDS), is a significant cause of morbidity and mortality in critically ill patients. ALI/ARDS in humans is characterized by a severe acute

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

inflammatory response in the lungs and is associated with mortality ranging from 40% to 60%. Every year more than 150,000 new cases of ARDS are reported in the US alone (Johnson and Matthay, 2010). Current treatment for ALI is only supportive and there is need for improved treatment options.

Several studies in animal models of ALI/ARDS have shown beneficial effects of mesenchymal stem (stromal) cells (MSCs) administration in treating ALI/ARDS (Fang et al., 2010; Gupta et al., 2007; Lee et al., 2009; Mei et al., 2007; Xu et al., 2008; Xu et al., 2007). The beneficial effects of MSC therapy in ALI models include increased survival, significantly decreased lung damage and also decreased levels of proinflammatory cytokines in lungs.

MSCs are plastic adherent multipotent cells that also possess self-renewal and immunomodulatory properties. Initially MSCs were isolated from the bone marrow (BM), but later on it was discovered that MSCs exist in all tissues including the lungs (da Silva Meirelles et al., 2006; Lama et al., 2007; Ricciardi et al., 2012). MSCs express mesenchymal markers such as CD44, CD90 and CD105 but lack the expression of hematopoietic markers, CD34 and CD45. MSCs also show reduced expression of major histocompatibility and co-stimulatory molecules (Le Blanc et al., 2008). Thus, allogenic MSCs have been successfully used as therapy in animal disease models and in clinical settings. Due to their extensive proliferative capacity, differentiation and immunoregulatory properties, MSCs are attractive as cellular therapy for inflammatory and autoimmune diseases, and regenerative medicine. Currently, MSCs are being investigated in 756 clinical trials as a therapy for several human conditions. (<https://clinicaltrials.gov/ct2/results?cond=&term=mesenchymal+stem+cells&cntry1=&state1=&recrs=>)

Previously we demonstrated that MSCs can be isolated from the lungs of pigs (Khatri et al., 2015). Similar to human lung MSCs and pig BM-MSCs, pig lung MSCs express mesenchymal markers such as CD44, CD73, CD90, and CD105, and do not express hematopoietic markers, CD34, CD45 (Khatri et al., 2015; Ricciardi et al., 2012). Pig lung MSCs were multipotent and differentiated into adipocytes, osteocytes and lung epithelial cells. Pig lung MSCs also had immunomodulatory properties and inhibited T cell proliferation and production of IFN γ and TNF- α by T cells and dendritic cells, respectively (Khatri et al., 2015).

In this manuscript, we isolated and characterized MSCs derived from bronchoalveolar lavage fluid (BAL) of pigs and examined their therapeutic potential in treating *E. coli*/LPS-induced ALI in a pig model. Pigs closely resemble humans in terms of anatomy, physiology and immunology. Therefore, pigs are a useful model for several human diseases. (Cozzi et al., 1997; Ibrahim et al., 2006; Rogers et al., 2008a; Rogers et al., 2008b; Saif et al., 1996; Yuan and Saif, 2002). Additionally, body size of pigs is also similar to humans and their life span is long enough to examine the therapeutic efficacy, safety and long-term effects of stem cell transplantation (Chang et al., 2008; Lim et al., 2010; Shake et al., 2002; Sonoyama et al., 2006; Zhou et al., 2011). Our data demonstrate that BAL-MSCs possess self-renewal and differentiation properties and are equally efficient as BM-MSCs in treating endotoxin-induced ALI in a pig model.

2 MATERIALS AND METHODS

2.1 Isolation of porcine BAL-MSCs

Lungs and bone marrow were harvested from 2-6 week-old conventional Large White-Duroc crossbred pigs obtained from the OSU herd (n=6). MSCs from BM were isolated as described earlier (Khatri et al., 2009; Khatri and Saif, 2013). Lungs were harvested from the pigs and BAL cells were collected as described previously (Khatri et al., 2010). BAL cells were counted by trypan blue dye exclusion method and suspended in Dulbecco's Modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Atlanta Biologicals) and 1% antibiotics (C-DMEM). One million BAL cells were seeded in T-25 cm² flasks (Nunc). After 48 hours (hrs), non-adherent cells were removed and cells were fed with fresh medium every 3–4 days until cells were 80-90% confluent approximately in 14-18 days. Confluent monolayers of MSCs were sub-cultured using 0.25% trypsin-EDTA (Gibco). MSCs were passaged at least 2 times to remove contaminating alveolar macrophages and MSCs passaged between 2 and 5 times were used for *in vitro* characterization and pig experiments.

We determined the self-renewal potential of BAL-MSCs by colony forming unit-fibroblast (CFU-F) assay as described previously (Khatri et al., 2009). For this, 200 BAL-MSCs were plated in each well of a 6-well plate. The cells were cultured in C-DMEM for 14 days and medium was changed every 3–4 days. After the incubation, cells were washed and stained with Wright-Giemsa stain (Fisher Diagnostics). Colonies greater than 3 mm and consisting of approximately 50 cells were counted.

2.2 BAL-MSCs phenotyping by flow cytometry

The expression of mesenchymal markers (CD29, CD44, CD90 and CD105) and hematopoietic markers (CD34 and CD45) on BAL-and BM-MSCs was examined by flow cytometry (Khatri et al., 2010; Khatri et al., 2015). BM-MSCs were included as controls. The following primary antibodies specific to pig or cross-reactive human antibodies were used: mouse anti-pig CD29 (IgG1), mouse anti-pig CD44 (IgG2b), mouse anti-pig CD90 (IgG1, VMRD), mouse anti-pig CD45 (IgM, VMRD), mouse anti-pig/human CD105 (IgG2a, GeneTex), and FITC Mouse Anti-human CD34 (IgG1, BD Biosciences). MSCs were incubated with predetermined dilution of these antibodies for 30 min at 4° C. After the incubation, cells were washed and stained with specific secondary antibodies. Cells were then acquired using a FACS Aria II (BD Biosciences) flow cytometer and analyzed using FlowJo (Tree Star, Ashland, OR, USA) software (Khatri et al., 2010).

2.3 Differentiation of BAL-MSCs into adipocytes, chondrocytes and osteocytes

In vitro differentiation ability of culture expanded BAL-MSCs into adipocytes, chondrocytes and osteocytes was examined as previously described (Khatri et al., 2009). For adipogenesis, BAL-MSCs were cultured in adipogenic induction medium consisting of C-DMEM, 1 µM dexamethasone, 10 µg/ml insulin, and 0.2 mM indomethacin for 2 weeks. The cells were fed with fresh medium every 3-4 days. Generation of lipid droplets indicative of adipogenesis was detected by 0.3% Sudan III staining.

To induce the differentiation of BAL-MSCs into osteocytes, these cells were cultured in C-DMEM, 100 nM dexamethasone, 10 mM β -glycerophosphate and 0.05 mM L-ascorbic acid-2-phosphate for 3 weeks. Calcium deposition in differentiated osteocytes was detected by Alizarin S staining (Khatri et al., 2009).

For chondrogenic differentiation, BAL-MSCs were incubated for 3 weeks in C-DMEM medium supplemented with 50 mM ascorbic acid, 0.5 mg/ml insulin (Sigma) and 10 ng/ml recombinant human transforming growth factor- β 1 (Life Technologies). Cultures were fed with fresh medium every 3-4 days. Sulfated glycosaminoglycans in differentiated cultures were detected by 1% Alcian blue staining.

2.4 Experimental design to examine the therapeutic effects of BAL-MSCs in LPS-induced ALI in pigs

Five-week-old conventional Large White-Duroc crossbred pigs were obtained from the OSU herd. Maintenance of pigs and all experimental procedures were conducted in accordance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University (Protocol#2014A00000040).

Twelve 5 week-old pigs were divided into 4 groups. Groups 1-3 (n=3 pigs each) were inoculated intratracheally with *E. coli* LPS (1 mg/kg bw) and pigs in group 4 (n=2) were included as controls and were inoculated intratracheally with DMEM (Khatri et al., 2010). Twelve hrs after LPS-inoculation, pigs in group 1 and 2 were administered intratracheally with BM-MSC (2×10^6 /kg bw) and BAL-MSC (2×10^6 /kg bw) respectively and pigs in group 3 were similarly inoculated with DMEM. Forty eight hrs after the MSC administration, pigs were euthanized and lungs were obtained. Gross lung pneumonic lesions in whole lung were examined and BAL was collected as described previously (Khatri et al., 2010; Thomas et al., 2015). Smears were prepared from BAL cells and neutrophils were counted after staining the slides with Wright-Giemsa stain. Lung lysates prepared from left lung were used for the detection of cytokines by ELISA. Whole right lung was collected in 10% buffered formalin for histological analysis. Paraffin embedded sections were examined for bronchiolar epithelial changes, peribronchiolar inflammation and interstitial pneumonia and lesions were scored from 0-3 as described previously (Jung et al., 2009; Richt et al., 2003).

2.5 Detection of cytokines in lungs

Inflammatory cytokines, TNF- α and CXCL10 levels in lung lysates of pigs were examined by ELISA as described previously (Khatri et al., 2010).

2.6 Labeling of MSCs with PKH-26 dye

To track the engraftment of BM- and BAL-MSCs in the lungs of pigs, these cells were labeled with lipophilic fluorescent dye, PKH-26 (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions. Approximately 99% of MSCs were labeled with PKH-26 red dye as determined by examination under the fluorescent microscope (Olympus, Japan).

2.7 Engraftment of PKH-26 labeled MSCs in lungs and their transdifferentiation into lung epithelial cells

Eleven 5 week-old pigs were divided into 4 groups as described above. Groups 1-3 (n=3 pigs each) were inoculated intratracheally with *E. coli* LPS (1 mg/kg bw) and pigs in group 4 (n=2) were included as controls and were inoculated intratracheally with DMEM. Twelve hrs after LPS-inoculation, pigs in group 1 and 2 were administered intratracheally with PKH-26 labeled BM-MSCs (2×10^6 /kg bw) and BAL-MSCs (2×10^6 /kg bw) respectively and pigs in group 3 were similarly inoculated with DMEM. Forty eight hrs after the LPS inoculation, pigs were euthanized and lungs were harvested and frozen sections were prepared. Engraftment of MSCs in lungs was determined by detecting PKH-26 labeled (red) MSCs in sections under a fluorescent microscope (Olympus, Japan) and cells in five microscopic fields were counted for each animal (x40).

To examine the transdifferentiation of administered MSCs into lung epithelial cells, frozen lung sections were stained using rabbit anti-human surfactant protein C [(SPC; a type II pneumocyte marker, Millipore)] antibody to detect type II epithelial cells and cells showing double staining for PKH-labeled MSC (red) and SPC positive cells (green) were examined. To confirm the mesenchymal phenotype of PKH-labeled MSC, engrafted MSCs in lungs were also stained with mesenchymal markers (CD44).

2.8 Statistical analysis

A two-tailed Student *t*-test or One-way ANOVA followed by Tukey test was used to compare significant differences between groups. A P value of $P < 0.05$ was considered statistically significant.

3 RESULTS

3.1 Characteristics of porcine BAL-MSCs

We isolated MSCs from BAL fluid of 2-6 week-old SPF pigs (n=6). Isolated BAL-MSCs showed the ability to adhere to plastic surface and showed MSC-specific fibroblast like morphology (Fig. 1A). To examine the frequency of MSCs in BAL cells, we plated one million BAL cells in T25 cm² flask. After 14-18 days of incubation, we observed 40 ± 22 (n=5) MSC colonies per one million BAL cells, indicating that MSCs constitute approximately 0.004% of total BAL cells in pigs.

Next we examined the self-renewal ability of BAL-MSCs by CFU-F assay. We cultured 200 BAL-MSCs in each well of 6-well plate. After 14 days of incubation MSCs formed 154 ± 28 (n=3) colonies indicating that BAL-MSCs possess self-renewal ability (Fig. 1B). In Fig. 1C morphology of a typical MSC colony comprising of a cluster of fibroblast-like cells after 14 days of culture is shown.

3.2 Expression of mesenchymal markers on Porcine BAL-MSCs

To confirm the mesenchymal lineage of BAL-MSCs, we examined the surface expression of mesenchymal and hematopoietic markers on BAL-MSCs by flow cytometry. BM-MSCs are known to express mesenchymal markers and were included as positive controls. BM- and

BAL-MSCs expressed mesenchymal markers (CD29, CD44, CD90 and CD105) but expression of hematopoietic markers CD34 and CD45 was not detected on either of MSCs confirming their mesenchymal lineage (Fig. 2A and B).

3.3 Differentiation of BAL-MSCs into adipocytes, osteocytes and chondrocytes

Classical MSCs demonstrate ability to differentiate into multiple cell types. We examined if BAL-MSCs are multipotent and can differentiate into adipocytes, osteocytes and chondrocytes when cultured in differentiation medium.

BAL-MSCs differentiated into adipocytes as evidence by the detection of red lipid droplets in the cytoplasm of differentiated adipocytes upon staining with Sudan III (Fig. 3A). BAL-MSCs cultured in chondrogenic induction medium formed compact homogeneous structures and deposition of proteoglycans in differentiated cultures was demonstrated by Alcian blue staining (Fig. 3C). Osteocyte differentiation was evident by the presence of tightly packed nodule-like structures and by the detection of calcium deposition by Alizarin S staining (Fig. 3E).

3.4 Effect of MSCs administration on attenuation of LPS-induced ALI in pigs

To induce ALI, pigs were inoculated intratracheally with 1 mg/kg of *E. coli* LPS. Twelve hrs after LPS inoculation, pigs were intratracheally administered with BM-or BAL-MSCs (2×10^6 cells/kg) and lungs were examined 48 hrs after MSCs administration. Significant increase ($P < 0.05$) in percent of neutrophils was observed in BAL cells of LPS-inoculated pigs as compared to control pigs and BM-or BAL-MSC treatment caused significant decrease ($P < 0.05$) in neutrophils in the BAL cells (Fig. 4). Pigs inoculated with LPS showed red or dark red areas of consolidation in cranial and middle lobes. No gross lung lesions were visible in control, BAL-and BM-MSCs treated pigs (Fig. 5).

Microscopically, lungs of pigs inoculated with LPS showed marked infiltration of neutrophils and macrophages in the alveoli, thickening of interalveolar septa due to infiltration of macrophages and neutrophils indicative of interstitial inflammation, infiltration of neutrophils in the bronchi, and lymphocytes and plasma cell infiltration in the periphery of the bronchi was observed (Fig. 6). Mild infiltration of inflammatory cells in alveoli and bronchi was observed in the lungs of pigs inoculated with LPS and treated with BM-or BAL-MSCs (Fig. 6). No microscopic lesions were observed in the lungs of control pigs. We also determined if BM-and BAL-MSCs transplantation altered the production of inflammatory cytokine in lungs. Significantly less ($P < 0.05$) production of TNF- α and CXCL10 in lung lysates of pigs injured with LPS and treated with BM- or BAL-MSCs as compared to only LPS-inoculated pigs was observed. There was no significant difference ($P > 0.05$) in the levels of inflammatory cytokines in lung lysates of pigs transplanted with BM-or BAL-MSCs (Fig. 7). These findings indicated that similar to BM-MSCs, BAL-MSCs possessed immunoregulatory properties and decreased infiltration of inflammatory cells and production of proinflammatory cytokines in LPS-induced ALI.

3.5 Engraftment and trans-differentiation of BM- and BAL-MSc in LPS-injured lung

To monitor the engraftment of transplanted MSCs in lungs we labeled MSCs with PKH-26 dye. MSCs were administered intratracheally 12 hrs after LPS inoculation. Pigs were euthanized 48 hrs after MSCs administration and PKH-labeled MSCs were detected in lung sections of pigs (Fig. 8A). The mean number of engrafted BAL-MSCs in LPS-injured lungs was significantly higher ($P<0.05$) than the BM-MSCs (Fig. 8B)

Next we examined if engrafted MSCs differentiated into lung epithelial cells. Type II lung epithelial cells were stained in lung sections to determine if they differentiated from PKH-26 labeled transplanted MSCs. As shown in Fig. 9, engrafted BM- and BAL-MSCs did not differentiate into type II epithelial cells as evidenced by absence of double stained MSCs (red) and type II epithelial cells (green).

4 DISCUSSION

In this study, we isolated multipotent MSCs from the BAL fluid of pigs. Similar to BM-MSCs, porcine BAL-MSCs expressed mesenchymal markers and hematopoietic stem cell markers were not detected on their surface. BAL-MSCs also possessed self-renewal and differentiation properties and differentiated into adipocytes, chondrocytes and osteocytes. Importantly, similar to BM-MSCs, BAL-MSCs demonstrated the ability to attenuate LPS-induced ALI in pigs and showed *in vivo* anti-inflammatory properties.

Previously, MSCs have been isolated from human lungs (Ricciardi et al., 2012; Rolandsson Enes et al., 2016) and BAL (Lama et al., 2007) and pig lungs (Khatri et al., 2015). Similar to BM-MSCs and human and mouse BAL-MSCs, porcine BAL-MSCs expressed mesenchymal markers and lacked the expression of hematopoietic markers (Lama et al., 2007; Tong et al., 2016). Additionally, like MSCs from other tissues, porcine BAL-MSCs were multipotent and differentiated into adipocytes, chondrocytes and osteocytes.

Bone marrow-derived MSCs are very well characterized and have been extensively studied as a therapy for several human conditions in animal models and in human clinical trials. However, the isolation of MSCs from bone marrow involves painful invasive procedure whereas collection of BAL is comparatively less invasive and BAL-MSCs were recently shown to express lung specific FOXF1 and HOXB5 genes which were absent in BM-MSc (Rolandsson Enes et al., 2016). Our data indicate that the frequency of MSCs (0.004%) in BAL cells is comparable to the frequency of MSCs (0.001%) in bone marrow cells. The BAL-MSCs also possess all the properties of MSCs as defined by the International Society for Cellular Therapy such as expression of mesenchymal markers, self-renewal, differentiation and immunoregulatory properties. Additionally, *in vitro* studies indicated that lung-specific MSCs possess higher ability to differentiate into lung epithelial cells as compared to BM-MSCs (Ricciardi et al., 2012). Our results of this study demonstrating comparable efficacy of BAL-MSCs to BM-MSCs in treating ALI and additional publication (Tong et al., 2016) showing superior therapeutic efficacy of BAL-MSCs as compared to BM-MSCs in treating ALI in mice model, suggest that BAL-MSCs may be effective in treating acute lung inflammatory diseases in humans.

In this study, the engraftment of BAL-MSCs was significantly higher than BM-MSCs. However, both cell types were equally effective in attenuating LPS-induced lung injury in pigs despite the significant difference in the number of engrafted cells. Numerous studies demonstrated that the beneficial effects of BM-MSC transplantation are due to the release of paracrine factors as few BM-MSCs engraft at the site of tissue injury (Rasmusson, 2006). However, Badri et al, (Badri et al., 2011) demonstrated that human lung MSCs engrafted and persisted in mice lung up to 6 months after transplantation suggesting that engraftment of lung specific MSCs in lungs is higher than BM-MSCs as also observed in our study. Since BM-MSCs were effective in treating ALI in our pig model, we assume that the number of engrafted BM-MSCs, even though significantly less than the BAL-MSCs, and likely soluble factors released by these cells were sufficient enough to mediate beneficial effects of these cells in our pig model of ALI. Both BM-and BAL-MSCs also demonstrated immunoregulatory properties and inhibited infiltration of inflammatory cells and decreased the production of inflammatory cytokines in the lungs of pigs treated with MSCs.

While examining the transdifferentiation of transplanted BM- and BAL-MSCs into lung epithelial cells, in our ALI pig model, neither BM- or BAL-MSCs differentiated into lung epithelial cells. Differentiation potential of MSCs is known to be governed by their tissue origin and we were expecting that because of familiar milieu, engraftment of BAL-MSCs will be higher in injured lung and engrafted MSCs may differentiate into lung epithelial cells. Ricciardi et al, (Ricciardi et al., 2012) demonstrated in *in vitro* studies that compared to BM-MSCs, lung-MSCs possess greater ability to differentiate into lung epithelial cells. Additionally, in mouse model, Badri et al (Badri et al., 2011) demonstrated long term engraftment of human lung MSCs in normal murine lung and showed that the engrafted cells communicate with resident bronchial and alveolar epithelium cells by gap junction formation. Although it is well established that BM-MSCs mediate their beneficial effects by the release of soluble factors independent of their engraftment or differentiation to repair tissue injury (Rasmusson, 2006), a few reports have also shown that, in animal models of lung injury administered BM- and umbilical cord blood-derived MSCs differentiated into lung epithelial cells (Sueblinvong et al., 2008; Wang et al., 2005). In current study, BAL-MSCs engraftment was indeed higher than BM-MSCs, however, we speculate that the duration of experiment was too short to observe differentiation of engrafted MSCs into lung epithelial cells. In future studies we will examine the therapeutic efficacy, engraftment, immunoregulation and, proliferation and differentiation of BAL-MSCs into lung epithelial cells in COPD and other chronic lung disease models where experiment duration will be longer than the current study.

We used pigs as a model to examine therapeutic potential of MSCs in ALI. Pigs are ideal as preclinical animal model for human diseases as pigs very closely resemble to humans in anatomy, physiology, immunology, metabolism, and pharmacokinetics (Soerensen et al., 2012). Moreover, the pathogenesis of ALI and several other human diseases in pigs is identical to that observed in humans. These properties make pig an excellent model to examine the therapeutic potential, safety, toxicity, dosing and engraftment of MSCs (Casal and Haskins, 2006). In this study, utilizing the pig model of LPS-induced ALI we demonstrated that (i) BAL-MSCs were effective as a therapy for the treatment of LPS-induced ALI (ii) BAL-MSCs were as effective as BM-MSCs in treating ALI (iii) the

engraftment of BAL-MSCs in LPS-injured lung was higher than BM-MSC (iv) MSC dose of 2×10^6 cells/kg was effective as therapy and (v) allogenic MSCs were safe to use. These preclinical data may be valuable in designing future human clinical trials.

In summary, we isolated MSCs from the BAL of pigs. Isolated MSCs possessed self-renewal and differentiation properties and were therapeutically effective in treating LPS-induced ALI a pig model. Our data demonstrating the therapeutic potential of BAL-MSCs in treating ALI in large animal model may help in designing human clinical trials for ALI using MSC-derived from human BAL.

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References

- Badri L, Walker NM, Ohtsuka T, Wang Z, Delmar M, Flint A, Peters-Golden M, Toews GB, Pinsky DJ, Krebsbach PH, Lama VN. Epithelial interactions and local engraftment of lung-resident mesenchymal stem cells. *Am J Respir Cell Mol Biol*. 2011; 45(4):809–816. [PubMed: 21378261]
- Casal M, Haskins M. Large animal models and gene therapy. *Eur J Hum Genet*. 2006; 14(3):266–272. [PubMed: 16333317]
- Chang C, Niu D, Zhou H, Zhang Y, Li F, Gong F. Mesenchymal stroma cells improve hyperglycemia and insulin deficiency in the diabetic porcine pancreatic microenvironment. *Cytotherapy*. 2008; 10(8):796–805. [PubMed: 18979304]
- Cozzi E, Tucker AW, Langford GA, Pino-Chavez G, Wright L, O'Connell MJ, Young VJ, Lancaster R, McLaughlin M, Hunt K, Bordin MC, White DJ. Characterization of pigs transgenic for human decay-accelerating factor. *Transplantation*. 1997; 64(10):1383–1392. [PubMed: 9392299]
- da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci*. 2006; 119(Pt 11):2204–2213. [PubMed: 16684817]
- Fang X, Neyrinck AP, Matthay MA, Lee JW. Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin-1. *J Biol Chem*. 2010; 285(34):26211–26222. [PubMed: 20554518]
- Gupta N, Su X, Popov B, Lee JW, Serikov V, Matthay MA. Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol*. 2007; 179(3):1855–1863. [PubMed: 17641052]
- Ibrahim Z, Busch J, Awwad M, Wagner R, Wells K, Cooper DK. Selected physiologic compatibilities and incompatibilities between human and porcine organ systems. *Xenotransplantation*. 2006; 13(6): 488–499. [PubMed: 17059572]
- Johnson ER, Matthay MA. Acute lung injury: epidemiology, pathogenesis, and treatment. *J Aerosol Med Pulm Drug Deliv*. 2010; 23(4):243–252. [PubMed: 20073554]
- Jung K, Renukaradhya GJ, Alekseev KP, Fang Y, Tang Y, Saif LJ. Porcine reproductive and respiratory syndrome virus modifies innate immunity and alters disease outcome in pigs subsequently infected with porcine respiratory coronavirus: implications for respiratory viral co-infections. *J Gen Virol*. 2009; 90(Pt 11):2713–2723. [PubMed: 19656969]

- Khatri M, Dwivedi V, Krakowka S, Manickam C, Ali A, Wang L, Qin Z, Renukaradhya GJ, Lee CW. Swine influenza H1N1 virus induces acute inflammatory immune responses in pig lungs: a potential animal model for human H1N1 influenza virus. *J Virol*. 2010; 84(21):11210–11218. [PubMed: 20719941]
- Khatri M, O'Brien TD, Chattha KS, Saif LJ. Porcine lung mesenchymal stromal cells possess differentiation and immunoregulatory properties. *Stem Cell Res Ther*. 2015; 6:222. [PubMed: 26560714]
- Khatri M, O'Brien TD, Sharma JM. Isolation and differentiation of chicken mesenchymal stem cells from bone marrow. *Stem Cells Dev*. 2009; 18(10):1485–1492. [PubMed: 19382875]
- Khatri M, Saif YM. Influenza virus infects bone marrow mesenchymal stromal cells in vitro: implications for bone marrow transplantation. *Cell Transplant*. 2013; 22(3):461–468. [PubMed: 23006541]
- Lama VN, Smith L, Badri L, Flint A, Andrei AC, Murray S, Wang Z, Liao H, Toews GB, Krebsbach PH, Peters-Golden M, Pinsky DJ, Martinez FJ, Thannickal VJ. Evidence for tissue-resident mesenchymal stem cells in human adult lung from studies of transplanted allografts. *J Clin Invest*. 2007; 117(4):989–996. [PubMed: 17347686]
- Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet*. 2008; 371(9624):1579–1586. [PubMed: 18468541]
- Lee JW, Fang X, Gupta N, Serikov V, Matthay MA. Allogeneic human mesenchymal stem cells for treatment of *E. coli* endotoxin-induced acute lung injury in the ex vivo perfused human lung. *Proc Natl Acad Sci U S A*. 2009; 106(38):16357–16362. [PubMed: 19721001]
- Lim JH, Piedrahita JA, Jackson L, Ghashghaei T, Olby NJ. Development of a model of sacrocaudal spinal cord injury in cloned Yucatan minipigs for cellular transplantation research. *Cell Reprogram*. 2010; 12(6):689–697. [PubMed: 21108536]
- Mei SH, McCarter SD, Deng Y, Parker CH, Liles WC, Stewart DJ. Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. *PLoS Med*. 2007; 4(9):e269. [PubMed: 17803352]
- Rasmusson I. Immune modulation by mesenchymal stem cells. *Exp Cell Res*. 2006; 312(12):2169–2179. [PubMed: 16631737]
- Ricciardi M, Malpeli G, Bifari F, Bassi G, Pacelli L, Nwabo Kamdje AH, Chilosi M, Krampera M. Comparison of epithelial differentiation and immune regulatory properties of mesenchymal stromal cells derived from human lung and bone marrow. *PLoS One*. 2012; 7(5):e35639. [PubMed: 22567106]
- Richt JA, Lager KM, Janke BH, Woods RD, Webster RG, Webby RJ. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. *J Clin Microbiol*. 2003; 41(7):3198–3205. [PubMed: 12843064]
- Rogers CS, Abraham WM, Brogden KA, Engelhardt JF, Fisher JT, McCray PB Jr, McLennan G, Meyerholz DK, Namati E, Ostedgaard LS, Prather RS, Sabater JR, Stoltz DA, Zabner J, Welsh MJ. The porcine lung as a potential model for cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2008a; 295(2):L240–263. [PubMed: 18487356]
- Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB Jr, Zabner J, Prather RS, Welsh MJ. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science*. 2008b; 321(5897):1837–1841. [PubMed: 18818360]
- Rolandsson Enes S, Andersson Sjolund A, Skog I, Hansson L, Larsson H, Le Blanc K, Eriksson L, Björner L, Scheduling S, Westergren-Thorsson G. MSC from fetal and adult lungs possess lung-specific properties compared to bone marrow-derived MSC. *Sci Rep*. 2016; 6:29160. [PubMed: 27381039]
- Saif LJ, Ward LA, Yuan L, Rosen BI, To TL. The gnotobiotic piglet as a model for studies of disease pathogenesis and immunity to human rotaviruses. *Arch Virol Suppl*. 1996; 12:153–161. [PubMed: 9015112]

- Schraufnagel, DE. Breathing in America: Diseases, Progress and Hope. New York: American Thoracic Society; 2010.
- Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, Pittenger MF, Martin BJ. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg.* 2002; 73(6):1919–1925. discussion 1926. [PubMed: 12078791]
- Soerensen KE, Skovgaard K, Heegaard PM, Jensen HE, Nielsen OL, Leifsson PS, Olsen HG, Aalbaek B, Kristensen AT, Kjelgaard-Hansen M, Wiinberg B, Iburg TM. The impact of *Staphylococcus aureus* concentration on the development of pulmonary lesions and cytokine expression after intravenous inoculation of pigs. *Vet Pathol.* 2012; 49(6):950–962. [PubMed: 22461225]
- Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Wang S, Shi S. Mesenchymal stem cell-mediated functional tooth regeneration in swine. *PLoS One.* 2006; 1:e79. [PubMed: 17183711]
- Sueblinvong V, Loi R, Eisenhauer PL, Bernstein IM, Suratt BT, Spees JL, Weiss DJ. Derivation of lung epithelium from human cord blood-derived mesenchymal stem cells. *Am J Respir Crit Care Med.* 2008; 177(7):701–711. [PubMed: 18063840]
- Thomas M, Wang Z, Sreenivasan CC, Hause BM, Gourapura JR, Li F, Francis DH, Kaushik RS, Khatri M. Poly I:C adjuvanted inactivated swine influenza vaccine induces heterologous protective immunity in pigs. *Vaccine.* 2015; 33(4):542–548. [PubMed: 25437101]
- Tong L, Zhou J, Rong L, Seeley EJ, Pan J, Zhu X, Liu J, Wang Q, Tang X, Qu J, Bai C, Song Y. Fibroblast Growth Factor-10 (FGF-10) Mobilizes Lung-resident Mesenchymal Stem Cells and Protects Against Acute Lung Injury. *Sci Rep.* 2016; 6:21642. [PubMed: 26869337]
- Wang G, Bunnell BA, Painter RG, Quiniones BC, Tom S, Lanson NA Jr, Spees JL, Bertucci D, Peister A, Weiss DJ, Valentine VG, Prockop DJ, Kolls JK. Adult stem cells from bone marrow stroma differentiate into airway epithelial cells: potential therapy for cystic fibrosis. *Proc Natl Acad Sci U S A.* 2005; 102(1):186–191. [PubMed: 15615854]
- Xu J, Qu J, Cao L, Sai Y, Chen C, He L, Yu L. Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *J Pathol.* 2008; 214(4):472–481. [PubMed: 18213733]
- Xu J, Woods CR, Mora AL, Joodi R, Brigham KL, Iyer S, Rojas M. Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. *Am J Physiol Lung Cell Mol Physiol.* 2007; 293(1):L131–141. [PubMed: 17416739]
- Yuan L, Saif LJ. Induction of mucosal immune responses and protection against enteric viruses: rotavirus infection of gnotobiotic pigs as a model. *Vet Immunol Immunopathol.* 2002; 87(3–4): 147–160. [PubMed: 12072229]
- Zhou L, Wang W, Liu Y, de Castro JF, Ezashi T, Telugu BP, Roberts RM, Kaplan HJ, Dean DC. Differentiation of induced pluripotent stem cells of Swine into rod photoreceptors and their integration into the retina. *Stem Cells.* 2011; 29(6):972–980. [PubMed: 21491544]

Fig. 1. Characteristics of porcine BAL-derived MSCs

(A) Porcine BAL-MSCs show fibroblast-like morphology typical of MSCs (x20). (B) Two hundred BAL-MSCs were cultured in each well of a 6-well plate for 14 days. Medium was changed every 3-4 days and colonies were stained with Wright-Giemsa stain. (C) Photograph shows morphology of wright-Giemsa stained single colony from panel B (x20).

Fig. 2. Expression of mesenchymal markers on BAL-MSCs

Single cell suspension of porcine BM- and BAL-MSCs were examined for the expression of mesenchymal markers (CD29, CD44, CD90 and CD105) and hematopoietic markers (CD34 and CD45) by flow cytometry. Similarly to BM-MSC, BAL-MSC expressed mesenchymal markers and lacked the expression of hematopoietic markers. Dotted line represents isotype control; Solid line represents antibody-specific staining.

Fig. 3. *In vitro* differentiation potential of porcine BAL-MSCs

Culture expanded BAL-MSCs were examined for their ability to differentiate into mesenchymal lineage cells. **(A) Adipocyte differentiation.** BAL-MSCs were cultured in adipogenic induction medium for 14 days. Differentiated adipocytes showed red lipid droplets in the cytoplasm of differentiated adipocytes when stained with 3% Sudan III ($\times 40$). **(B)** No lipid droplets were observed in MSCs cultured in control DMEM medium. **(C) Chondrocyte differentiation.** BAL-MSCs were incubated in chondrogenic induction medium for 3 weeks. Homogenous proteoglycans indicative of chondrogenesis were observed by Alcian blue staining. **(D)** Chondrogenesis was not observed in cultures incubated with control DMEM medium. **(E) Osteocyte differentiation.** BAL-MSCs were cultured in osteogenic induction medium for 21 days, calcium deposits indicative of osteogenic differentiation were detected by Alazirin Red staining. **(F)** No calcium deposits were detected in BAL-MSCs cultured in control DMEM medium.

Fig. 4. MSCs administration caused decrease in the number of neutrophils in BAL of LPS-injured pigs

Groups of commercial SPF pigs were inoculated with *E. coli* LPS. After 12 hrs, BAL-or BM-MSCs were administered intratracheally. Forty-eight hrs after MSC administration pigs were euthanized and number of neutrophils in BAL cells was determined. The data are expressed as % neutrophils in total BAL cells. Each bar represents means $\pm$ SD of percent neutrophils from 3 pigs. Asterisks indicate significant differences LPS and LPS+BAL-MSC or LPS+BM-MSC groups ($P < 0.05$).

Fig. 5. MSCs therapy attenuated LPS-induced gross lung lesions in pigs

Five-week-old commercial pigs were inoculated with *E. coli* LPS. After 12 hrs, BAL- or BM-MSCs were administered intratracheally. Forty-eight hrs after MSC administration pigs were euthanized and lungs were examined for gross pathological lesions. Pneumonia as evidenced by the presence of dark-red areas of consolidation was observed in the lungs of pigs inoculated with LPS. Gross lung lesions were not observed in control, BM-MSC or BAL-MSC-administered pigs. Arrows indicate areas of pneumonic lesions in the lungs of LPS-inoculated pigs.

Fig. 6. MSC therapy attenuate LPS-induced microscopic lung lesions in pigs

Five-week-old commercial pigs were inoculated with *E. coli* LPS. After 12 hrs, BAL-or BM-MSC were administered intratracheally. Forty-eight hrs after MSCs administration pigs were euthanized and Hematoxylin and Eosin stained lung sections were examined for microscopic lesions. **(A) Microscopic lung lesions.** Lung sections from control pig show normal alveoli and clear air spaces. Lung sections from LPS-inoculated pigs show extensive infiltration of neutrophils and macrophages in alveoli and thickened alveolar wall whereas lung sections from LPS+BM-MSC and LPS+BAL-MSC administered pigs show mild infiltration of inflammatory cells ($\times 200$). **(B) Mean microscopic lung lesions.** Lung sections were scored from 0-3 depending on the severity in changes in bronchiolar epithelium, peribronchiolar inflammation and interstitial pneumonia. Data in each bar are

means of microscopic lung lesions score of 3 pigs $\pm$ SD. Asterisks indicate significant differences between LPS and LPS+BAL-MSC or LPS+BM-MSC groups ($P < 0.05$).

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Fig. 7. BAL-MSCs therapy decrease proinflammatory cytokine production in LPS-injured lungs of pigs

ALI was induced in 5 week-old pigs by intratracheal inoculation of *E. coli* LPS. Twelve hrs later, pigs were administered intratracheally with BM-MSCs or BAL-MSCs. Pigs were euthanized 48 hrs after MSC administration and lungs were harvested. TNF- α and CXCL10 production in lung lysates was determined by ELISA. Values in each bar are means $\pm$ SD of cytokine concentrations in pg/g of lung from 3 pigs. Asterisks indicate significant differences between LPS and LPS+BAL-MSC or LPS+BM-MSC groups ($P < 0.05$).

Fig. 8. Engraftment of MSCs in the lungs of LPS-injured pigs

ALI was induced in 5 week-old pigs by intratracheal inoculation of *E. coli* LPS. Twelve hrs later, PKH-26 labeled BM-MSCs or BAL-MSCs were administered intratracheally. Forty eight hrs after MSC administration pigs were euthanized and lungs were harvested to prepare frozen sections. **(A)** Photograph show engraftment of PKH-26 labeled MSCs (red) in the lungs ($\times 20$). Engraftment of BAL-MSCs in lungs was significantly higher as compared to BM-MSCs. **(B)** Engrafted MSCs in lung sections were counted in five microscopic fields from each pig/group. Each bar represents mean $\pm$ SD of MSCs in 5 microscopic fields each from 3 pigs. Asterisks indicate significant differences between LPS and LPS+BAL-MSC or LPS+BM-MSC groups ($P < 0.05$).

Fig. 9. No transdifferentiation of transplanted MSCs into lung epithelial cells in lungs of LPS-injured pigs

ALI was induced in 5 week-old pigs by intratracheal inoculation of *E. coli* LPS. Twelve hrs later, PKH-26 labeled BM-MSCs or BAL-MSCs were administered intratracheally. Forty eight hrs after MSCs administration pigs were euthanized and lungs were harvested to prepare frozen sections. Lung sections of BAL- and BM-MSCs administered pigs were stained for the detection of type II lung epithelial cells using anti-surfactant protein C (SPC; a type II pneumocyte marker) antibody and goat anti-rabbit Alexa Fluor 488 as secondary antibody (SPC positive cells, green) and PKH-26 labeled engrafted MSCs (red). Nuclei were stained with DAPI (blue). Magnification: $\times 400$. No SPC positive staining was detected in engrafted MSC suggesting that there was no transdifferentiation of BAL-or BM-MSC into type II lung epithelial cells.