Airway transplantation of adipose stem cells protects against bleomycin-induced pulmonary fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease with poor prognosis. Adipose-derived stem cells (ADSC) have demonstrated regenerative properties in several tissues. The hypothesis of this study was that airway transplantation of ADSC could protect against bleomycin (BLM)-induced pulmonary fibrosis (PF). Fifty-eight lungs from 29 male Sprague-Dawley rats were analyzed. Animals were randomly divided into five groups: a) control (n=3); b) sham (n=6); c) BLM (n=6); d) BLM+ADSC-2d (n=6); and e) BLM+ADSC-14d (n=8). Animals received 500 µL saline (sham), 2.5 UI/kg BLM in 500 µL saline (BLM), and 2×10^6 ADSC in 100 µL saline intratracheally at 2 (BLM+ADSC-2d) and 14 days (BLM+ADSC -14d) after BLM. Animals were sacrificed at 28 days. Blinded Ashcroft score was used to determine pulmonary fibrosis extent on histology. Hsp27, Vegf, Nfkβ, IL-1, IL-6, Col4, and Tgfβ1 mRNA gene expression were determined using real-time quantitative-PCR. Ashcroft index was: control=0; sham=0.37±0.07; BLM=6.55±0.34 vs sham (P=0.006). BLM vs BLM+ADSC-2d=4.63±0.38 (P=0.005) and BLM+ADSC-14d=3.77±0.46 (P=0.005). BLM vs sham significantly increased Hsp27 (P=0.018), Nfkβ (P=0.009), Col4 (P=0.004), Tgfβ1 (P=0.006) and decreased IL-1 (P=0.006). BLM+ADSC-2d vs BLM significantly decreased Hsp27 (P=0.009) and increased Vegf (P=0.006), Nfkβ (P=0.009). BLM+ADSC-14d vs BLM significantly decreased Hsp27 (P=0.028), IL-6 (P=0.013), Col4 (P=0.002), and increased Nfkβ (P=0.040) and Tgfβ1 (P=0.002). Airway transplantation of ADSC significantly decreased the fibrosis rate in both early and established pulmonary fibrosis, modulating the expression of Hsp27, Vegfa, Nfkβ, IL-6, Col4, and Tgfβ1. From a translational perspective, this technique could become a new adjuvant treatment for patients with IPF.

Significance of this study

What is already known about this subject?
Idiopathic pulmonary fibrosis (IPF) is the most frequent interstitial lung disease; it is chronic, irreversible, and associated with poor prognosis, as no satisfactory medical treatment exists. Thus, important areas for research in IPF, such as the study of new treatments for both fibrotic and inflammatory stages as well as genetic studies aimed at identifying new targets that allow us to modify IPF outcomes, have been highly recommended by the US National Heart, Lung and Blood Institute.

What are the new findings?
This experimental study is the first evaluation of airway transplantation of adipose-derived stem cells. We report protective effects of these cells against inflammatory and fibrotic stages in a bleomycin-induced pulmonary fibrosis model. We describe their capacity to decrease the pulmonary fibrotic rate and significantly regulate the expression of genes involved in the pathogenesis of pulmonary fibrosis.

How might these results change the focus of research or clinical practice?
Although further research is needed, from a translational point of view, airway transplantation of adipose-derived stem cells is a potential new adjuvant treatment for patients suffering from pulmonary fibrosis.

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common diffuse interstitial lung disease and is associated with poor prognosis, with a median survival time of 2.5–5 years after diagnosis.[1] It is chronic and irreversible and is characterized by a progressive destruction of lung architecture with scar formation.[2 3] IPF can occur at any age, although it is most common between ages 40 and 70 years, which is why it is believed to be related to lung aging and oxidative stress (OS).[4 5]

Currently, limited medical treatments are capable of modifying in some ways the outcome of IPF and avoid progression towards respiratory failure, which significantly reduces patient

quality and quantity of life.[6 7] For advanced stages, lung transplantation is the most effective therapeutic alternative, although it is restricted for most patients.[8]

Stem cells (SC) have demonstrated their ability to repair damaged tissue.[9] Adipose-derived stem cells (ADSC) can regenerate injured tissues such as bone, cartilage, liver, and myocardium, as well as lung lesions in smokers.[10] In 2014, Sang Hoon Lee *et al*[11] , in an experimental model of bleomycin (BLM)-induced pulmonary fibrosis (PF), showed that intraperitoneal ADSC administration inhibited inflammatory processes, fibrotic phenomena, apoptosis, and Tgfβ1 expression. In 2015, Tashiro *et al*[12] showed that BLM-induced PF in aged mice could be blocked by young-donor ADSC due to changes in collagen turnover and markers of inflammation.

Recently, Ghadiri *et al*[13] reported that SC could be considered a therapeutic strategy for IPF treatment, as they can repair and replace the damaged lung tissue due to their capacity to proliferate and differentiate into alveolar cells, the cells initially damaged in the fibrotic process.

Based on these findings, our objective was to assess the potential role of ADSC in IPF. Our hypothesis was that airway transplantation of ADSC could ameliorate BLM-induced PF in an experimental model.

MATERIALS AND METHODS

Study design

This study was approved by the institutional animal care committee and followed the European Convention guidelines on the use and protection of animals. A total of 29 male Sprague-Dawley rats (weight 300–360 g) were used under optimal stabling conditions. Animals were randomly assigned into five groups: a) control (n=3), where bilateral lung samples were taken immediately from healthy lungs; b) sham (n=6), where 500 µL saline was administered via intratracheal injection; c) BLM (n=6), where intratracheal injection of BLM was administered; d) BLM+ADSC-2d (n=6), receiving intratracheal BLM initially followed by intratracheal injection of ADSC at 2 days; e) BLM+ADSC-14d (n=8), in which an initial intratracheal injection of BLM was later followed by intratracheal ADSC at 14 days. In groups b), c), d), and e), animals underwent general anesthesia, and bilateral lung samples from medial segments of upper and lower lobes were collected 28 days after BLM administration. Lung samples were stored at –80°C for histological and genomic studies. We did not consider more than one confirmatory experiment since histopathological lesions of bleomycin-induced PF have been well described and well reproduced.[14]

BLM-induced PF model

Animals were anesthetized with subcutaneous injection of 0.25 mg/kg medetomidine (Domtor, Pfizer, USA) and intraperitoneal injection of 50 mg/kg ketamine (Ketolar, Pfizer, USA), orotracheally intubated with a 14-gauge catheter and mechanically ventilated (Servo 900, Siemens AG, Munich, Germany) and placed at 45° over a surgical (table 1). After that, 2.5 UI/k of BLM in 500 µL of saline was administered via intratracheal injection as was described by Brown *et al*[14] and animals were immediately ventilated for 5 min to facilitate drug distribution. Animals received a subcutaneous injection of 0.25 mg/kg atipamezole (Antisedan, Pfizer, USA) to reverse anesthesia. Following recovery from anesthesia, rats were returned to their cages and given food and water normally (figure 1).

ADSC transplantation

Two Sprague-Dawley donor animals (300 g) underwent general anesthesia with an intraperitoneal injection of 75 mg/kg of ketamine (Ketolar). ADSC isolation was made from inguinal adipose tissue. After mechanical disruption, the resuspended pellet underwent enzymatic digestion with collagenase type I at 37°C under stirring for 30 min. After multiple steps of washing in sterile phosphate-buffered saline, the cell pellet was resuspended and seeded in treated cell-culture flasks with Dulbecco's Modified Eagle Medium plus 10% fetal bovine serum and 1% penicillin/streptomycin. Non-adherent cell fraction (adipocytes, endothelial cells, etc) was removed after 24 hours, and both expansion and purification of ADSC were performed by successive trypsinizations. ADSC were characterized by flow cytometry according to Dominici *et al*[15] and frozen in liquid nitrogen, where they remained until use. A single dose of 2×10^6 ADSC in 100 µL was administered via intratracheal injection under general anesthesia at 2 or 14 days after BLM as previously described.

Clinical outcome

Signs of stress, assessment of behavior and animal welfare according to the international classification of Federation of European Laboratory Animal Science Associations[16] were assessed daily. Animals were weighed at baseline and 28 days.

Table 1 Gene names and primer sequences (5′–3′) used for real-time PCR

Genbank accession no.	Description	Gene symbol	Forward primer	Reverse primer
NM_001276711	Nuclear factor kappa-light-chain-enhancer of activated B cells	Nfkβ	GTATGGCTTCCCGCACTATGG	TCGTCACTCTTGGCACAATC
NM_001164708	Heat shock protein-1 27 KDa	Hsp27	GGCACACTCACGGTGGAGGC	GGGAGGGCTGGTGACGGCTA
NM_021578	Transforming growth factor beta 1	Tgfβ1	TGAGTGGCTGTCTTTTGACG	TGGGAGTGATCCCATTGATT
NM_001287114	Vascular endothelial growth factor-A	Vegfa	CCAGGCTGCACCCACGACAG	CGCACACCGCATTAGGGGCA
NM_053953	Interleukin-1	IL-1	GGAGGCCATAGCCCATGATT	TCCTTCAGCAACACAGGCTT
NM_012589	Interleukin-6	IL-6	CATTCTGTCTCGAGCCCACC	GCTGGAAGTCTCTTGCGGAG
NM_001135759	Collagen type IV	Col4	GCCAAGTGTGCATGAGAAGA	AGCGGGGTGTGTTAGTTACG
NM_001248320	Peptidyl-prolyl isomerase A	PPIasa	AGCACTGGGGAGAAAGGATT	AGCCACTCAGTCTTGGCAGT

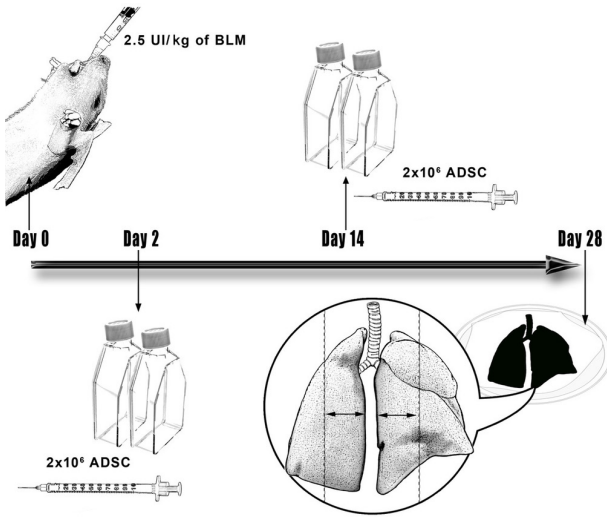

Figure 1 Treatment schedule. Rats were treated with one dose of bleomycin (BLM) or saline via intratracheal injection. Adipose-derived stem cells (ADSC) were intratracheally injected at 2 or 14 days after BLM exposure. Animals were sacrificed at 28 days after BLM and medial segments of upper and lower lobe of each lung were collected for histological and genomic study (double-headed arrows).

Histological evaluation

Euthanasia was performed 28 days after BLM administration. Lung samples were fixed in 4% paraformaldehyde and were later embedded in paraffin and cut at 3 mm thickness, stained with hematoxylin and eosin and examined under a light microscope. An expert pathologist (JLAM) blinded to the experimental history of the specimens performed pathological studies. Pulmonary fibrosis rate was evaluated according to Ashcroft classification,[17] from 0 (no fibrosis) to 8 (complete fibrosis). For the last three decades, the Ashcroft classification has been used for quantification of lung fibrosis in experimental models. The results obtained here correlate with those obtained using the latest techniques, such as microcomputed tomography.[17 18]

Microbiological test

In order to rule out a possible external inflammatory component, we decided to incorporate specific microbiological evaluations, since the distortion of the pulmonary architecture produced by PF can affect mucociliary activity and secondary stagnation of mucus, which could facilitate the colonization for microorganisms that can produce an exaggerated inflammatory response. Lung samples were placed in BD Fluid Thioglycollate Medium (Becton Dickinson, USA) at 35°C for 7 days. Cultures were performed on blood, chocolate, and MacConkey agar (BBL, BD Diagnostic Systems, USA) at 35°C in 5% CO_2 atmosphere for 48 hours (aerobic bacteria). Brucella agar supplemented with vitamin K1 and hemin (BBL, BD Diagnostic Systems) was incubated in a 35°C anaerobic atmosphere for 4 days (anaerobic bacteria). The ViteK V.2 (BioMérieux, USA) system was used for identification and susceptibility testing of isolated bacteria.

Gene expression analysis by real-time quantitative-PCR

The mRNA expression levels of genes involved in cellular stress and IPF were measured using real-time quantitative-PCR (qPCR) (Online Data Supplement). Total RNA was isolated by homogenization of frozen tissue using a Polytron PT-2000 (Kinematica AG) according to the GITC-phenol/chloroform.[19] Briefly, 1 µg of total RNA was treated with RNase-free DNase I (Promega) to remove genomic DNA and then was reverse-transcribed using the iScript reverse transcriptase kit (Roche, Mannheim, Germany). A total of 2 mL of cDNA served as a template in a 13 µL qPCR reaction mix containing the primers and SYBR Green PCR Master Mix (Diagenode, Belgium). Quantification of gene expression was performed with an ABI PRISM 7000 SD RT-PCR according to the manufacturer's protocol. The amplified PCR products were subjected to electrophoresis on a 1.5% agarose gel to confirm predicted sizes. Data were extracted, and amplification plots were generated with ABI SDS software. Exon-specific primers were designed using the Primer V.3 program.[20] The level of individual mRNA measured by qPCR was normalized to the level of the housekeeping genes cyclophilin and ribosomal 28S by using the method by Pfaffl.[21] For graphing purposes, the relative expression levels were scaled according to the control and sham group.

Statistical analyses

Data were expressed as group mean±SD. Comparisons among categorical variables were assessed by X^2 test. Differences in gene expression levels were tested with the Mann-Whitney U test since they did not follow normal distribution under the Kolmogorov-Smirnov test. Data management and statistical analysis were performed using the statistical package SPSS V.15.0 (SPSS, Chicago, Illinois, USA). Weight differences between each group were described as median, 25th percentile and 75th percentile. A P-value <0.05 was considered to be statistically significant.

RESULTS

Postoperative outcome

No mortality was observed. No significant differences in temperature or signs of pain (piloerection, dacryorrhea, immobilization, closed eyes) were observed. Transient mild respiratory distress was observed after BLM administration in groups (c), (d), and (e).

Significant differences in weight gain between the baseline and the end of the study were observed in the BLM (P=0.03), BLM+ADSC-2d (P=0.03), and BLM+ADSC-14d (P=0.008) groups, with higher values evidenced in the ADSC groups. Significant differences were observed when comparing BLM vs BLM+ADSC-2d (P=0.028) and BLM+ADSC-14d (P=0.012), since recovery of weight was faster and higher in the ADSC groups.

Macroscopic description of lungs

The lungs of the control and sham animals appeared healthy. The BLM group showed bilateral lesions characterized by dark red patchy areas with a harder consistency and lack of lung expansion. There were fewer and smaller lesions with an adequate lung expansion in the groups treated with ADSC (figure 2). No adhesions were observed.

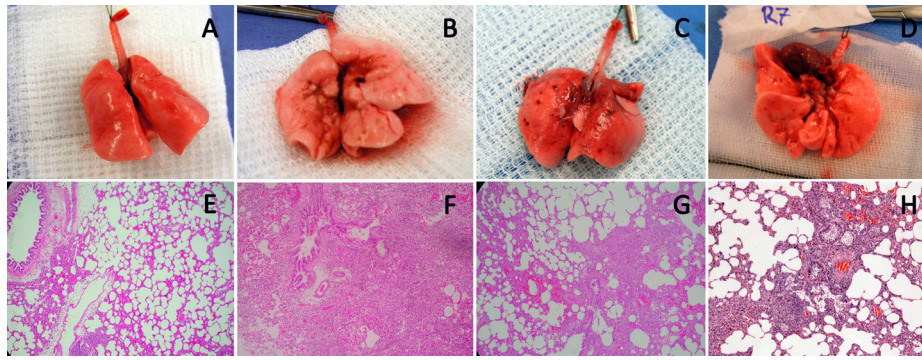

Figure 2 Macroscopic and histological view (10x) of the lungs. (A) Sham group: showed lungs with normal appearance; (B) BLM group: showed bilateral lesions, characterized by dark red patchy areas and harder consistency and lack of expansion; (C) BLM+ADSC-2d and (D) BLM+ADSC-14d: showed fewer and smaller lesions presenting with adequate lung expansion; (E) sham group: showed an isolated slight thickening of the alveolar walls with normal lung morphology; (F) BLM group: showed complete parenchymal consolidation with almost complete disappearance of the alveolar spaces and large areas of fibrosis; (G) BLM+ADSC-2d and (H) BLM+ADSC-14d: showed partial recovery of the airspace and partial restoration of the normal structure of the alveolar walls. ADSC, adipose-derived stem cells; BLM, bleomycin.

Histology

The control group showed no alterations. The sham group showed isolated, slight thickening of alveolar walls with normal lung morphology. The BLM group exhibited complete parenchymal consolidation with near-complete disappearance of alveolar spaces, vast areas of fibrosis and occupation of alveolar spaces by alveolar macrophages, alveolar epithelial cells with prominent nuclei, fibroblastic proliferation with architectural distortion and images of alveolar bronchiolization, some red blood cells, and few lymphocytes.

A partial, 29.4%–42.5% recovery of the airspace without complete restoration of the normal structure of alveolar walls was observed in the BLM+ADSC-2d and BLM+ADSC-14d groups. Reactive changes of alveolar epithelium with signs of bronchiolization were also observed. No necrosis was observed in any of the animals (figure 2).

Ashcroft score

Control animals showed a score of 0, and the sham group had scores of 0.37±0.07. The BLM group showed the highest score (6.55±0.34), which was statistically significant when compared with sham animals (P=0.006). The BLM+ADSC-2d group showed a score of 4.63±0.38, which was significant when compared with the BLM group (P=0.005). The BLM+ADSC-14d group had a score of 3.77±0.46, which was significant when compared with the BLM group (P=0.005) (figure 3). The score of the BLM+ADSC-14 group was significantly lower compared with the BLM+ADSC-2d group (P=0.012).

Microbiological study

Cultures of lung samples were negative for aerobic and anaerobic bacteria in all groups.

Analysis of gene expression by qPCR

No significant differences in the mRNA expression of Hsp27 (P=0.522), Vegf (P=0.631), or Nfkβ (P=0.715) were observed between sham animals versus controls, while a significant increase of expression of IL-6 (P=0.004), Col4 (P=0.016), IL-1 (P=0.006), and a decrease of Tgfβ1 (P=0.006) were observed in the sham group (figure 4A).

A significant increase of expression of Hsp27 (P=0.018), Nfkβ (P=0.009), Col4 (P=0.004), and Tgfβ1 (P=0.006), and decreased IL-1 (P=0.006) were observed in the BLM group vs sham (figure 4B). ADSC treatment at 2 days post-BLM administration versus BLM significantly decreased the expression of Hsp27 (P=0.009) and increased the expression of Vegf (P=0.006) and Nfkβ (P=0.009) (figure 4C). ADSC treatment at 14 days post-BLM versus BLM significantly decreased the expression of Hsp27 (P=0.028), IL-6 (P=0.013), Col4 (P=0.002), and increased the expression of Nfkβ (P=0.040) and Tgfβ1 (P=0.002) (figure 4D). Gene expression comparisons not specifically mentioned were not significant.

DISCUSSION

There are few effective and available treatments for IPF. The only two drugs currently approved by the US FDA for treatment of IPF—pirfenidone and nintedanib—are based

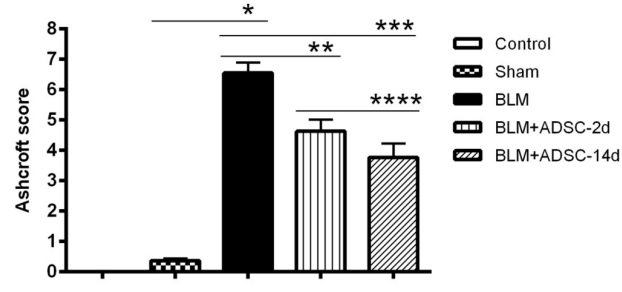

Figure 3 Ashcroft score. Sham group: showed a score of 0.37±0.07. BLM group: showed the highest score of 6.55±0.34 (P=0.006 when compared with the sham group)*. BLM+ADSC-2d: showed a score of 4.63±0.38 (P=0.005 when compared with BLM group)**. BLM+ADSC-14d: showed the lowest score, of 3.77±0.46 (P=0.005 when compared with BLM group)*** (P=0.012 when compared with BLM +ADSC-2d) ****. ADSC, adipose-derived stem cells; BLM, bleomycin.

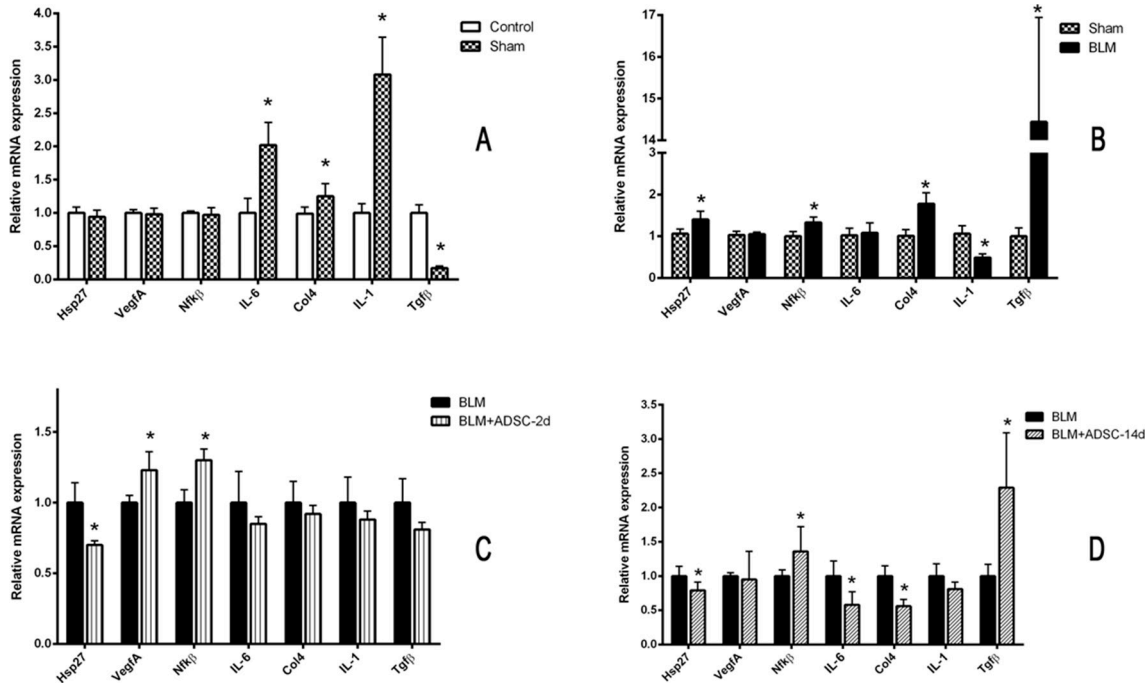

Figure 4 Gene expression. (A) Control vs sham: significantly increased mRNA expression of IL-6 (P0.006), IL-1 (P=0.006), Col4 (P=0.016), and decreased Tgfβ (P=0.006). (B) BLM vs sham: significantly increased Hsp27 (P=0.018), Nfkβ (P=0.009), Col4 (P=0.004), Tgfβ (P=0.006), and decreased IL-1 (P=0.006). (C) BLM+ADSC-2d vs BLM: significantly decreased Hsp27 (P=0.009) and increased VegfA (P=0.006) and Nfkβ (P=0.009). (D) BLM+ADSC-14d vs BLM: significantly decreased Hsp27 (P=0.028), IL-6 (P=0.013), Col4 (P=0.002), and increased Nfkβ (P=0.040) and Tgfβ (P=0.002). ADSC, adipose-derived stem cells; BLM, bleomycin.

on their efficacy in slowing the decline in lung function, although they are not able to avoid fibrosis progression and the final effects in survival are unknown yet.[22] The National Heart, Lung, and Blood Institute recommendations for IPF research[23] include the study of new treatments for fibrotic stages and the beginning of the inflammatory stages and genetic studies aimed at identifying new targets that allow us to modify IPF outcome.

In this sense, for the first time, intratracheal transplantation of ADSC has significantly ameliorated the lung damage in both early inflammatory and fibrotic stages in a BLM-induced PF model. The significant reduction in both numbers of fibrotic lesions and the fibrosis rate in ADSC groups at 2 and 14 days suggest that ADSC can protect or decrease the fibrotic process in lung tissue.

It has been described that lung injury starts in alveolar cells.[24] Previous studies have described intravenous or intraperitoneal administration of ADSC[11 12 25 26] to ameliorate PF in a BLM-induced PF model. The need for doses higher than 5×10^6 of ADSC described in these studies may be related to the blocking in the pulmonary capillaries, preventing access to the alveolar space.[27] However, we reached the alveolar space directly through airway transplantation, enabling us to use less than half the number of intravenous doses described in these studies. From a translational point of view, ADSC airway transplantation could decrease time to transplantation, shortening the time required for ADSC expansion, the expense of the procedure and the saline volume needed to be administered, as we suspect that saline could be responsible for the minimal histological changes and significant expression of genes related to inflammatory processes observed in sham animals.

On the other hand, it has been reported[12] that fibrotic lung lesions may resolve spontaneously in young mice in a BLM-induced PF model; it has even been suggested that this variable must be taken into account when evaluating and translating the experimental results to clinical trials. However, in our study with young rats aged 3–4 months as both ADSC donors and recipients, we could not verify this point, as all animals in the BLM group showed high Aschroft scores. The genetic differences between species may have influenced this issue, as our study was conducted in rats and not mice.[12] In any event, better assessment of this point will require specifically addressed studies.

Cells have different defense mechanisms, which include heat shock proteins (Hsp) to protect structural proteins from the effects of stress or apoptosis. Hsp27 is a small protein involved in folding and refolding proteins and in the degradation of those that have been irreversibly denatured. These actions of Hsp27 are due to (1) its ability to modulate OS and stimulate the expression of antioxidant enzymes which act as a cytoprotective agent; (2) the inhibition apoptosis and protection of actin filaments from fragmentation; and (3) its essential role in tissue differentiation, thermotolerance, and cytoprotection under OS conditions. However, despite these protective properties, Wettstein *et al*[28] described that this protein is directly related to PF and that its inhibition blocks its development. In fact, other authors such as Vidyasagar *et al*[29] pointed to Hsp27 as a biomarker of disease and a potential therapeutic target.

Although protein levels do not always follow gene expression profiles and therefore their functional consequences are unknown, in our study, we have observed that BLM increased Hsp27 expression, and ADSC treatment significantly reduced it to normal levels. This indicates that this protein is either directly involved in the development of PF or is expressed in the damaged lung in an attempt to protect cells from OS so that its expression decreases with ADSC treatment since ADSC decrease OS, inflammatory phenomena, and lung injury. This protective effect of Hsp27 is supported by two previous works by our research group. The first was a DNA microarray study of chronic rejection in lung transplantation,[30] in which Hsp27 was involved in the protection of the cytoskeleton and the inhibition of apoptosis. The second study described the protective effect of ozone therapy on chronic rejection after lung transplantation, which was also associated with decreased Hsp27 expression.[31] We believe that the decreased Hsp27 expression seen in the ADSC treatment groups is likely related to lesser need of expression, as less lung damage was evidenced. However, further studies are required to establish the precise role of Hsp27 in PF.

ADSC transplantation at 2 days significantly increased Vegf expression, probably because the role of the reduction of vascularization is more relevant in the initial stages of fibrosis. As a result, Vegf could promote: (1) anti-inflammatory processes mediated by ADSC; (2) migration of monocytes and macrophages to the inflammatory foci, where they remove cellular debris in early stages of the disease; and 3) stimulation of the neovascularization and differentiation of the ADSCs into endothelial cells dependent on Vegf when RhoA/ROCK signaling pathway Tgfb-dependent is not active.[32] The lack of significant Vegf expression at 14 days may indicate that the role of ischemia in advanced fibrotic stages is less relevant or more time is needed to detect a significant increase in its expression, especially considering that Vegf expression takes place at 21 days after SC transplantation,[33] or its expression is inactivated by the activation of RhoA/ROCK signaling Tgfb-dependent.

Tgfb is a secretory protein that performs various functions in the cell, such as control of cell growth, cell proliferation, differentiation processes, and apoptosis.[34] Tgfb can stimulate fibroblast and myofibroblast proliferation directly inducing IPF,[35] and its activities have been closely related to the generation of obstructive pulmonary disease and pulmonary hypertension. However, its expression was significantly increased with ADSC-14d transplantation, and we hypothesize that this could be related to additional non-profibrotic actions. Interestingly, in a significant number of cells, Tgfb is capable of mediating apoptosis, inducing OS response, which seems to be associated with the activity of TIEG transcription factor (Tgfβ1-inducible early response gene)[36] and this could be another possible ADSC-induced mechanism of action over fibrotic cells. An additional explanation could be its recognized dual immune-modulatory actions.[37] In this sense, the role of ADSC-related Tgfβ1 expression may be predominantly more immunosuppressive (via CD4+ regulatory T cells) than an inductor of fibroblast/myofibroblast proliferation. This hypothesis is further supported by the significant decrease in the proinflammatory IL-6 we simultaneously found in the ADSC-14d group.

Nfkβ is a protein complex that controls DNA transcription and is involved in the cellular response to stimuli such as OS, cytokines, UV radiation, oxidized LDL, and bacterial or viral antigens.[38] It is a nuclear transcription factor with significant proinflammatory activity involved in a wide variety of biological functions, including the synthesis of proinflammatory cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-8. Its activation has been associated with numerous inflammatory diseases, including IPF,[39] cancer, autoimmune diseases, septic shock, viral infections, and altered immune development.[40] Methods to inhibit Nfkβ signaling have a great therapeutic interest in oncological and inflammatory diseases.[41 42] When mesenchymal SC are exposed to TNF, endotoxins, and hypoxia, they increase Nfkβ, extracellular signal-regulated kinase, and c-Jun kinases N-terminal activation inhibiting the expression of Vegf, fibroblast growth factor 2, and hepatocyte growth factor.[43] In our study, we have observed a significant increase in the expression of Nfkβ in the BLM group with respect to its proinflammatory action in PF. However, a significant increase was also seen in the groups treated with ADSC-2d and ADSC-14d. We have no further explanation for this unexpected finding. Maybe, actions of Nfkβ are dose-dependent: Nfkβ stimulation below a threshold-level leads to proinflammatory effects, while Nfkβ stimulation above a threshold-level leads to other predominant (or more protective) effects.

IL-6 is a cytokine produced by cells of the innate immune system, macrophages, T cells, endothelial cells, and fibroblasts as a potent inducer of acute-phase response. IL-6 release is induced by IL-1 and increases in response to TNF. Its proinflammatory and profibrotic actions have been described in experimental models of BLM-induced PF.[44] IL-6 has also been known as IFN-R2 or B-cell stimulating factor-2, and it is identical to hepatocyte-stimulating factor, proving its ability to stimulate hepatocytes to produce acute-phase proteins such as fibrinogen and α-1-antitrypsin.[45] In our study, ADSC-14d transplantation significantly reduced the expression of IL-6 up to the level of the controls. This finding could be related to the decreased fibrotic grading observed in the ADSC-14d group, although further studies are required to assess its role.

The Col4 gene encodes a protein of the basement membrane serving as a structural constituent of the extracellular matrix is the major component of the basement membrane. This collagen is involved in the differentiation of epithelial cells, the organization of the basal membrane, morphogenesis of blood vessels, and some alterations have been associated with many diseases including IPF as well.[46 47] Rats that received pretreatment with a specific inhibitor of the inducible enzyme that produces nitric oxide (iNOS) showed lower expression levels of Col4.[48 49] Furthermore, the signaling generated by iNOS enhances the expression of Tgfβ1, TIMP-1, and Hsp47 in the pulmonary fibroblasts playing an important role in the IPF. In our study, its expression was significantly lower in the BLM+ADSC-14d group concerning antifibrotic and regenerative effects of ADSC.

Our study has some potential limitations which could be solved in further research by evaluating OS parameters and developing a chronic BLM-induced PF model to sacrifice rats at different times after ADSC transplantation so as to

better understand the evolution of the complex relationship between the various regulatory and regenerative processes mediated by ADSC. Also, funding limitations did not allow us to carry out additional techniques or assessments such as the measurement of protein levels, additionally.

We conclude that airway transplantation of ADSC has significantly decreased the fibrosis rate and lung injury induced by BLM in both inflammatory and established fibrotic stages and simultaneously modulated the expression of Hsp27, Nfkβ, Vegf, Tgfβ, IL-6, and Col4 gene expression. From a translational point of view, airway transplantation of ADSC could be a potential treatment in humans that may be used to prevent or treat IPF (and maybe other pulmonary fibrosis as secondary to chemotherapy, radiotherapy, etc) for which limited non-surgical-treatment does not exist. Further translational research will confirm whether there is an actual benefit.

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Contributors NS-R, PL, DLF, and BC conceived, designed, and oversaw all of the studies, collection of results, interpretation of the data, and writing of the manuscript and were responsible for the primary undertaking, completion, and supervision of all experiments. PL, DLF, and NS-R conducted animal surgeries, stem cell procedures, sample collections, and gene analysis. JLAM and DLF conducted histological studies. MDF-P, MGA, JC, and LMLR assisted in methodological design and statistical analysis. MGA, LMLR, JC, FB, and WR reviewed and critically edited the manuscript.

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