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Human embryonic stem cells recover in vivo acute lung inflammation bleomycin-induced

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Abstract. Idiopathic pulmonary fibrosis (IPF) is characterized by alveolar epithelial cell injury, type II cell activation, apoptosis and bronchiolar epithelial cell proliferation, accumulation of extracellular matrix and fibroblasts. No current animal model recapitulates all of these cardinal manifestations of the human disease. However, bleomycin instillation in mice lung by intranasal way (ITN) represents the best experimental model of pulmonary fibrosis in which alveolar pneumocytes type II (ATII) are usually depleted. The aim of this study was to test the possibility to recover acute lung fibrosis after transplantation of human embryonic type II derived-pneumocytes in a murine model of bleomycin-induced damage. Our results indicate the striking "clinical" beneficial effect of differentiated HUES-3 cells into ATII in terms of lung function, weight loss and mortality in injured mice, suggesting this stem cell therapy as a promising, systemic and specific treatment of human pulmonary fibrosis. *(Sarcoidosis Vasc Diffuse Lung Dis 2013; 30: 177-185)*

Key words: intranasal bleomycin administration, lung fibrosis mouse model, human embryonic stem cells, cell therapy

Introduction

Intratracheal or intraperitoneal instillation of bleomycin, an antineoplastic antibiotic drug acting through the formation of a complex with oxygen and metals such as Fe, leading to the production of oxygen radicals, DNA breaks, and ultimately cell death. Exposure to bleomycin, administered either intravenously and intratracheally, is the most widely used animal model of lung acute damage and fibrosis (1). While intravenous injection primarily damages pulmonary endothelia, direct instillation into the airways causes bronchiolar and epithelial injury. Generally administered as single intratracheal instillation, bleomycin is followed by a well-described sequence of events: an early neutrophilic alveolitis followed by lymphocyte accumulation. A fibrotic response ensues, characterized by patchy fibrosis without of hyaline membrane formation whose extent is proportional to extent of initial damage (2). Not with standing its limitations with epitomizing the usual interstitial pneumonia pattern that characterizes idiopathic pulmonary fibrosis, the bleomycin model is the most routinely used model of acute lung injury and idiopathic pulmonary fibrosis (IPF) (3). A model of repetitive bleomycin lung injury has been recently described resulting in more prominent lung fibrosis, with alveolar epithelial cell hyperplasia and the typical pattern of temporal heterogeneity of fibrotic lesions. In fact histological evaluation of lung

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revealed in this model an extensive fibrosis and extracellular matrix deposition with a prominence of hyperplastic alveolar epithelial cells lining the areas of fibrosis. Areas of fibrosis appeared with marked septal thickening areas of collagen deposition (3, 4).

In the context that alveolar type II pneumocytes (ATIICs) can undergo proliferation and differentiation to replace injured alveolar type I cells, thus playing a central role in the natural regenerative process of the injured alveolus (5), we adopted an intranasal (ITN) repetitive bleomycin administration to test possibility to repair acute lung damage and fibrosis by injection of human embryonic cell derived-type II pneumocytes into the airways of bleomycin-damaged mice.

Methods

Animals and experimental groups

A total of 80 female nude mice aged 8-9 weeks (Charles River, Wilmington, MA, USA) were used. They were divided into a group of 25 mice treated with saline solution (Ctrl) and a group of 50 mice intranasal administrated (ITN) with bleomycin (Sanofi Aventis) (4, 6). Briefly, bleomycin was resuspended in steril phosphate buffered saline at 5U/ml and stored at 4°C. Stock was freshly diluted and daily intranasally delivered drop wise using a pipetman in supine position for two weeks (Figure 1). Mice were sacrificed for damage evaluation after 7, 14 and 21 days (Damage). A group of 14 damaged animals received $2.5x10^6$ cells at day 14 by intratracheal instillation (Damage+cells), while the remaining a group of 5 mice were used to evaluate cell teratogenicity.

Animal care was carried out in accordance with European Economic Community Council Directive 86/109, OJL 358, Dec 1 1987 and with NIH Guide for the Care and Use of Laboratory Animals.

Embryonic stem cell differentiation

Human embryonic cell line HUES-3 (Melton Laboratory-Harvard University) (karyotype 46,XY) were cultured according to the manufacturer's protocol (7). For *in vitro* differentiation into alveolar pneumocytes type II cells (HUES-3-ATII Cells), HUES-3 cells were treated with Small Airway Growth Medium (SAGM; Clonetics) for 3 days (8,9). Teratogenicity was evaluated, as previously reported (9). The use of human embryonic stem cells was approved by the Bioethical Committee of the Tor Vergata University Hospital (Rome, Italy).

Histological examination

Lungs were fixed in formalin (24 hours), sectioned and embedded in paraffin. Five μm-thick serial sagittal sections of the entire lungs were cut and stained with Haematoxylin&Eosin or Masson's trichrome to analyze the extent of lung inflammation; the severity of fibrosis was evaluated at 200x magnification according to the Ashcroft method (6). Fibrosis was graded from 0 (normal lung) to 4 (severe distortion of structure, large fibrous areas, and honeycomb lesions). The mean score was calculated as reported.

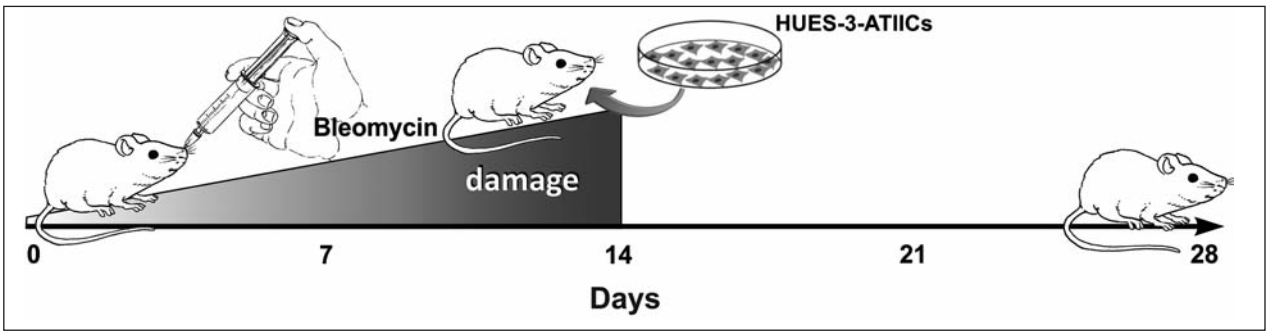

Fig. 1. Timeline of experimental protocol: Nude mice daily received bleomycin drug by intranasal instillation for 14 days. At day 14 some mice intratracheally received human embryonic stem cells pre-differentiated into type II alveolar pneumocytes (HUES-3-ATIICs). Mice were sacrificed before and after cells injection for experimental analysis.

Real-time RT-PCR

Quantitative Real-Time RT-PCR was performed for inflammatory markers interleukin 6 (IL-6), Tumor Necrosis Factor alpha (TNF-α), Monocyte Inflammatory Protein 2 (MIP-2), Collagen Type 1 alpha 1(Col1-α1) and CXC chemokine ligand 12 (CXCL12). GAPDH (glyceraldehyde-3¬phosphate dehydrogenase) was used as housekeeping gene (9). Quantitative values were normalized with respect to those obtained in control healthy mice injected with saline solution.

Measurement of inflammatory cytokine

For homogenization, the tissue was thawed, weighed and 1 μL of RIPA buffer was added for every 0.1gr of tissue. The tissue was then homogenized at 20.0 rpm for 5 min. The homogenates were centrifuged at 12000 rpm for 10 min at 4°C. After centrifugation, supernatants were aliquot in 500 μL and stored at -80°C until analysis. The proteins were measured by Bradford method. All samples were assayed for TNF-α, Interleukin 1 alpha (IL-1α), Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6), Interleukin 10 (IL-10), Interleukin 4 (IL-4) and Matrix metalloproteinase 9 (MMP9) by using commercially available ELISA kits (GeneTex, Inc. San Antonio, Texas). Standard curves and controls were performed according to the guidelines of the producer. Data were analyzed by a semi-automated biochip system (Evidence Investigator).

Immunohistochemistry

Detection of human nuclear antigen (HuNu) was performed on lung paraffin sections using a primary streptavidin-conjugated antibody (HuNu; 1:100; Chemicon) diluted in PBT, as reported (9). For immunofluorescence, samples were incubated with human pro-SP-C (1:2000; Chemicon, Temecula, CA, USA) (8). Images were acquired using a Zeiss (Zeiss, Thornwood, NY, USA) Axioplan 2 microscope.

Gas exchange properties

Blood arterial oxygen saturation was recorded in time course using a small rodent oximeter sensor mounted on the thigh of each experimental animal (MouseOX; STARR Life Sciences, Oakmont, PA, USA). Data were collected for a minimum of 10 sec without any error code and the measurement was repeated six times within a 3-min period.

Statistical Analysis

Data were expressed as mean values ±SEM for multiple independent experiments for statistical analysis the unpaired Student's *t*-test was used. A p value lower than 0.05 was regarded as significant. Kaplan-Maier curve was calculated using the log rank test.

Results

HUES-3 cell injection counteracts bleomycin-induced lung fibrotic remodeling

Histological analysis was carried out to evaluate the effects of HUES-3 cell injection on the lung damage following 14 days of bleomycin administration. In damaged mice, a initially focal and peribronchiolar damage was evident after 7 days, including oedematous changes, hyaline membranes and aggregation of polymorphonuclear neutrophils in both alveoli and interstitium (Figure 2A), according with the literature (2, 4). Inflammatory infiltrates became progressively confluent at 14 days, typically involving large areas of lung parenchyma. By day 21, fibrotic alveolitis was clearly evident. Epithelial-lined cystic spaces and collagen deposition were also present (Figure 2A). In contrast, in HUES-3-ATII cell-treated mice at the same time we observed a reduction of lung parenchymal collagen deposition, as also documented by semi-quantitative evaluation ($p \leq 0.05$, Figure 2B), strongly supporting a positive effect of HUES-3-ATII cell injection against bleomycin-induced lung fibrotic remodeling.

HUES-3-ATII cells injection reduced inflammatory cytokines

In order to evaluate the anti-inflammatory effect of HUES-3 cell injection, we evaluated cytokine gene expression by RT-PCR analysis. As reported in Figure 3A, experimental data showed that both IL-6

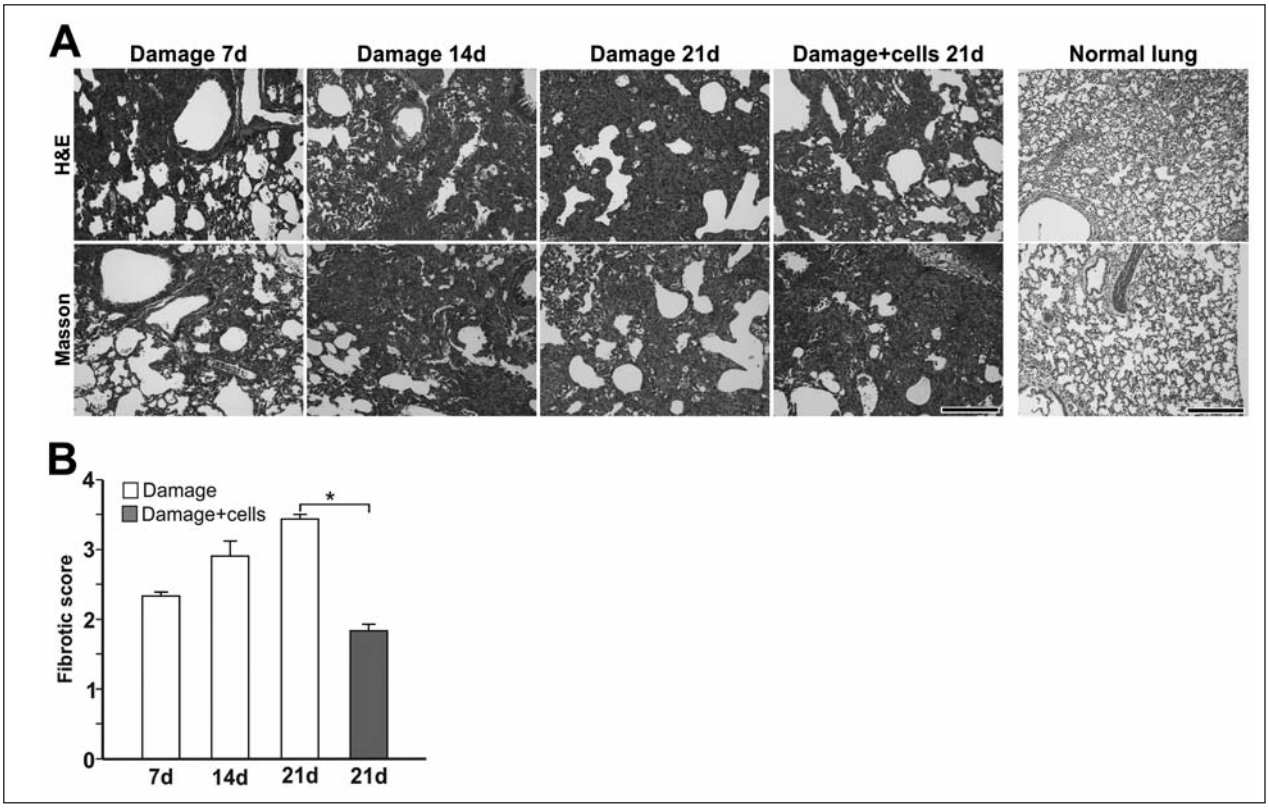

Fig. 2. (A) Haematoxylin&Eosin (H&E) and Masson trichrome staining (Masson) of mouse lungs 7, 14 and 21 days after bleomycin damage, after HUES-3 alveolar type II cell injection (Damage+cells) and healthy mouse lungs tissue (Normal lung). Note in normal mice H&E staining shows regular alveolar structure of lung parenchyma and Masson trichrome staining documents scarce collagen accumulation in perivascular and peribronchial spaces, and not within interstitium. Scale bars: 500 μm and 100 μm. (B) Quantitative evaluation of parenchymal fibrotic changes in mice treated with bleomycin (Damage) and with cells at 21stday (Damage+cells). Fibrotic score (Ashcroft score) was obtained with a continuous numerical scale for determining the degree of fibrotic changes. Data are expressed as mean ± SEM for multiple independent experiments. Statistical comparison was made using unpaired Student's t-test. *($p \leq 0.05$).

and MIP-2 increased to a maximum value already 7 days after damage, specifically 13 and 19 times higher than control uninjured lungs. In cell-injected strongly reduced IL-6 and MIP-2 transcript compared to bleomycin alone lungs (**$p \leq 0.03$; ***$p \leq 0.02$). Consistently with pro-inflammatory and pro-fibrotic cytokine expression, the level of the tissue collagen marker Col1-α1 showed a progressive increase during the damage induction (9.5 fold increase respect to uninjured control). A strong and statistically significant reduction of Col1-α1 mRNA expression was evidenced after HUES-3-ATII Cells injection (***$p \leq 0.02$), strongly supporting an important anti-fibrotic action. The same finding was detected for TNF-α and CXCL12, which was already described as involved in the trafficking of circulating fibrocytes to a fibrotic lungs (10) (Figure 3A).

At the same time, protein level of inflammatory cytokines was quantified by ELISA in ITN-damaged lungs receiving or not HUES-3-ATII Cells injection (Figure 3B). All cytokines analyzed were markedly increased in bleomycin-treated lungs compared to uninjured controls. Expression of examined cytokines as significantly reduced in HUES-3-ATII Cells -injected lungs. In particular, TNF-α, IL-1α and IL-1β rapidly increased to a maximum level soon 7 days after bleomycin treatment, while the increase of IL-6, IL-10 and IL-4 was delayed and became progressive after the damage. Highest levels of inflammation were detected for TNF-α 7 days after bleomycin treatment (5.91 pg/ml vs uninjured control 0.56 pg/ml; $p < 0.01$), for IL-10 (5.47 pg/ml vs 0.9 pg/ml; $p \leq 0.03$) and for IL-4 after 21 days (5.87 pg/ml vs1.7 pg/ml; $p \leq 0.04$). A strong reduction was

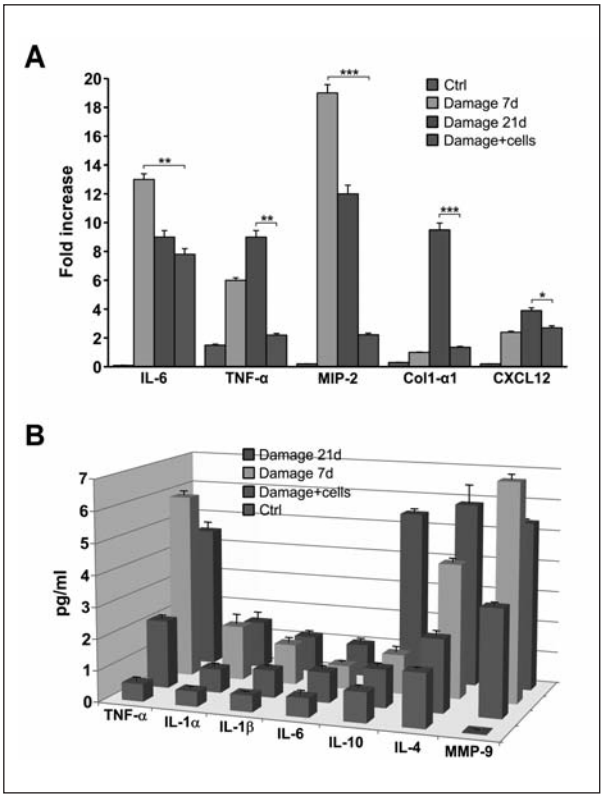

Fig. 3. (A) Real-time RT-PCR analysis of inflammatory cytokines of bleomycin-treated and cells injected lungs. An evident reduction of cytokine expression was found after HUES-3-ATII cells injection (Damage +cells at day 21) respect to damaged mice (Damage 7d and Damage 21d). The fold increase of each transcript is calculated comparing to control healthy mice (Ctrl). All values obtained are the mean of at least three independent experiments performed in triplicate (*p≤ 0.05;**p≤ 0.03;***p≤ 0.02). (B) ELISA test for the measurement of inflammatory cytokines in bleomycin-treated and cells injected lungs.
Data are expressed as mean ± SEM for multiple independent experiments.

evidenced after HUES-3-ATII Cells, with values of 2.17 pg/ml (p≤0.03 vs bleomycin control), 1.21 pg/ml (p≤0.01), and 2.31 pg/ml (p<0.02) for TNF-α, IL-10 and IL-4, respectively. Anti-inflammatory cytokine IL-6 protein level showed an opposite trend compared to TNF-α, as already described (11) MMP9 is mainly involved in tissue remodeling associated with pathological situations such as acute lung injury (12). MMP9 expression increased after bleomycin-induced damage (0.03pg/ml in uninjured control; 6.95pg/ml 7 days after bleomycin, p<0.05; 5.38 pg/ml 21 days after damage, p<0.03) and significantly decreased in HUES-3-ATII Cells injected lungs (3.39pg/ml; p≤0.04), revealing a marked reduction of inflammatory scenario.

Long-term beneficial effect of HUES-3-ATII Cell injection in bleomycin-mice lungs

To assess the human ATII cells engraftment into mouse lungs, we looked for the presence human markers in treated mice 4 weeks after cell administration. Expression of Human Nuclear antigen HuNu (Figure 4A; arrows) was performed using the streptavidin-polyADP ribose polymerase diaminobenzedine peroxidase system and confirmed the presence of human cells within murine tissue. The same cells were immunohistochemically detected in serial sections by human Surfactant Protein-C (SP-C), being the latter exclusively secreted by type II pneumocytes (Figure 4B; arrows). Molecular analysis was also performed in the same tissue and confirmed the presence of human DNA (data not shown) (9).

In parallel, teratogenic potential of differentiated cells, HUES-3-ATII Cells, was evaluated and ruled out by injecting these cells into Nude mice analyzing the animals 1 year after HUES-3-ATII Cells administration by histological examination (data not shown).

Survival curve was evaluated comparing bleomycin damaged mice, damaged mice in which cells have been injected and control healthy mice in which only saline solution has been administrated (Figure 5A). All damaged mice died within 23 days, specifically after14 days of bleomycin treatment surviving mice represented 50% of the total, according to data reported in literature (13). Strikingly, all mice receiving HUES-3 cells survive up to six weeks. Moreover, daily weight measurement showed a strong correlation with the survival rate, since a striking impressive recovery of weight (a mean of about 17 grams 14 days after bleomycin treatment and about 28 grams in cell-receiving mice) was observed.

Lung functionality was also measured by analyzing gas exchange capacity in cell transplanted and untransplanted mice during the induction of damage. Blood oxygen saturation level measurement showed an improvement of oxygenation only in HUES-3-ATII cell-treated mice and not in damaged control mice (Figure 5B), with a functional recovery of about 15%.

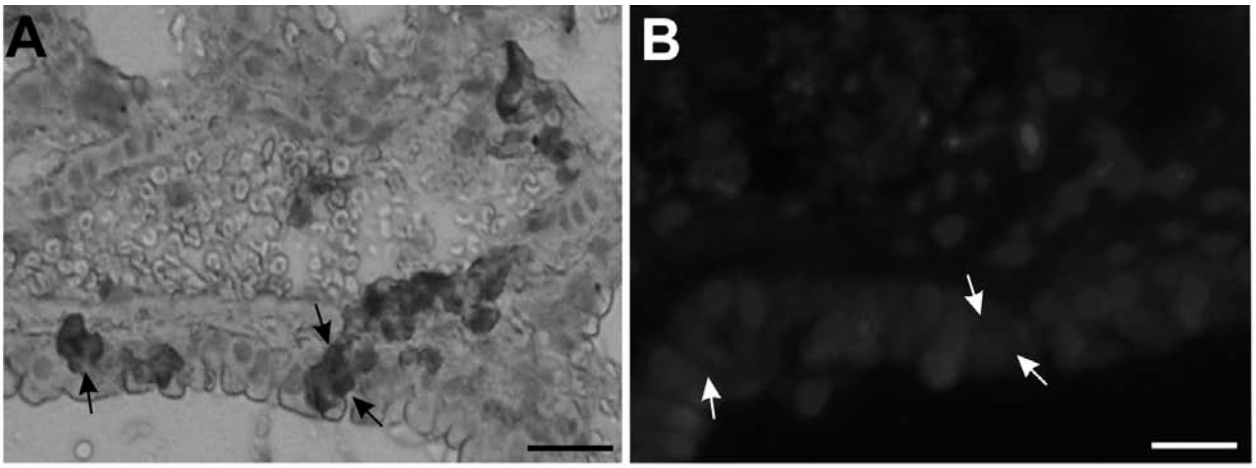

Fig. 4. (A) Immunostaining for human nuclear antigen (HuNu) was performed using the streptavidin-polyADP ribose polymerase (PARP) diaminobenzidine (DAB) peroxidase system on representative sections of bleomycin-treated lung from HUES-3 alveolar type II (ATII) cell-injected mice (Damage+cells). Arrows show positive cells. (B) Subsequent section in which the same cells (arrows) were shown to be positive for human surfactant protein C (SP-C) antibody (red) at higher magnification. Scale bars: (A) 100 μm; (B) 30 μm.

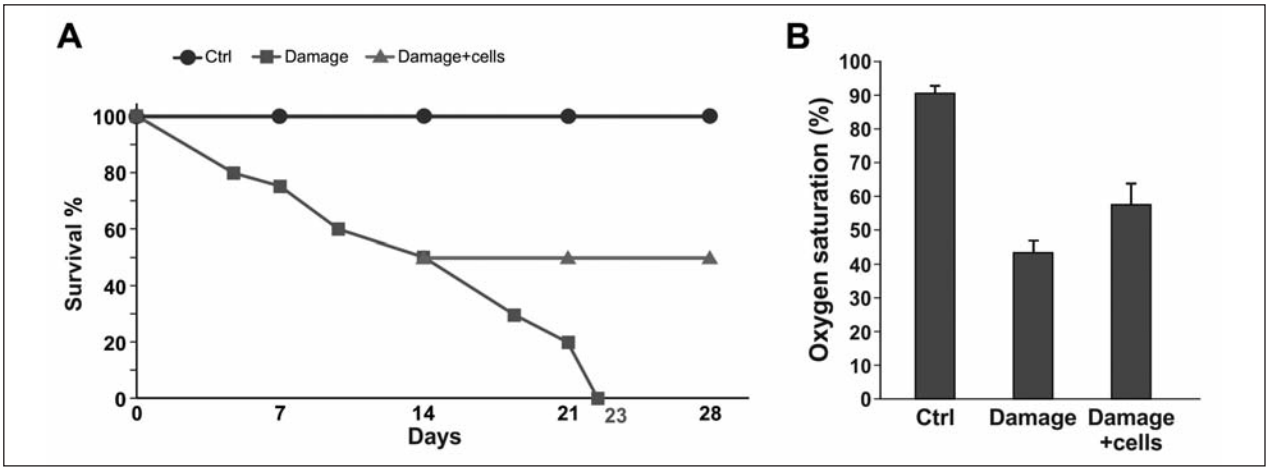

Fig. 5. (A) Survival rate was measured by Kaplan Maier curve from the first day of bleomycin treatment in damaged mice (Damage), in HUES-3-ATII cells-injected mice (Damage+cells) and in control healthy mice (Ctrl). Mice progressively died during bleomycin treatment (until 23rd day), except for those receiving cells that instead survive. (B) Blood arterial oxygen saturation levels were daily recorded during bleomycin damage. After HUES-3-ATII cells injection (Damage+cells), a recovery of arterial oxygen saturation levels was recorded. Significance level is indicated by p=0.02 (comparison of Damage with Damage+cells

Discussion

Bleomycin is a well known fibrogenic agent, provoking an initial adult respiratory distress syndrome-like injury with subsequent strong fibro-proliferative response. Bleomycin is the most widely used drug for chemical induction of pulmonary fibrosis in animal models. It is highly reproducible, inducing a fibrotic histologic remodeling similar to that observed in human lung fibrosis (14).

Idiopathic pulmonary fibrosis (IPF) is caused by the injury and death of alveolar epithelial cells, followed by an abnormal activation of reparative processes leading to fibroblast recruitment (15). Pulmonary fibrosis, homogenously distributed within the lung, with some accentuation of the basal re-

gions, which may be due to preferred intranasal distribution to these areas, was documented by a severe loss of lung compliance, increased soluble collagen and lung hydroxyproline content, a typical reticular fibrosis pattern, and the histologic finding of fibroblast invasion and matrix deposition.

Our histological data revealed that at 14 days lung parenchymal inflammation is present and a not very well established fibrosis; instead, a clear fibrotic stage was documented at 21days. Regarding molecular and biochemical analyses, the inflammatory response becomes evident in tissues by day 7 together with the fibrotic one that becomes apparent at day 21. As for cytokines, IL-6, TNF-α, MIP-2 at the tissue level, the early phase is characterized by patchy neutrophilic alveolitis and the late phase by patchy areas of fibrosis, but there is no formation of hyaline membranes. The extent of fibrosis is proportional to the severity of the injury. ITN method mimics the most severe and pathological conditions of human pulmonary fibrosis, as demonstrated by biochemical and molecular analyses performed to measure protein and transcripts levels (16). Since drug therapy is ineffective to restore a non-pathological pulmonary condition, we verified a new strategy based on cell therapy in an experimental model of lung fibrosis.

A number of studies have been carried out using mesenchymal cells, or cells derived from fetal membrane of human term placenta or also from amniotic fluid in order to repair murine lung epithelium damaged by injury or disease (17-23). These cells expressed some markers of alveolar epithelium following injection into mice and showed a significant anti-fibrotic and anti-inflammatory effect in bleomycin model of injury, most probably due to transient paracrine action of injected cells. Regarding human embryonic stem cells (hESCs), a few papers demonstrated their capacity in differentiating into functional ATIICs (8, 9, 24). The first one reported an *in vitro* differentiation of hESCs into a mixed population of cells containing a small percentage of ATII Cells, and so not suitable for transplantation because carrying a significant risk of producing teratomas after transplantation (8). To solve this problem, a pure population of ATIICs was successively yielded by generating stable transfected hESC lines that, once transplanted in a mouse model of bleomycin induced lung injury, were able to functionally repair the epithelium of the acutely injured alveolus, without causing teratoma formation (24).

As recently published, Spitalieri and colleague showed that human embryonic stem cells (HUES-3) were not only capable of differentiating into type II pneumocytes, but were also able to repair lung injury in a silica experimental model of acute pulmonary fibrosis. Molecular, immunohistochemical functional analyses and ultrastructural examination, demonstrated that a type II pneumocyte phenotype was obtained by differentiating HUES-3 cells (9, 25, 26).

Based on these results, we propose to test the capacity of human embryonic cells to functionally recover lung fibrosis induced in Nude mice by bleomycin administration.

At day 14 (at the end of bleomycin treatment) HUES-3-ATII Cells have been inoculated into damaged mice when lung fibrosis microenvironment had just appeared, as assessed by histological analysis. Treated mice were sacrificed and a recovery of lung function was evidenced by molecular and histological analyses as well as in *vivo* functional evaluation. Increased inflammatory cytokines transcription is observed after bleomycin-induced injury. In particular, IL-6 is significantly elevated in the bleomycin-injured lung, where it may act in synergy with TNF-α (Figure 3A) to perpetuate inflammation and fibrosis (11,27-30). We observed a marked reduction of the expression of IL-6, TNF-α and MIP-2 (suggestive of the pathogenesis of lung injury) only in damaged lungs of mice receiving HUES-3-ATII Cells. These anti-inflammatory effects were also reported when fetal membrane-derived cells and human amnion epithelial cells were transplanted (21,22), suggesting several mechanisms of action used by these cells in reducing inflammation and fibrosis in injected mice.

The mechanism by which HUES-3-ATII Cells resettle the damaged lung remains to be elucidated.

However, when HUES-3-ATII Cells were injected in healthy mice, no human cells were detected there after (data not shown), indicating that differentiated stem cell homing required to the damaged lung the production of inflammation derived factors, such as cytokines. In this regard, it has been shown that the temporal and spatial increase in the expression of CXCL12 in the epithelium of the injured lung provides a gradient for CXCR4-expressing circulating progenitor epithelial cells during re-epithe-

lialization of the basement membrane (10, 31, 32). In our context CXCL12 resulted to be over-expressed in the injured lungs, decreasing after HUES-3-ATII cells injection.

Moreover the expression of the fibrosis marker Col1-α1 was significantly reduced only in differentiated stem cell-transplanted mice indicating a damage repair (24) in which a partial recovery of fibrosis could be suggested (33).

A similar pattern was observed for protein expression before and after cell administration in damaged lungs. MMP9, proteins of the matrix metalloproteinase (MMP) family, are believed to be the main physiological mediators of extracellular matrix degradation. In adult healthy tissue, low level MMP expression mediates normal matrix remodeling, while during inflammation and injury, large amounts of MMPs are produced presumably to repair damaged extracellular matrix. As for all the other markers, MMP9 protein increased during drug treatment, higher soon after 7 than 21 days, but its levels further decreased after cells injection.

Several studies reported the occurrence of epithelial-mesenchymal transition (EMT) within lung murine fibrosis caused by repetitive lung injury with bleomycin. Polarized epithelial cell, which normally interacts with basement membrane via its basal surface, undergoes multiple biochemical changes that enable it to assume a mesenchymal cell phenotype (34, 35). Some biomarkers have been identified to demonstrate the passage of a cell through an EMT. Fibroblast-specific protein 1 (FSP1), α-SMA, and collagen are specific markers for mesenchymal products generated by the EMTs occurring during the development of fibrosis in various organs, such as kidney, liver, lung, and intestine (36-38). These cells continued to exhibit epithelial-specific morphology and molecular markers but showed concomitant expression of the mesenchymal marker and α-SMA. Colocalization of these two sets of distinct markers defines an intermediate phenotype of EMT, indicating cells that have passed only partly through an EMT. Such cells are likely to represent the intermediate stages of EMT, when epithelial markers continue to be expressed but new mesenchymal markers have already been acquired(39).

To monitor if HUES-3-ATII cells eventually underwent EMT process, we performed a dual staining with human SPC and α-SMA markers evidencing the absence of cells expressing both markers within lung section of cell-treated mice (data not shown). Although EMT occurence cannot be completely excluded, these results allowed us to assign a beneficial role to HUES-3-ATII Cells during the damage recovery.

Histological analyses were also performed on lung sections of transplanted animals to detect if any human cells still reside into murine lung tissue. Interestingly, as it has been shown that the turnover of the adult mammalian alveolar epithelium is about 3 to 4 weeks (40), the observation that transplanted HUES-3-ATII Cells are still present as we identified in the lungs of bleomycin injured mice after 4 and up to 10 weeks from administration, suggests their engraftment as well as a long term beneficial effect of HUES-3-ATII Cells. In other words human differentiated cells exerted their effects engrafting human tissue and also secreting anti-inflammatory factors, as already reported in literature (8, 9, 24, 41).

Finally analysis measuring the percentage of oxygen saturation demonstrated after cell administration a discrete recovery of lung function as well as survival rate reported how cells protected mice from an irreparable death occurring in all damaged but untreated animals.

These results indicated stem cell treatment as an innovative strategy for the treatment of airway diseases, devoid of teratogenic risks, as demonstrated by us and other groups using *in vivo* studies in Nude models (24, 41, 42).

References

1. Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008; 295: L379-99.
2. Shen AS, Haslett C, Feldsien DC, Henson PM, Cherniack RM. The intensity of chronic lung inflammation and fibrosis after bleomycin is directly related to the severity of acute injury. Am Rev Respir Dis 1988; 137: 564-71.
3. Degryse AL, Lawson WE. Progress toward improving animal models for idiopathic pulmonary fibrosis. Am J Med Sci 2011; 341: 444-9.
4. Degryse AL, Tanjore H, Xu XC, et al. Repetitive intratracheal bleomycin models several features of idiopathic pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 2010; 299: L442-52.
5. Bishop AE. Pulmonary epithelial stem cells. Cell Prolif 2004; 37: 89-96.
6. Ashcroft T, Simpson JM,Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. J Clin Pathol 1988; 41: 467-70.
7. Cowan CA, Klimanskaya I, McMahon J, et al. Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med 2004; 350: 1353-6.

8. Samadikuchaksaraei A,Bishop AE. Derivation and characterization of alveolar epithelial cells from murine embryonic stem cells in vitro. Methods Mol Biol 2006; 330: 233-48.
9. Spitalieri P, Quitadamo MC, Orlandi A, et al. Rescue of murine silica-induced lung injury and fibrosis by human embryonic stem cells. Eur Respir J 2012; 39: 446-57.
10. Phillips RJ, Burdick MD, Hong K, et al. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest 2004; 114: 438-46.
11. Smith RE, Strieter RM, Phan SH, Lukacs N,Kunkel SL. TNF and IL-6 mediate MIP-1alpha expression in bleomycin-induced lung injury. J Leukoc Biol 1998; 64: 528-36.
12. Corbel M, Boichot E, Lagente V. Role of gelatinases MMP-2 and MMP-9 in tissue remodeling following acute lung injury. Braz J Med Biol Res 2000; 33: 749-54.
13. Izbicki G, Segel MJ, Christensen TG, Conner MW, Breuer R. Time course of bleomycin-induced lung fibrosis. Int J Exp Pathol 2002; 83: 111-9.
14. Moeller A, Ask K, Warburton D, Gauldie J, Kolb M. The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? Int J Biochem Cell Biol 2008; 40: 362-82.
15. Chilosi M, Doglioni C, Murer B, Poletti V. Epithelial stem cell exhaustion in the pathogenesis of idiopathic pulmonary fibrosis. Sarcoidosis Vasc Diffuse Lung Dis 2010; 27: 7-18.
16. du Bois RM Strategies for treating idiopathic pulmonary fibrosis. Nat Rev Drug Discov 2010; 9: 129-40.
17. Ortiz LA, Gambelli F, McBride C, et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci U S A 2003; 100: 8407-11.
18. Sueblinvong V, Loi R, Eisenhauer PL, et al. Derivation of lung epithelium from human cord blood-derived mesenchymal stem cells. Am J Respir Crit Care Med 2008; 177: 701-11.
19. Carraro G, Perin L, Sedrakyan S, et al. Human amniotic fluid stem cells can integrate and differentiate into epithelial lung lineages. Stem Cells 2008; 26: 2902-11.
20. Moodley Y, Atienza D, Manuelpillai U, et al. Human umbilical cord mesenchymal stem cells reduce fibrosis of bleomycin-induced lung injury. Am J Pathol 2009; 175: 303-13.
21. Cargnoni A, Gibelli L, Tosini A, et al. Transplantation of allogeneic and xenogeneic placenta-derived cells reduces bleomycin-induced lung fibrosis. Cell Transplant 2009; 18: 405-22.
22. Moodley Y, Ilancheran S, Samuel C, et al. Human amnion epithelial cell transplantation abrogates lung fibrosis and augments repair. Am J Respir Crit Care Med 2010; 182: 643-51.
23. Tzouvelekis A, Antoniadis A,Bouros D. Stem cell therapy in pulmonary fibrosis. Curr Opin Pulm Med 2011; 17: 368-73.
24. Wang D, Morales JE, Calame DG, Alcorn JL,Wetsel RA. Transplantation of human embryonic stem cell-derived alveolar epithelial type II cells abrogates acute lung injury in mice. Mol Ther 2010; 18: 625-34.
25. Baritussio A, Bellina L, Carraro R, et al. Heterogeneity of alveolar surfactant in the rabbit: composition, morphology, and labelling of subfractions isolated by centrifugation of lung lavage. Eur J Clin Invest 1984; 14: 24-9.
26. Fang X, Song Y, Hirsch J, et al. Contribution of CFTR to apical-basolateral fluid transport in cultured human alveolar epithelial type II cells. Am J Physiol Lung Cell Mol Physiol 2006; 290: L242-9.
27. Olszewski MA, Huffnagle GB, McDonald RA, et al. The role of macrophage inflammatory protein-1 alpha/CCL3 in regulation of T cell-mediated immunity to Cryptococcus neoformans infection. J Immunol 2000; 165: 6429-36.
28. Li XY, Donaldson K, Brown D,MacNee W. The role of tumor necrosis factor in increased airspace epithelial permeability in acute lung inflammation. Am J Respir Cell Mol Biol 1995; 13: 185-95.
29. Gosset P, Lassalle P, Vanhee D, et al. Production of tumor necrosis factor-alpha and interleukin-6 by human alveolar macrophages exposed in vitro to coal mine dust. Am J Respir Cell Mol Biol 1991; 5: 431-6.
30. Driscoll KE, Carter JM, Howard BW, Hassenbein D, Janssen YM, Mossman BT. Crocidolite activates NF-kappa B and MIP-2 gene expression in rat alveolar epithelial cells. Role of mitochondrial-derived oxidants. Environ Health Perspect 1998; 106 Suppl 5: 1171-4.
31. Murdoch C. CXCR4: chemokine receptor extraordinaire. Immunol Rev 2000; 177: 175-84.
32. Ceradini DJ, Kulkarni AR, Callaghan MJ, et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 2004; 10: 858-64.
33. Zhao F, Zhang YF, Liu YG, et al. Therapeutic effects of bone marrow-derived mesenchymal stem cells engraftment on bleomycin-induced lung injury in rats. Transplant Proc 2008; 40: 1700-5.
34. Kalluri R,Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 2003; 112: 1776-84.
35. Kalluri R,Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009; 119: 1420-8.
36. Zeisberg EM, Potenta SE, Sugimoto H, Zeisberg M,Kalluri R. Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. J Am Soc Nephrol 2008; 19: 2282-7.
37. Zeisberg M, Yang C, Martino M, et al. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem 2007; 282: 23337-47.
38. Okada H, Ban S, Nagao S, Takahashi H, Suzuki H, Neilson EG. Progressive renal fibrosis in murine polycystic kidney disease: an immunohistochemical observation. Kidney Int 2000; 58: 587-97.
39. Zeisberg M,Neilson EG. Biomarkers for epithelial-mesenchymal transitions. J Clin Invest 2009; 119: 1429-37.
40. Haase MG, Klawitter A, Bierhaus A, Geyer P, et al. Inactivation of AP1 proteins by a nuclear serine protease precedes the onset of radiation-induced fibrosing alveolitis. Radiat Res 2008; 169: 531-42.
41. Banerjee ER, Laflamme MA, Papayannopoulou T, Kahn M, Murry CE, Henderson WR, Jr. Human embryonic stem cells differentiated to lung lineage-specific cells ameliorate pulmonary fibrosis in a xenograft transplant mouse model. PLoS One 2012; 7: e33165.
42. Wetsel RA, Wang D,Calame DG. Therapeutic potential of lung epithelial progenitor cells derived from embryonic and induced pluripotent stem cells. Annu Rev Med 2011; 62: 95-105.