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The Pathology of Bleomycin induced Fibrosis is Associated with Loss of Resident Lung Mesenchymal Stem cells which Regulate Effector T-cell Proliferation

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Abstract

Tissue resident mesenchymal stem cells (MSC) are important regulators of tissue repair or regeneration, fibrosis, inflammation, angiogenesis and tumor formation. Here we define a population of resident lung mesenchymal stem cells (luMSC) that function to regulate the severity

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of bleomycin injury via modulation of the T-cell response. Bleomycin induced loss of these endogenous luMSC and elicited fibrosis (PF), inflammation and pulmonary arterial hypertension (PAH). Replacement of resident stem cells by administration of isolated luMSC attenuated the bleomycin-associated pathology and mitigated the development of PAH. In addition, luMSC modulated a decrease in numbers of lymphocytes and granulocytes in bronchoalveolar fluid and demonstrated an inhibition of effector T cell proliferation in vitro. Global gene expression analysis indicated that the luMSC are a unique stromal population differing from lung fibroblasts in terms of proinflammatory mediators and pro-fibrotic pathways. Our results demonstrate that luMSCs function to protect lung integrity following injury however when endogenous MSC are lost this function is compromised illustrating the importance of this novel population during lung injury. The definition of this population in vivo in both murine and human pulmonary tissue facilitates the development of a therapeutic strategy directed at the rescue of endogenous cells to facilitate lung repair during injury.

Keywords

resident lung mesenchymal stem cells; pulmonary fibrosis; pulmonary hypertension; stem cells

INTRODUCTION

Pulmonary fibrosis (PF) affects a significant number of individuals worldwide, with current estimates of patients in the United States at over 500,000 ((1)PF Foundation website). The incidence of PF is expected to increase as the population ages (2). PF may occur secondary to other conditions such as interstitial lung disease, connective tissue disease, and viral infection, although it may also appear without a known cause. PF is characterized by a loss of functional alveolar surfaces for gas exchange and is often complicated by pulmonary arterial hypertension (PAH). The pathology of both involves the influx of T lymphocytes and other inflammatory cells to the lung (3, 4). Alterations in the phenotypes of these T cells, including effector and regulatory subsets, modulate the inflammatory responses in lung (3, 4). Currently there are no effective treatments for PF or PAH, however, stem cell-based therapies are increasingly being explored (1).

Previous studies have focused on the potential therapeutic impact of bone marrow mesenchymal stem cells (BM-MSC) or stromal cells on a variety of lung conditions. These cells have been isolated from BM, expanded *ex vivo* and reintroduced into patient populations and various rodent models of disease. Their reported effects have largely been beneficial, including documented antiinflammatory, proangiogenic and reparative properties, as opposed to contributing to further disease pathology. The administration of BM-MSCs has specifically reported beneficial effects in acute lung injury (ALI), neonatal chronic lung disease or injury, bleomycin-induced pulmonary fibrosis, radiation induced lung injury, and monocrotaline induced pulmonary hypertension (5–16). BM-MSCs are distinct from circulating bone marrow derived mesenchymal cells (BM-MCs) that are hematopoietic in origin, and may be termed fibrocytes. These latter cells are recruited to tissue stroma over the course of adult lifetime, (17) as well as to sites of injury and hypoxia where they lose hematopoietic characteristics and differentiate into mesenchymal cell lineages such as fibroblasts, myofibroblasts, endothelium, stroma and adipocyte progenitors (18, 19). BM-MCs are localized in fibroblast foci during PF and are also located in the remodeled media and adventitial vascular layers associated with PAH (18–22). Thus, hematopoietic-derived mesenchymal cells appear to *contribute* to disease development compared to BM-MSC, that *ameliorate* disease. Although, the hematopoietic derived mesenchymal cell is similar to BM-MSC in terms of surface markers and multilineage

differentiation potential, it is likely their resident tissue niche specifies function during the development of disease.

Our laboratory and that of Summer et al. have isolated a population of lung-resident mesenchymal stem cells (LuMSC) using flow cytometry to detect Hoechst 33342 vital dye efflux. We demonstrated that these cells had multilineage differentiation potential (osteocyte, adipocyte and chondrocyte) and characteristics of 'stemness' including high telomerase activity (23, 24). We hypothesized that loss of LuMSC in response to bleomycin injury is in part responsible for the pathology and that replacement of this population would attenuate injury via regulation of T-cell proliferation. In the present study we rigorously define a population of resident lung MSC which can be isolated based upon cell surface determinants as well as localized in vivo. BM transplantation studies confirmed the resident lung origin of the adult LuMSC. Following bleomycin injury we defined a decrease in the resident LuMSC population via identification of H33342^{low}CD45^{neg} by flow cytometry and in vivo by immunostaining to detect the multidrug resistance transporter ABCG2 (25, 26). For the first time ABCG2 has been validated as a marker for this LuMSC population in both murine and human lung tissue. We also explored the potential for replacement of the LuMSC with exogenously administered cells to attenuate bleomycin-induced PF and associated PAH via the regulation of effector T cell proliferation. LuMSC protected against lung injury in the absence of detectable engraftment in tissue while lung fibroblasts had no effect. The inhibition of T-cell proliferation was limited to the LuMSC and not fibroblasts. With these anti-inflammatory effects, LuMSC may represent a resident lung stromal cell type important in the maintenance of tissue integrity. These results illustrate the importance of further defining a role for these cells in vivo during tissue homeostasis and disease.

Materials and Methods

Modeling of bleomycin fibrosis and associated pulmonary arterial hypertension

All procedures and treatments were approved by the Institutional Animal Care and Use Committee. Bone marrow transplants were performed as previously described using GFP donor mice and C57Bl6J recipients (17). Bleomycin injury was performed using C57Bl6J female mice (27). Mice were euthanized at 14 or 35 days following bleomycin treatment. Lung MSC or lung fibroblast cells (FB) were injected via tail vein (150,000 or 250,000 cells in 100µl PBS). The mice were randomized and distributed as 3–5 mice per cage for study. PBS treated BMtx mice n=4; Bleomycin treated BMtx mice n=6.

The evaluation of fibrosis was performed by identifying fibrotic findings in each section which were scored using the criteria of Ashcroft and colleagues (28). The number of test subjects per group were as follows: control n=5, day 14 bleomycin n=7, lung MSC +PBS n=3, lung fibroblast (FB) + PBS n=3, lung MSC + Bleomycin d14 n=5, lung FB + Bleomycin d14 n=7. PAH was documented by measurement of right ventricular systolic pressure (RVSP) and quantification of muscularization as an indirect measure of pulmonary artery pressure and remodeling as previously described (27, 29). The number of test subjects per RVSP group were as follows: control n=6, day 14 bleomycin n=5, lung MSC +PBS 6, lung fibroblast (FB) + PBS n=3, lung MSC + Bleomycin d14 n=19, lung FB + Bleomycin d14 n=9. For muscularization analysis the number of test subjects per group were as follows: control n=4, day 14 bleomycin n=4, lung MSC +PBS n=3, lung fibroblast (FB) + PBS n=3, lung MSC + Bleomycin d14 n=4, lung FB + Bleomycin d14 n=4. The results are presented as the average number of muscularized actin positive vessels per field of view. The total number of mice in each group for all experimental analyses were: control n=15, day 14 bleomycin n=25, lung MSC +PBS n=9, lung fibroblast (FB) + PBS n=3, lung MSC + Bleomycin d14 n=22, lung FB + Bleomycin d14 n=20.

Phenotypic and Functional Analyses of luMSC

LuMSCs were isolated, differentiated and cultured as described previously (23) from wt C57Bl6/J mice, B6.129S7-Gt(ROSA)26Sor/J or C57Bl6 ABCG2-GFP (25). Two to three separate analyses were performed on 30,000 to 50,000 cells per sample. LuFB were isolated from aliquots of this single cell suspension using the same culture conditions. BAL was collected as previously described (28). Single cell suspensions of Hoechst stained lung tissue or BAL were stained with antibodies to the cell surface markers indicated in the figure legends (Supplemental Table 1). Gates were set using fluorescent minus one (FMO) controls. Phenotypic analyses were repeated twice independently. Gating strategies included FSC/SSC, dead cell exclusion with either propidium iodide or DAPI, red blood cell exclusion with Ter119 and doublet discrimination. Controls for flow cytometry included Hoechst stained BM, unstained cells and cell suspensions incubated with conjugated isotype matched control antibodies. Controls for analysis of GFP positive lung cells included a wildtype and GFP mouse lung (Supplemental Figure 6). Hoechst^{low}CD45^{neg} luMSC were sorted using a Legacy Moflo cell sorter with Summit 4.3 software (Beckman Coulter, Miami, FL). Sort mode was set to Purify 1. BAL and luMSC were analyzed on a CyAn ADP flow cytometer (Beckman Coulter, Miami, FL). The number of test subjects per BAL group were as follows: control 7, day 14 bleomycin 7, luMSC +PBS 3, luFB + PBS 3, luMSC + Bleomycin d14 19, luFB + Bleomycin d14 3. Multilineage differentiation and characterization of luMSC to adipocyte, chondrocytes and osteoblast lineages was performed as previously described (13, 17, 23).

B-galactosidase staining of lung tissue and Lung MSC—Lung tissues were harvested on day 14 following bleomycin injury and introduction of cells, inflated with a 1:1 OCT:30% sucrose mixture and snap frozen. LuMSC and luFB were isolated from C57Bl6/ROSA^{lacZ} mice and expanded in culture using chamber slides (Supplemental Figure 7). Frozen sections (12 μ M) or cell chamber slides of experimental lung tissue were fixed with 4% paraformaldehyde for 30 minutes and LacZ staining was performed as previously described (17). 75–100 slides were analyzed per lung sample. The number of test subjects per group were as follows: control 4, day 14 bleomycin 4, luMSC + Bleomycin d14 6.

Immunohistochemistry

Paraffin embedded mouse lung sections treated using a standard processing method for immunostaining (17). Human lung tissue was collected at Vanderbilt University, Nashville TN under the IRB#060203 PI: Barbara Meyrick. Incubation with primary antibodies was performed using proliferating cell nuclear antigen (PCNA), CD3, smooth muscle alpha actin (SMA) (1:500; DAKO, Ft. Collins, CO) or ABCG2 (1:100; BD Pharmingen, SanDiego CA) overnight followed by Alexa 488 or 594 fluorescent secondary antibody (1:500, Invitrogen, Carlsbad, CA). Coverslips were mounted with DAPI (Vector Laboratories, Burlingame, CA).

Imaging

All images were captured with a Nikon Eclipse 90i upright epifluorescence microscope. Objective lenses included a Nikon 4 $\times$ (WD 20) and 10 $\times$ (DICN1). Fluorochromes used included DAB, DAPI, Alexa 488 and Alexa 594. The camera used to capture the images was a Nikon DS-Fi1 using the Nikon NIS elements AR 3.10 acquisition software.

Transcriptome Analysis

Total RNA was prepared with PicoPure RNA Isolation Kit reagents (Arcturus Bioscience Inc., Moutnain View, CA) from two independently isolated cultures of luMSC (n=2) or luFB (n=2). Complimentary DNA generated from amplified RNA was hybridized to

duplicate Affymetrix (Santa Clara, CA) Mouse gene 1.0st chips. Array analysis and qRT-PCR validation was performed as described (17). qRT-PCR assays were performed in triplicate and levels of analyzed genes normalized to glyceraldehyde-3-phosphate dehydrogenase abundance (primer list is provided in Supplemental Table 2).

T cell proliferation assay

The T cell proliferation assays were performed as described (30) with the following modifications: CD4⁺ T cells from spleens of TCR transgenic mice, OT-II with T cells that recognize the ovalbumin 323–339 peptide, were purified by depleting CD8⁺, CD19⁺, MHC-II⁺ and CD25⁺ cells. Antigen presenting cells (APC) were isolated by positively sorting MHC-II⁺ cells. They were then fixed and loaded with 0.5 µg/ml or 5 µg/ml OVA323 peptide. LuMSC or luFB (6.25×10^4) were plated with growth arrested APC (1×10^6) in 96 well plates. Purified CD4⁺ 1×10^5 cells, typically greater than 95% pure were labeled with CFSE, washed and added to APC/cells. Cells were incubated for 4 days then stained with anti anti-CD40 (Cy5) and CD4 (APC) and assayed using a Miltenyi MacsQuant 7 channel flow cytometer and the FloJo analysis software. Proliferation was measured as decrease in mean fluorescent intensity of CFSE compared to background, T cells labeled with CFSE and not exposed to APC. This assay was performed twice independently.

Statistical Analysis

Data analyzed by one-way ANOVA followed by Tukey HSD post-hoc analysis using JMP version 5.0.12. Significance was defined as p value < 0.05*, p value < 0.01** or p value < 0.001 ***.

RESULTS

Defining the Hoechst^{dim} CD45^{neg} resident lung MSC

We and others have previously defined a lung MSC population that can be enriched by flow cytometric sorting of Hoechst 33342 stained lung tissue to identify a Hoechst^{dim} side population (SP) of cells (23, 24). To date information is lacking as to the true origin of these cells as tissue specific or bone marrow derived as well as the role of these cells in vivo during tissue homeostasis, injury and repair. Largely these shortcomings are due to an inability to localize these luMSC in tissue. We address this issue in our current studies which further characterize the luMSC and define ABCG2 as a marker appropriate to study these cells in vivo. In addition we localize these cells to the alveolar-capillary barrier in both murine and human tissue.

Common between the lung mesenchymal populations is their characteristic cell surface expression of MSC markers (CD73, CD105, CD106, CD 44, Thy1/CD90, CD133 and Sca1) and lack of hematopoietic and vascular markers, including CD45, F480, c-kit and CD146 (Supplemental Figure 1). The MSC also differentiate into three characteristic mesenchymal lineages: osteoblast, chondrocyte and adipocyte (Supplemental Figure 1). However the tissue-specific origin of these cells has not been defined. Therefore we employed bone marrow transplant analysis of lethally irradiated wild type (wt) C57Bl/6 mice using genetically marked GFP donor marrow to determine whether the adult luMSC were of local or bone marrow origin. Engraftment of recipient mice was found to be greater than 85% by analysis of peripheral blood 15 weeks post-transplant. At that time, mice were treated with a single intratracheal dose of bleomycin or PBS control. 35 days post injury the lungs were isolated, digested to single cell suspension and co-stained to evaluate CD45 and GFP expression in the SP/Hoechst^{dim} population (gated region) (Figure 1A). We found that the lung MSC population (Hoechst^{dim} CD45^{neg}) cells were GFP negative, indicating a resident tissue origin of luMSC (Figure 1B). In contrast approximately 30% of the Hoechst^{dim}

CD45^{pos} cells were derived from the GFP positive bone marrow in both control and bleomycin-injured lungs. Interestingly the subpopulation of SP cells (Hoechst^{dim}) decreased 2.9-fold 35 days following bleomycin injury.

Loss of the luMSC is associated with the pathology of bleomycin injury

We then performed a time course experiment to analyze the effects of bleomycin treatment on the SP population and found a significant decrease on days 7 and 14 following bleomycin relative to the uninjured control mice (Figure 2A). Taken these analyses further we localized these cells in vivo by immunolocalization of the multidrug resistant transporter ABCG2. ABCG2 confers the Hoechst^{dim}SP phenotype by facilitating the efflux of Hoechst 33342 dye (25, 26). As proof of principle we confirmed the expression of ABCG2 in lung SP using flow cytometric analysis of lung cell suspensions obtained from mice in which GFP expression is driven by the ABCG2 promoter (25) (Supplemental Figure 2). Essentially ABCG2 expression was enriched and detectable in the SP and undetected when analyzing the single cell suspension of lung (or whole lung tissue). Therefore ABCG2 protein expression was adequate determinant to localize these cells in lung tissue sections 14 days following intratracheal administration of PBS or bleomycin (Figure 2B). Immunostaining confirmed a decrease in ABCG2 positive cells 14 days following bleomycin treatment. These cells were localized to the alveolar-capillary interface of the distal lung by dual staining to detect ABCG2 and PECAM-1 (Figure 2C). To determine whether this ABCG2 population had clinical relevance from rodent to patient tissue ABCG2 immunostaining was used define this population of cells to the interstitium of control human lung tissue (Figure 2D). We may therefore use the population of cells further characterized in these studies to extrapolate their potential function and importance in human lung.

Replacement of the luMSC population attenuates bleomycin fibrosis and PAH in vivo

Other laboratories have reported that BM-MSC treatment attenuated remodeling and inflammation in rodent models of PF and ALI (6–10, 12, 14–16, 22, 31). Here we sought to analyze the effects of luMSC on bleomycin-mediated PF with PAH. Bleomycin or PBS was instilled intratracheally in wildtype mice followed by an intravenous injection of PBS, genetically labeled (lacZ expressing) male luMSC (1.5 or 2.5×10^5), or lung fibroblast (luFB) cells. Histological differences in the degree of fibrosis were evident at day 14 post injury when comparing the bleomycin treated and bleomycin + luMSC groups (Figure 3A). The bleomycin+luMSC treated group was similar in appearance to the untreated control group, while the bleomycin+ luFB appeared similar to the bleomycin treated group with fibrotic pathological changes. The degree of fibrosis was measured using the Ashcroft scoring system (28, 32). LuMSC administration decreased the degree of fibrosis by 1.7 fold (Figure 3B). This protective effect was absent in the luFB treated group. Treatment with luMSC significantly decreased weight loss associated with bleomycin, and increased survival from 50% at 14 days with bleomycin alone to 80% in the setting of bleomycin and luMSCs. Additionally, luMSC decreased PAH measured by RVSP in parallel with decreasing muscularization of small caliber (0–100 μ M) pulmonary vessels (Figure3C).

Immunoregulatory properties of luMSC

The role of T cells in PF has been controversial in terms of disease pathogenesis. In PAH, however, alterations in the circulating subpopulations of CD4 and CD8 cells and their accumulation in tissue suggest a dysfunction of the immune response (3, 4). BM-MSC and BAL-derived MSC decrease inflammatory cell trafficking to the alveolar space, and limit T cell proliferation (15, 33). To determine whether the beneficial impact of luMSC on PF might be due to changes in the populations of inflammatory cells present in the alveolar space following bleomycin injury, BAL was collected in animals concurrent with tissue collection, and analyzed by flow cytometry. The peak inflammatory response detectable in

BAL occurred on day 14 following injury and was consistent with published literature (Supplemental Figure 3). LuMSC treatment decreased T cell (Thy1/CD90), granulocyte (GR1) and B cell (B220) population (Figure 4 A,B). No significant change was detected in monocyte/macrophage analysis between groups (CD11b/F480).

Qualitative analysis of T cell localization and proliferation in lung tissue following bleomycin injury was performed by immunostaining of lung tissue collected on days 7 and 14 (post-injury) to detect T cells and PCNA. T cells were localized *in vivo* using the pan T cell marker CD3 which detects both CD4 and CD8 positive cells, its expression overlapping with Thy1/CD90. Thy1/CD90 interacts with CD3 via the T cell receptor complex, the expression of which may be found on both resting and activated T cells. Therefore we anticipate that the trends observed in Thy1/CD90 observed in the BAL following bleomycin and cell treatment will be recapitulated by CD3. Examining expression of CD3 would allowed the detection of a difference in T cell numbers, while PCNA expression, proliferation. On days 7 and 14 following bleomycin injury, CD3 positive cells were increased relative to the PBS treated controls and decreased with luMSC treatment (Figure 4C). CD3 positive cells localized to the parenchyma as well as fibrotic regions. Few CD3 / PCNA positive cells were present on days 7 and 14 in the lung tissue (Supplementary Figure 4) which may suggest that the regulation of the T cell response to bleomycin occurs outside the lung.

To further substantiate a role for luMSC in the regulation of the T cell response we performed *in vitro* proliferation analyses. LuMSC and luFB were co-cultured with growth-arrested antigen presenting cells and effector enriched T cells labeled with the membrane dye CFSE. T cells were isolated from OT2 mice and demonstrated a dose dependent proliferative response to ovalbumin ligand (OVA ; (30)). After 72 hours, with increasing doses of OVA, the presence of luMSC, not luFB, decreased T cell proliferation (Figure 5A). Interestingly, increasing the numbers of T effector cells resulted in an amplification of the anti-proliferative effect. No apoptosis was detected in any groups at 72 hours. Lung MSC also expressed the surface determinant CD80, a co-stimulatory molecule which is capable of facilitating lymphocyte interactions (Figure 5B,C).

LuMSC are a unique stromal cell population distinct from luFB

Global gene expression analysis was employed to determine whether the luMSC were indeed a distinct population of cells present in the pulmonary stroma relative to luFB. These two distinct populations were isolated from the same sets of pooled lung tissue and allowed the comparison of different stromal fractions from the same lung preparations. cDNA from each sample was hybridized to murine whole genome arrays. Principal component analysis showed that the luMSC were a significantly distinct population from luFB (Figure 6A). Each set of duplicate samples segregated to a different region of the diagram. Supervised hierarchical analysis of genes associated with immune response, extracellular matrix, blood vessel development / angiogenesis, and wnt/ Tgf β /stemness revealed that luFB expressed increased levels of inflammatory mediators (including TNF and IL-1), higher levels of pro-fibrotic and decreased angiogenic genes (Figure 6B, Supplemental Table 3). LuMSC demonstrated decreased expression of proinflammatory mediators, myofibroblast specification and extracellular matrix production (Supplemental Table 3). qRT-PCR analysis validated changes in specific gene expression identified by microarray analyses including genes associated with immune response (chemokine ligands 5, 12 and 14 (Cxcl5, 12 & 14), associated fibrosis associated genes including target genes of the wnt pathway (secreted frizzled related protein 1 (sfrp1), T-box20 (tbx20), periostin (postn), typeIII collagen (col3a1) and angiogenesis (flk-1))(Supplementary Figure 5). Both luMSC and luFB expressed the antiinflammatory molecule AIP6/TSG6 while IL1RN expression was restricted to the MSC (Supplementary Figure 5). These results were consistent with

functional differences observed between luMSC and luFB described in these studies. When compared to published BM-MSC gene lists luMSC and luFB share similar expression patterns (Supplemental Table 4) (34–37). The subcategories compared included adult MSC genes versus fetal, signature BM-MSC genes and stemness candidates.

DISCUSSION

We have identified an immunomodulatory role for a novel population of resident lung MSC during lung injury. We have identified ABCG2 as a marker appropriate to localize and study these cells in vivo in both murine and human tissue. We documented a decrease in endogenous luMSC following bleomycin injury, suggesting that endogenous luMSC fail to effectively participate in lung repair. Our work definitively demonstrates that rescue of luMSC with exogenous cells attenuates the inflammation, fibrosis and pulmonary arterial hypertension associated with bleomycin induced lung injury. One potential mechanism we explored to explain the decrease in inflammation is a decreased T-effector cell proliferation mediated by luMSC. This inhibitory effect is limited to the MSC and not lung fibroblasts. The studies described herein also highlight a resident lung origin for a population of luMSC distinct in function and transcriptome from lung fibroblasts. Our work suggests that “rescuing” endogenous luMSCs to initiate repair in the setting of lung injury could mitigate the need for cell transplant. The ability to study these cells in vivo in both murine and human tissue provides a novel tool for the study of lung MSC.

Multipotent MSC have been isolated and characterized from various tissues (38–40). MSC with a phenotype similar to BM-MSC, but associated with pulmonary tissue have also been identified in the SP (23, 24), BAL from human lung allografts (41) and as a Sca-1 positive fraction of adult mouse lung (42). More recently we have demonstrated that over the course of a lifetime hematopoietic cells from myeloid lineage take up residence in stroma of adipose tissue, assume a phenotype indistinguishable from tissue resident MSC and differentiate to fat (17). Taking these data into consideration we analyzed the origin of the luMSC by bone marrow transplantation analysis. After 20 weeks in the presence or absence of injury we found the Hoechst^{low}CD45^{neg} cells were consistently GFP negative, therefore derived from adult lung tissue. It is interesting to note that Lama et al. (41) also identified the lung tissue origin of BAL derived MSC in patient allograft tissue following transplantation; however, an initial BM origin cannot be excluded at this time.

Given the phenotypic similarities of luMSC to BM-MSC and their resident tissue origin we hypothesized that luMSC would participate in the repair process of lung following bleomycin injury. LuMSC administered intravenously at the time of injury decreased the degree of fibrosis and collagen deposition in lungs as determined by Ashcroft scoring 14 days following intratracheal bleomycin exposure. Measurement of right ventricular systolic pressures demonstrated the attenuation of PAH and associated vascular remodeling. A caveat to these studies was the ability of luFB treatment to decrease muscularization in response to bleomycin treatment to levels similar to luMSC. While the positive effects of luMSC may be attributed to the regulation of effector T cell proliferation, the effect of luFB observed in these studies may be explained by differences in vessel SMC thickness, as well as differences in immunomodulation or differences in additional paracrine factors produced between the FB and MSC resulting in SMC contraction or proliferation. Essentially lung FB were included in these studies as a lung derived mesenchymal or stromal cell type lacking the stem cell properties which would characterize them as MSC. Further characterization of differences between luFB and luMSC is necessary in order to elucidate the factors that distinguish therapeutic effects of lung MSC. Surprisingly, similar to BM-MSC, protective effects occurred in the absence of lung engraftment. One may hypothesize that a luMSC would differentiate into lung tissue types during repair. However the issue of niche for these

cells to reside during injury may be an obstacle to their engraftment or differentiation. Additionally, the possibility for isolation and culture procedures altering these cells' function cannot be discounted.

Another important observation of our studies was the immunosuppressive effect of luMSC. LuMSC administration down-regulated the appearance of inflammatory cells in the BAL and was associated with an anti-proliferative effect on effector T cells in vitro. In patients with IPF, and in animal models of fibrosis, the response of the lung environment has been reported to be profibrotic, antifibrotic or have no effect depending on the phenotype of T cells present (4). To determine whether the anti-inflammatory effect of luMSC was related to the regulation of T cell proliferation previously reported for BM-MSC and BAL derived MSC (33) we performed co-culture analysis. We demonstrate that luMSC significantly decreased effector T cell proliferation while luFB had no effect. Interestingly, with increasing concentrations of T cells the inhibition of proliferation was more pronounced suggesting that the T cells were effectively communicating with each other. MSC have also been shown to induce apoptosis of activated T cells (43), however, we did not identify apoptosis as a mechanism of regulation in our assays. A paracrine anti-inflammatory role for BM-MSC has been described for the decrease in macrophage secretion of TNF (7, 15) and T cell proliferation (33) via the soluble mediators IL-1RN, AIP6 and PGE₂, respectively. However, these and other previous reports have characterized a lack of T cell costimulatory molecules on the MSC, whereas we have identified the presence of CD80 receptor protein and mRNA in these luMSC and luFB under basal conditions. The presence of CD80 suggests that although a paracrine role has been described for MSC, cell-cell contact with T cells may also mediate important effects by these populations. It is also important to note that T cell proliferation was not observed in lung tissue following bleomycin injury on days 7 or 14, suggesting that the luMSC likely regulates the T cell response in an extrapulmonary site. LuMSC likely represent a resident pulmonary stromal cell type similar to BM-MSC which exhibit anti-inflammatory effects via regulation of T effector proliferation. Both luMSC and luFB may also regulate additional T cell subpopulations which merits further study.

In light of our data demonstrating that luMSC but not luFB attenuate bleomycin induced lung injury, it is interesting to consider differences between these stromal cell populations within the pulmonary tissue. Transcriptional profiling indicated that the luMSC differ from lung fibroblasts in decreased expression of genes involved in inflammation and fibrosis. LuFB express increased levels of selected genes such as Cxcl14, and fibrosis related genes periostin (44), type III collagen as well as the wnt/βcatenin targets Tbx20 (45) and sfrp1(46). Both luMSC and luFB express AIP6/TSG6 which has anti-inflammatory properties (47, 48). MSC present in lung tissue can be activated following cardiac injury to increase the secretion of AIP6 decreasing inflammation, infarct size and improving cardiac function (47). The expression of IL1RN, another key mediator in the anti-inflammatory repertoire of BM-MSC(15), is limited to luMSC and not FB. Interestingly, when compared to published BM-MSC gene lists luMSC and luFB exhibited similar expression patterns (34–37). It is therefore likely that the differences between the lung mesenchymal cell types, luMSC and luFB, are a key in our understanding of their functional differences.

Characteristics of luMSC define their similarity to BM-MSC and their ability to protect from injury via regulation of T cell proliferation. Taken together these results suggest that resident luMSC may function to regulate pulmonary tissue repair. If one accepts this hypothesis, questions that remain are the true function of these cells within the context of the lung, and identification of their niche which presumably dictates function (49). The luMSC present in the recipient lung do not appear sufficient for protection or repair following bleomycin injury. This deficiency may be due to an impaired function, a decrease in their number

following injury, or a combination of both. Rescuing the deficient endogenous luMSC population via the administration of exogenous luMSC attenuated injury. Bleomycin injured lung tissue is known to stimulate the proliferation and recruitment of BM-MSC both in vitro and in vivo (10, 12, 16). Injury may therefore affect administered luMSC differently than endogenous cells. Administered luMSC may also function outside the lung to suppress the injury, and the number of cells administered is likely an important factor where a “threshold dose” of luMSC may be required for sufficient repair. Our results demonstrate that exogenously administered luMSCs decrease lung injury while endogenous cell function is compromised and suggest a therapeutic strategy for rescue of endogenous cells to facilitate lung repair during injury. The repeatability of isolation of this well-characterized population of cells and the ability to identify them in vivo allows this venue of study. The existence of this cell population in human lung tissue also suggests the use of mouse models to study luMSC may have an impact on understanding their role in human disease processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Adult lung MSC are not derived from bone marrow hematopoietic cells
 BM transplants were performed with whole BM from GFP-expressing donor mice. At 15 weeks post-transplantation mice were treated with a single intratracheal dose of PBS or bleomycin. (A) 5 weeks following injury lungs were harvested and the SP identified by flow cytometry. (B) The SP was further analyzed to detect GFP and CD45 labeled subpopulations. PBS treated BMtx mice n=4; Bleomycin treated BMtx mice n=6.

Figure 2. Bleomycin injury depletes the endogenous lung MSC population

(A) Analysis of the lung SP population over time following bleomycin injury demonstrated a decrease in the endogenous luMSC. Representative scattergrams are shown for each transplant (PBS or Bleomycin day 35) and time point (Days 35, 7 & 14) and the average percentage is indicated. ** $p < 0.01$. Immunostaining was performed on lung sections to detect ABCG2 positive lung SP 14 days following intratracheal administration of PBS or bleomycin (B). (C) Cells were localized to the alveolar capillary interface using dual staining to detect ABCG2 (green) and PECAM-1 (red). Double labeled cells appear yellow. (D) Additional immunostaining to detect ABCG2 was performed to localize these cells to the interstitium of control human lung tissue. Detection was performed using DAB colorimetric substrate which labeled immunoreactive cells brown. 100x Mag.

Figure 3. Lung derived MSC replacement attenuates bleomycin injury

Mice were treated with intratracheal bleomycin or PBS and intravenous injection of luMSC, luFB or PBS and harvested 14 days following injury. (A) Histologic sections were obtained from each group and luMSC transfer + bleomycin resembled the PBS control while bleomycin and bleomycin + luFB demonstrate increased fibrosis (40x mag). (B) Body weight (BW) of mice and survival decreased with bleomycin treatment and was rescued by luMSC. Fibrosis scored using the Ashcroft method was also attenuated by luMSC infusion not luFB. (C) PAH as measured by RVSP and muscularization of vessels was increased with bleomycin and significantly decreased with luMSC. There was no mortality, fibrosis or PAH in the control groups. The number of test subjects per group for histological evaluation, body weight (BW) and mortality analyses were as follows: control n=9, day 14 bleomycin n=9, lung MSC +PBS n=6, lung fibroblast (FB) + PBS n=3, lung MSC + Bleomycin d14 n=19, lung FB + Bleomycin d14 n=18. The number of test subjects per RVSP group were as follows: control n=6, day 14 bleomycin n=5, lung MSC +PBS 6, lung fibroblast (FB) + PBS n=3, lung MSC + Bleomycin d14 n=19, lung FB + Bleomycin d14 n=9. For muscularization and Ashcroft analysis the number of test subjects per group were as follows: control n=4, day 14 bleomycin n=4, lung MSC +PBS n=3, lung fibroblast (FB) + PBS n=3, lung MSC + Bleomycin d14 n=4, lung FB + Bleomycin d14 n=4. The results are presented as the average number of muscularized actin positive vessels per field of view.

Figure 4. LuMSC modulate inflammatory cells present in BAL and lung tissue

Mice were treated with intratracheal bleomycin or PBS and intravenous injection of luMSC, luFB or PBS and harvested 7 and 14 days following injury. (A) BAL was collected from for flow cytometric analysis of T cell (Thy1/CD90), granulocyte (GR1), monocyte/macrophage (CD11b/F480) and B cell (B220) significant change was detected in analysis between groups. Data is presented as the mean percentage of total CD45 cells present in BAL. Control groups receiving cells did not exhibit inflammation in BAL significantly different from the PBS control. (B) Representative scattergrams of BAL analysis demonstrate a corresponding decrease in GR1 and CD90 positive cells from bleomycin injured mice that received luMSC treatment. (C) Immunofluorescent staining confirmed an increase in CD3 positive T cells following bleomycin injury on days 7 and 14 relative to the PBS control and luMSC treatment. The number of test subjects per BAL group were as follows: control 7, day 14 bleomycin 7, luMSC +PBS 3, luFB + PBS 3, luMSC + Bleomycin d14 19, luFB + Bleomycin d14 3. 100x Mag.

Figure 5. LuMSC inhibit antigen driven proliferative responses of effector T cells
 (A) luMSC or luFB (6.25×10^4) were plated with growth arrested OVA stimulated APC (1×10^6) in 96 well plates on followed by the addition of CFSE labeled T effector cells (1×10^5). The cells were cultured for 96 hours. Data is presented as the percentage of proliferating T cells based upon decreasing CFSE fluorescence intensity quantitated by flow cytometry. These studies were performed twice independently. (B,C) Representative flow cytometry and qRT-PCR analysis of luMSC and luFB expression of cell surface CD80 which would promote T cell interaction. The mean and standard error are presented.

Figure 6. The transcriptome of LuMSC define a unique subpopulation of stromal cells distinct from luFB

(A) LuMSC and luFB were isolated from mouse lungs by flow cytometry and expanded in culture. cDNA from each sample was hybridized to Affymetrix mouse whole genome microarrays. (A) Principal component analysis of array data showing clustering of LuMSC (SP1,2) separate from luFB (FB1,2). (B) Supervised hierarchical cluster analysis of genes involved in WNT or TGF β signaling and stemness, the immune response, blood vessel development and angiogenesis and extracellular matrix (ECM) following normalization of all data sets. Data show LuMSC relative to luFB have increased expression of angiogenic genes, decreased expression of genes involved in fibrosis (including wnt and Tgfb pathways) and inflammation (including up-regulation of inflammatory TNF and IL-1 pathways). Two independently isolated pooled cultures of LuMSC (n=2) or luFB (n=2) were used for these analyses.