Mesenchymal Stromal Cells in Bronchoalveolar Lavage as Predictors of Bronchiolitis Obliterans Syndrome

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Rationale: **Bronchoalveolar lavage fluid (BAL) from human lung allografts demonstrates the presence of a multipotent mesenchymal stromal cell population. However, the clinical relevance of this novel cellular component of BAL and its association with bronchiolitis obliterans syndrome (BOS), a disease marked by progressive airflow limitation secondary to fibrotic obliteration of the small airways, remains to be determined.**

Objectives: **In this study we investigate the association of number of mesenchymal stromal cells in BAL with development of BOS in human lung transplant recipients.**

Methods: **Mesenchymal colony-forming units (CFUs) were quantitated in a cohort of 405 BAL samples obtained from 162 lung transplant recipients. Poisson generalized estimating equations were used to determine the predictors of BAL mesenchymal CFU count.**

Measurements and Main Results: **Higher CFU counts were noted early post-transplantation; time from transplant to BAL of greater than 3 months predicted 0.4-fold lower CFU counts ($P = 0.0001$). BOS diagnosis less than or equal to 365 days before BAL was associated with a 2.11-fold higher CFU count ($P = 0.02$). There were 2.62- and 2.70-fold higher CFU counts noted in the presence of histologic diagnosis of bronchiolitis obliterans ($P = 0.05$) and organizing pneumonia (0.0003), respectively. In BAL samples obtained from BOS-free patients greater than 6 months post-transplantation (n = 173), higher mesenchymal CFU counts (≥10) significantly predicted BOS onset in both univariate (hazard ratio, 5.61; 95% CI, 3.03–10.38; $P < 0.0001$) and multivariate (hazard ratio, 5.02; 95% CI, 2.40–10.51; $P < 0.0001$) Cox regression analysis.**

Conclusions: **Measurement of mesenchymal CFUs in the BAL provides predictive information regarding future BOS onset.**

Keywords: bronchiolitis obliterans syndrome; acute rejection; bronchoalveolar lavage

Lung transplantation is the only viable option for many patients with end-stage lung disease (1). However, long-term survival after lung transplantation is limited by bronchiolitis obliterans (BO), a fibroproliferative disease of the terminal airways marked by infiltration with mesenchymal cells (2, 3). Progressive fibrotic narrowing and obliterans of the airways leads to an irreversible, relentless decline in lung function termed "bronchiolitis obliterans syndrome" (BOS) (4). BOS is seen in 51% of transplant recipients by 5.6 years and is the major cause of mortality after 1 year of transplantation (5). Early identification of this fibroproliferative process, allowing timely therapeutic interventions, continues to be a major challenge in this field.

Bronchoalveolar lavage (BAL) offers a unique tool to sample the internal milieu of the lung and its cellular and molecular components have been evaluated to investigate markers and predictors of BOS (6, 7). Proinflammatory cytokines (IL-8; regulated upon activation normal T-cell expressed and secreted; monocyte chemoattractant protein-1) (8–11), profibrotic growth factors (transforming growth factor-β, platelet-derived growth factor) (10, 12), and markers of extracellular matrix remodeling, such as matrix metalloproteases (13), have been reported to be increased in the BAL fluid in presence of BOS. Among cellular components, an increase in proportion of neutrophils is reported in BOS (9, 14, 15), but its predictive ability is limited by its lack of specificity (16).

We have recently demonstrated that cells obtained from BAL samples of human lung allografts, studied in culture in a plastic adherent condition, demonstrate growth of distinct fibroblastoid colony-forming units (CFUs) of mesenchymal progenitor cells with multilineage differentiation potential (17). Mesenchymal cells are the primary effector cells in fibroproliferation; however, the association of this novel mesenchymal cell population in BAL with the development of BOS has not been studied. In this study, we quantitate mesenchymal CFUs in a large prospective cohort of BAL samples obtained from lung transplant recipients and demonstrate that an increase in number of mesenchymal CFUs in BAL predicts development of BOS. Some of the results of these studies have been previously reported in the form of an abstract (18).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Bronchiolitis obliterans syndrome (BOS) is the major cause of poor outcomes after lung transplantation. While BOS is recognized as a fibrotic disease of the small airways, biomarkers of this fibroproliferative process, allowing its early detection, are lacking.

What This Study Adds to the Field

This study identifies the increased numbers of mesenchymal progenitor cells in the bronchoalveolar lavage fluid as a novel cellular predictor of BOS in human lung transplant recipients.

METHODS

Patient Population

Lung transplant recipients undergoing bronchoscopy at University of Michigan were enrolled in a prospective trial. The study was approved by the University of Michigan Institutional Review Board, and informed consent was obtained before participation in the study.

Between January 2005 and February 2010, 561 BAL samples were collected from 193 lung transplant recipients. A total of 405 BAL samples from 162 patients, which could be maintained in culture for 14 days allowing mesenchymal CFU measurement, were included in the analysis (Figure 1). Compared with the 156 samples that were not included in the analysis, these 405 samples included in the analysis were obtained at a similar time post-transplant ($P = 0.10$) and had a similar incidence of

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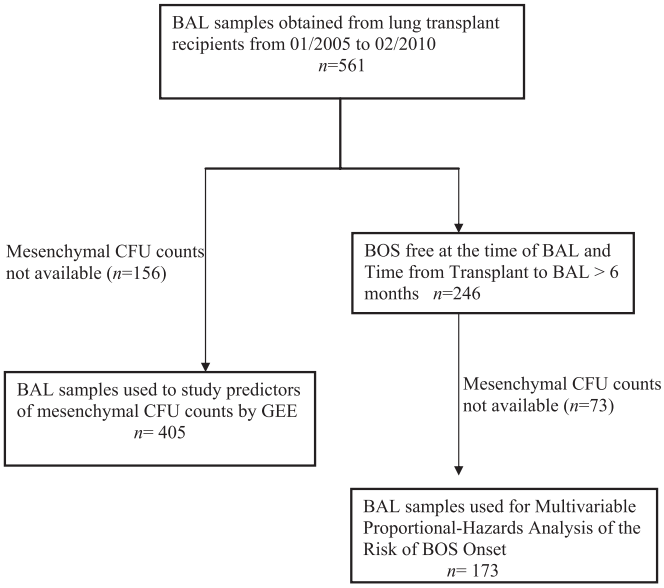

Figure 1. CONSORT diagram illustrating the selection of patients included in the data analysis. BAL = bronchoalveolar lavage; GEE = generalized estimating equation; BOS = bronchiolitis obliterans syndrome.

presence of BOS at the time of sampling ($P = 0.67$). Similarly, no differences were seen in the incidence of acute rejection (AR) ($P = 0.82$), lymphocytic bronchitis (LB) ($P = 0.71$), and BO or organizing pneumonia ($P = 0.86$) between the two groups. However, as expected, the BAL samples with failure to maintain 2-week cultures had higher incidence of positive bacterial ($P = 0.002$) and fungal cultures ($P = 0.05$).

BAL Mesenchymal CFU Assay

BAL samples were processed as previously described (17, 19). The numbers of mesenchymal CFUs in BAL were measured using methods similar to those described by Castro-Malaspina for bone marrow–derived cells (20). Briefly, recovered BAL fluid was filtered through a sterile strainer to remove noncellular particulate material, and the cell pellet recovered by centrifugation at 1,000 rpm for 5 minutes. Two million nucleated cells isolated from BAL were seeded in a 100-mm cell culture dish and incubated at 37°C in 5% CO_2 / 95% air in medium consisting of high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA); 100 U/ml penicillin/streptomycin (Invitrogen); and 0.5% fungizone (Invitrogen). Medium was changed every 3 days. Single separated fibroblastoid colonies are identified as early as 7 days after initial plating. Colonies were counted between Days 14 and 21 after initial plating.

Characterization of Mesenchymal CFUs

Mesenchymal CFUs from BAL of lung transplant recipients were expanded in culture and further characterized using methodology as previously described (17). BAL samples from five patients from each group (normal, evidence of AR on histology, and evidence of BOS on physiology) were studied. The cell surface phenotype of culture-expanded cells was analyzed by multiparameter flow cytometric analyses (fluorescence-activated cell sorter). Briefly, cells were trypsinized at passage 2; aliquotted at a concentration of 0.5×10^6 cells per milliliter; and stained for 30 minutes with either conjugated specific antibodies (BD biosciences, San Jose, CA) or isotype-matched control mouse IgGs at recommended concentration. Labeled cells were washed twice; resuspended in fluorescence-activated cell sorter buffer; and analyzed on fluorescence-activated cell sorter Calibur flow cytometer using the CellQuest software program (Becton Dickinson, Franklin Lakes, NJ).

To investigate multilineage differentiation potential, adipogenic and osteogenic differentiation was performed on culture-expanded cells as previously described (17). For adipogenic differentiation, confluent cells cultures in a 24-well plate were treated with adipogenic differentiation medium containing 10^{-6} M dexamethasone, 0.5 mM isobutylmethylxanthine, 10 μg/ml insulin, and 200 μM indomethacin in Dulbecco's modified Eagle medium high glucose media (0.5 ml per well). At 3 weeks cells were fixed with 10% formaldehyde (Fisher Scientific, Fair Lane, NJ) and incubated with fresh oil red O for 1 hour at room temperature; lipid droplets were visualized and photographed. Osteogenic differentiation was induced by incubating the cells with 10^{-8} M dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerophosphate in basal medium. After 21 days, cells were fixed and incubated with freshly made 2% alizarin red stain, pH 4.2 for 3 minutes. All reagents, unless specified, were from Sigma (St. Louis, MO). mRNA was isolated and real-time polymerase chain reaction performed as previously described (17).

Clinical Variables

The indication for bronchoscopy was defined as surveillance if it was performed routinely to rule out AR. Nonsurveillance bronchoscopies were performed when clinically indicated for such factors as dyspnea, decrement in lung function, or follow-up of previous episodes of AR. Transbronchial biopsies obtained at the time of BAL were examined according to established criteria (21). AR was defined as biopsy score of greater than or equal to A1. LB was defined as biopsy score of greater than or equal to B1. BOS was defined by physiologic testing according to the International Society of Heart and Lung Transplantation guidelines (4). Information on bacterial, fungal, and viral cultures was available on all BAL samples. Positive cytomegalovirus (CMV) was defined as CMV detection in BAL by shell vial (early antigen detection) or culture. Information on relative proportion of neutrophils, lymphocytes, macrophages, and eosinophils in the BAL was obtained from the results of fluid cell differential performed in the clinical pathology laboratory at the University of Michigan.

Statistical Analyses

The Wilcoxon signed-rank test was used to compare pair-wise differences in continuous CFU-F counts between aliquots from the same BAL sample. A one sample *t* test was used to test that CFU-F between serial dilutions decreased by 50% on average. Wilcoxon rank-sum tests were used to compare CFU-F counts between cases and controls at various time-points post–lung transplantation. Poisson generalized estimating equations (GEE) were used to determine which clinical variables predict CFU-F counts in BAL samples. GEE, a well-established strategy for analysis of correlated data (22), was used because multiple samples were obtained at different time points from the same subjects. GEEs were also used to determine association of various BAL cell populations (percent neutrophils, percent macrophages, and percent lymphocytes) with CFU-F counts. Time to BOS was modeled using Cox proportional hazards models with robust variance estimation (23, 24) used to account for patients contributing more than one event history from various BAL measurements. CFU group-specific adjusted times to BOS plots for the average patient profile were taken from corresponding Cox models.

RESULTS

Patient Population

A total of 405 BAL samples obtained from 162 lung transplant recipients comprised the study cohort. The patient population included 71 females and 91 males with a mean age of 51 years (range, 21–69 yr) at the time of transplantation. Major indications for transplantation included emphysema (n = 69); idiopathic pulmonary fibrosis (IPF) (n = 50); cystic fibrosis (n = 20); and other diagnoses (n = 23). Clinical variables at the time of BAL and the number of patients contributing these data in the study cohort are displayed in Table 1. Histologic information from concurrently performed trans-bronchial biopsies was available on 372 BAL samples.

Quantitation and Characterization of Mesenchymal CFUs in the BAL Obtained from Lung Transplant Recipients

The number of mesenchymal CFUs in the BAL was quantitated as described in the METHODS section. CFU counts per 2×10^6 cells plated in a 100-mm dish were reported. To determine that the

TABLE 1. CHARACTERISTICS OF BRONCHOALVEOLAR LAVAGE SAMPLES*

Clinical Variable	N (*number of patients contributing samples*)
Indication for bronchoscopy	
Surveillance	257 (120)
Nonsurveillance	148 (94)
Time from transplant to BAL	
0–3 mo	110 (78)
3–6 mo	76 (63)
6 mo–1 yr	77 (64)
1–2 yr	67 (51)
>2 yr	75 (59)
Biopsy at the time of BAL	
Acute rejection (≥A1)	52 (43)
Lymphocytic bronchitis (≥B1)	26 (25)
Bronchiolitis obliterans	4 (4)
Organizing pneumonia	8 (8)
Biopsy not performed	33 (29)
BOS at the time of BAL	
BOS-free at the time of BAL	359 (145)
Early post-BOS (time from BOS to BAL <365 d)	29 (19)
Late post-BOS (time from BOS to BAL ≥365 d)	17 (14)
Microbiology cultures from BAL	
Positive bacterial cultures	51 (36)
Positive cytomegalovirus viral cultures	17 (14)
Positive respiratory viral cultures	9 (9)

Definition of abbreviations: BAL = bronchoalveolar lavage; BOS = bronchiolitis obliterans syndrome.

* 405 samples from 162 patients.

quantitation technique is valid, cells obtained from 15 BAL samples were plated in duplicate (2×10^6 million cells per dish, two separate 100-mm dishes). Mesenchymal CFU counts were obtained for each dish in a given patient. There were no statistically significant differences between aliquots (Wilcoxon signed-rank test; $P = 0.94$). Further serial twofold dilutions were performed on five BAL samples. An average of 51% decrease in CFU counts (range, 40–60%) was noted between serial dilutions. The decrease is not significantly different from 50% according to one sample *t* test ($P = 0.44$). These analyses show reproducibility between measures of CFU count from a BAL sample and support the quantitative nature of the CFU assay.

Mesenchymal CFUs from representative BAL samples derived from lung transplant recipients were culture expanded and recharacterized. Similar to what has been previously described for multipotent mesenchymal stromal cells (17, 25, 26), culture-expanded CFUs from BAL of patients in all groups (normal, AR, and BOS) demonstrated expression of CD44, CD105, CD73, and CD90 (Figure 2), and lacked expression of CD45 and CD34 (data not shown). Multilineage differentiation to adipocytic and osteocytic lineages was demonstrable in all cell lines tested (Figure 3).

Predictors of Mesenchymal CFUs in the BAL

Mesenchymal CFU counts in the BAL fluid obtained from lung transplant recipients demonstrated significant variability (range, 0–90; mean, 10.34; SD, 15.41). The distribution of mesenchymal CFU counts in the BAL samples is shown in Figure 4.

Poisson GEEs were used to determine the clinical variables that predict mesenchymal CFU counts in BAL samples. Time from transplantation, presence of BOS, and a histologic diagnosis of BO or organizing pneumonia were significant predictors of number of mesenchymal CFUs in the BAL (Table 2). Time post-transplant of greater than 90 days was associated with a 0.40-fold lower CFU count (95% confidence interval [CI], 0.30–0.53; $P < 0.0001$) in the BAL. CFU counts of 2.62- and 2.70-fold higher were noted in the presence of histologic diagnosis of bronchiolitis obliterans (95% CI, 1.00–6.92; $P = 0.05$) and histologic diagnosis of organizing pneumonia (95% CI, 1.57–4.65; $P = 0.0003$), respectively, on concurrent biopsies. Higher CFU counts were also noted in BAL samples obtained less than or equal to 365 days after BOS onset (early post-BOS) (2.11-fold increase; 95% CI, 1.10–4.03; $P = 0.02$). Other histologic diagnoses, presence of late post-BOS (time from BOS to BAL >365 d), presence of positive bacterial cultures, CMV positivity, indication for bronchoscopy, and pretransplant diagnosis did not significantly predict number of mesenchymal CFUs in the BAL.

Analysis of mean CFU counts over time post-transplantation demonstrated a bimodal distribution with higher mean mesenchymal CFUs in BALs obtained at time post-transplant of less than or equal to 3 months and greater than or equal to 24 months (Figure 5). The late increase was not seen when BAL samples obtained in presence of BOS (n = 46) or within 6 months of BOS onset (n = 36) were excluded.

Cell count and differential were available on 374 BAL samples. No association was noted between mesenchymal CFU counts and relative proportion of other cellular populations (percent neutrophil, percent lymphocytes, and percent macrophage; $P = 0.30$, 0.61, and 0.42, respectively).

Increased Numbers of Mesenchymal CFUs in BAL as a Predictor of BOS Onset

Next, we investigated if increased mesenchymal CFU counts in BAL samples can predict future development of BOS. Patients were followed for development of BOS until February 12, 2010. For this analysis BAL samples that were obtained from patients who were greater than 6 months post–lung transplantation and had no evidence of BOS at the time of BAL were used (n = 246). A total of 73 BAL had missing CFU values. No difference was seen in time to BOS onset from BAL between the cohort used in the analysis (173 BAL from 107 patients) versus the cohort with missing CFU values ($P = 0.35$).

Mesenchymal CFU count in BAL significantly predicted subsequent BOS development (hazard ratio [HR], 1.04 for every one mesenchymal CFU increase in BAL; 95% CI, 1.03–1.06; $P < 0.0001$). For the purpose of clinical use, mesenchymal CFU was also studied as a categorical variable. High CFU count was defined as CFU greater than or equal to 10 per 2×10^6 nucleated cells. This threshold was based on results shown in Figure 5 and estimated means from parameter estimates obtained from the GEE model shown in Table 2. High CFU count (CFU ≥10 in BAL 6 mo after transplantation) was found to be a significant predictor of subsequent BOS development (HR, 5.61; 95% CI, 3.03–10.38; $P < 0.0001$). Kaplan-Meier curves shown in Figure 6A demonstrate time to BOS in lung transplant recipients grouped by number of CFU-Fs in BAL. Median time to development of BOS from a BAL sample demonstrating CFU-F count greater than or equal to 10 was 370 days.

In multivariate analysis (Table 3), after adjusting for presence of AR, LB, sex, type of transplantation (single vs. bilateral), pretransplant diagnosis (IPF, emphysema, or others) and time post-transplantation, high CFU-F count remained a significant predictor of BOS onset (HR, 5.02; 95% CI, 2.40–10.51; $P < 0.0001$). Cox model-based survival estimates are shown for an average patient profile in Figure 6B.

Of the 24 patients who developed BOS during the course of follow-up, 17 patients were initially diagnosed as BOS stage 1, two as BOS stage 2, and five as BOS stage 3. At 6 months after BOS onset, 12 patients progressed to higher grades of BOS, and 3 died of BOS-related complications. Eight patients were in the same stage of BOS at 6 months and improvement of BOS grade was noted in four patients.

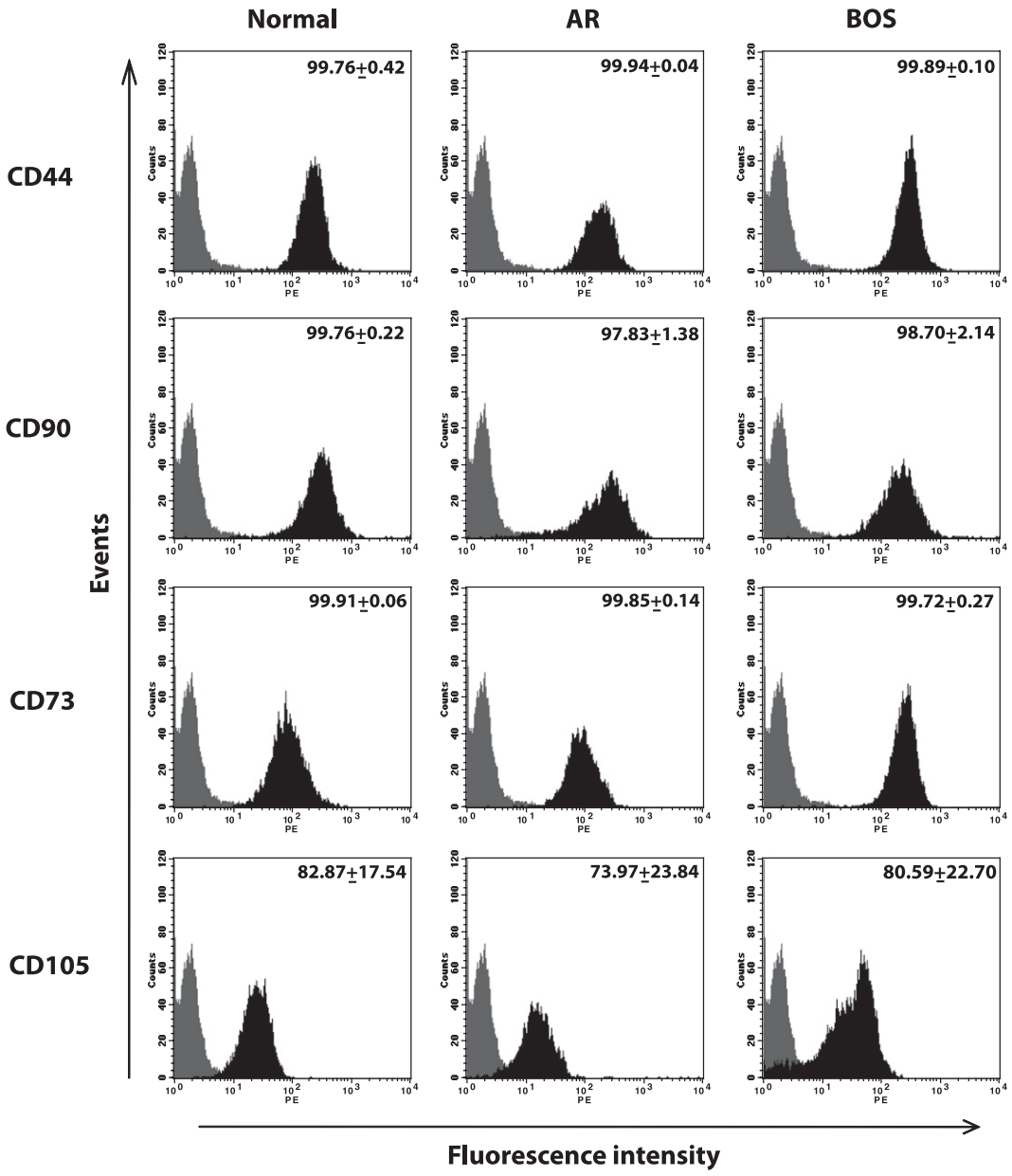

Figure 2. Flow cytometric analysis of cell surface markers on culture-expanded mesenchymal CFUs. Bronchoalveolar lavage (BAL) CFUs from lung transplant recipients (control, acute rejection [AR], and bronchiolitis obliterans syndrome [BOS]) were expanded in culture and immunostained for CD44, CD90, CD73, and CD105 surface markers with specific mAbs. Cells from the three groups were found to be predominantly positive for the surface markers studied. All histograms demonstrate specific mAbs in *black* and control isotype-specific IgGs in *gray*. The percentage of positive cells relative to the total number of cells analyzed (mean ± SD) is shown above the respective histograms. n = 5 individual patients in each group.

DISCUSSION

In this study we examined the relationship between a novel cellular component of BAL (number of mesenchymal stem cells [MSCs]) and development of BOS in human lung transplant recipients. We demonstrate that presence of BOS is associated with an increase in mesenchymal CFU counts in BAL fluid. Histologic diagnosis of BO or organizing pneumonia on concurrent trans-bronchial biopsies also predicted higher mesenchymal CFU counts in the BAL. Importantly, increased number of mesenchymal CFUs in BAL fluid was found to be a significant predictor of future BOS onset. Together, these data identify a novel cellular marker of chronic allograft rejection in human lung transplant recipients.

Quantitation of BAL mesenchymal CFU counts and its correlation with clinical variables in a large cohort of lung transplant recipients provides the first documentation of clinical predictors of MSC population in the BAL. Predictors of higher mesenchymal CFU counts in the BAL included diagnosis of BO or organizing pneumonia on histology, presence of BOS on pulmonary function testing, and time post-transplant less than or equal to 90 days. A very strong association was noted between number of mesenchymal CFUs in the BAL and histologic evidence of organizing pneumonia. Organizing pneumonia, a condition marked by presence of mesenchymal cells in the alveoli, can be commonly diagnosed by transbronchial biopsies. However, transbronchial biopsies have a low sensitivity for diagnosing BO, the histologic manifestation of BOS (27). A surrogate for a mesenchymal proliferative process in the BAL can have a potential role in differentiating a fall in FEV_1 from BO versus other confounding factors, such as hyperinflation or bronchial stenosis, hence identifying a more homogeneous BOS population. Higher BAL CFU counts were noted in the presence of histologic diagnosis of BO in our cohort. However, because of small numbers of cases with evidence of BO on biopsies, further studies validating this finding and establishing the clinical use of this marker in diagnosing BO in conjunction with clinical and physiologic parameters are warranted.

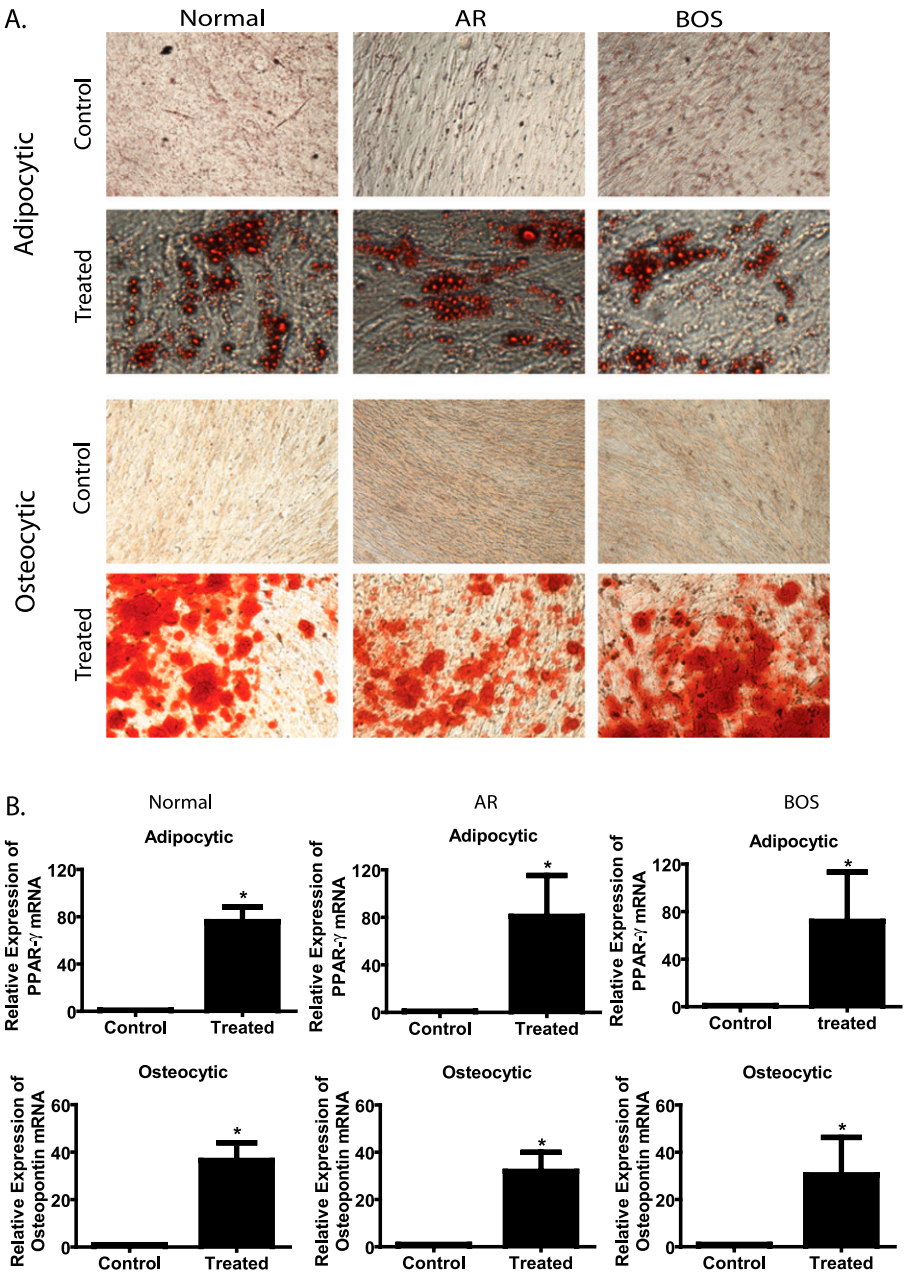

Figure 3. Multilineage differentiation potential of culture-expanded bronchoalveolar lavage (BAL) mesenchymal CFUs. (*A*) Cell lines generated from BAL CFUs of control, acute rejection (AR), or bronchiolitis obliterans syndrome (BOS) patients (n = 5 in each group) were investigated for the *in vitro* multilineage differentiation capacity. Accumulation of lipid droplets (indicating adipocytic differentiation) was demonstrated by staining with oil red O in treated cells. Osteocytic differentiation was indicated by calcium deposition as demonstrated by alizarin red staining (*red color*) in treated cells. No staining was observed in control untreated cells. (*B*) Real-time polymerase chain reaction was performed to analyze the expression of specific adipogenic and osteogenic related mRNAs under inductive culture conditions. Relative expression of PPAR-α mRNA (indicative of adipogenic activity) and osteopontin mRNA (indicative of osteogenic activity) are shown in control and treated conditions.

Diagnosis of BOS was found to be a significant predictor of MSC population in the allograft with twofold higher mesenchymal CFUs noted in BAL samples obtained with a year of BOS onset. This association was not seen in BAL samples obtained after a year of BOS onset. Study of course of FEV_1 after BOS onset has demonstrated that maximal decline in FEV_1 occurs within the first year after BOS onset (28). It can be speculated that the early time period after BOS onset marks a period of maximal fibroproliferation and hence this association with high mesenchymal CFUs. The present study was not powered to investigate if changes in CFU count after BOS onset correlate with the course of FEV_1 in these patients. A future prospective multicentric study is needed to investigate the prognostic ability of longitudinal changes in BAL CFU counts in patients with BOS.

Analysis of time post-transplant and CFU count demonstrated a very interesting bimodal distribution with higher mean mesenchymal CFU counts noted in BAL samples first within 3 months and then later after 24 months of lung transplantation. The presence of elevated MSC population early post-transplant, a period marked by intense cellular response to graft injury, is similar to what has been described for other cellular populations in the BAL (7). Elevated total cell count and neutrophils are seen in BAL during the first 3 months after lung transplantation (7). Whether this cellular response, in the form of increased mesenchymal cells accumulation in the first 3 months after lung transplantation, varies with the degree of ischemia reperfusion injury remains to be determined. The increase in MSC population in BAL at later time after lung transplantation was associated predominantly with development of BOS.

Analysis of a prospective cohort of BOS-free patients demonstrated that higher number of MSCs in BAL 6 months of lung transplantation significantly predicted future BOS onset. Mesenchymal CFU counts greater than or equal to 10 in BAL samples were associated with an approximately fivefold higher hazard of developing BOS. Predicting BOS early, before clinical compromise, is critical for instituting therapeutic modalities to prevent or

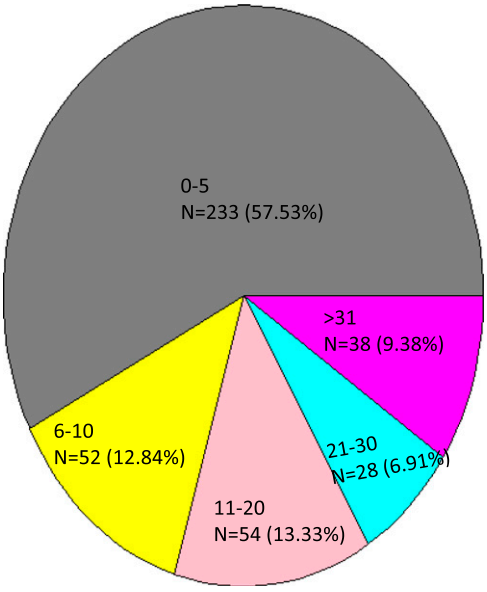

Figure 4. Distribution of mesenchymal CFU counts in bronchoalveolar lavage (BAL) samples obtained from lung transplant recipients. Numbers of mesenchymal CFUs in the BAL was quantitated by culturing BAL cells in plastic adherent condition. CFU counts per 2×10^6 cells plated in a 100-mm dish were reported (n = 405).

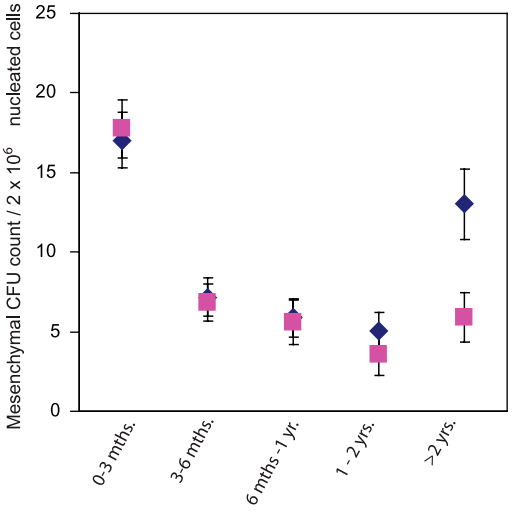

Figure 5. Relationship of mesenchymal CFU count bronchoalveolar lavage (BAL) samples and time post-lung transplantation. Mean CFU count over various time periods post-lung transplantation are demonstrated in all BAL samples (*blue diamonds*; n = 405). A bimodal distribution with higher mean mesenchymal CFUs in BALs obtained at time post-transplant of less than or equal to 3 months and greater than or equal to 24 months was seen. The late increase was not seen when BAL samples obtained in presence of BOS (n = 46) or within 6 months of BOS onset (n = 36) were excluded (*red squares*).

delay BOS onset. Information obtained from bronchoscopy, either BAL or transbronchial biopsies, has been evaluated to identify a simple predictor or biomarker of BOS. In retrospective analysis, diagnosis of a single or multiple episodes of AR and LB on transbronchial biopsies has been associated with BOS (29–33). Our analyses are restricted to patients who are already 6 months out from transplant and evaluated time to BOS onset from individual BAL. Similarly, another major difference in our approach was that we focused on the predictive ability of the current BAL and not cumulative information from all previous BALs. Thus, a patient with no AR at the time of the bronchoscopy performed as a part of this study might have had AR in the past. These differences could explain the lack of association of AR or LB with BOS in our model.

TABLE 2. MULTIVARIATE ANALYSIS OF THE PREDICTORS OF MESENCHYMAL CFU COUNTS IN BRONCHOALVEOLAR LAVAGE FLUID OF HUMAN LUNG TRANSPLANT RECIPIENTS (N = 372)

Variable	Estimate (*95% confidence interval*)*	*P* Value†
Time post-transplant >90 d	0.40 (0.30–0.53)	<0.0001‡
Early post-BOS (BOS to BAL ⩽365 d)	2.11 (1.10–4.03)	0.02‡
Late post-BOS (BOS to BAL >365 d)	1.47 (0.70–3.07)	0.31
Acute rejection (⩾A1)	0.95 (0.69–1.30)	0.75
Lymphocytic bronchitis (⩾B1)	0.80 (0.49–1.31)	0.38
Bronchiolitis obliterans	2.62 (1.00–6.92)	0.05‡
Organizing pneumonia	2.70 (1.57–4.65)	0.0003‡
Positive bacterial cultures	1.31 (0.74–2.32)	0.35
Positive cytomegalovirus cultures	0.62 (0.30–1.28)	0.20
Other virus	1.26 (0.47–3.37)	0.65
Nonsurveillance bronchoscopy (vs. surveillance)	1.08 (0.80–1.46)	0.62
Pretransplant diagnosis		
Idiopathic pulmonary fibrosis	0.70 (0.42–1.16)	0.16
Emphysema	0.81 (0.46–1.43)	0.47
Cystic fibrosis	0.70 (0.38–1.29)	0.26
Others	1.00§	NA§

Definition of abbreviations: BAL = bronchoalveolar lavage; BOS = bronchiolitis obliterans syndrome.

* Estimates indicate the multiplicative increase or decrease in expected CFU-F count according to the factor being true versus false, other factors held constant.

† *P* values generated from multivariate generalized estimating equation models accounting for correlation within patients.

‡ $P < 0.05$.

§ Reference population.

No significant association was seen in our cohort between mesenchymal CFUs in the BAL and positive CMV cultures. CMV infection and disease are considered a possible risk factor for development of BOS (4). It is important to note that in our cohort of patients with positive CMV cultures only one case had evidence of true CMV disease as shown by cytopathic changes on transbronchial biopsies. Thus, this study cannot comment on the association of mesenchymal CFUs and CMV pneumonitis. Similarly, although no correlation was seen between number of mesenchymal CFUs in BAL and positive bacterial cultures in the cohort from which CFU count could be obtained, there was increased incidence of positive bacterial culture in BAL with missing CFU data. This suggests that measuring the MSC population in the BAL by CFU count has limited predictability in the patient population with positive bacterial culture because this population is at increased risk of missing CFU data.

In multivariate analysis, after adjusting for presence of AR, LB, CMV, sex, type of transplantation (single vs. bilateral), pretransplant diagnosis (IPF, emphysema, or others), and time post-transplantation, high CFU count remained a significant predictor of BOS onset (HR, 4.73; 95% CI, 2.22–10.10; $P < 0.0001$). Although this ability of MSC numbers in the BAL to predict BOS onset suggests a potential for using this information in the clinical arena, a major limitation in our methodology of MSC quantitation is that it requires culturing BAL cells in plastic adherent conditions for 2 weeks. This technique, other than being time consuming, also leads to significant number of missing counts. Future investigation of a marker of MSCs that can be analyzed in a high-throughput manner is required to translate these finding to the clinical arena.

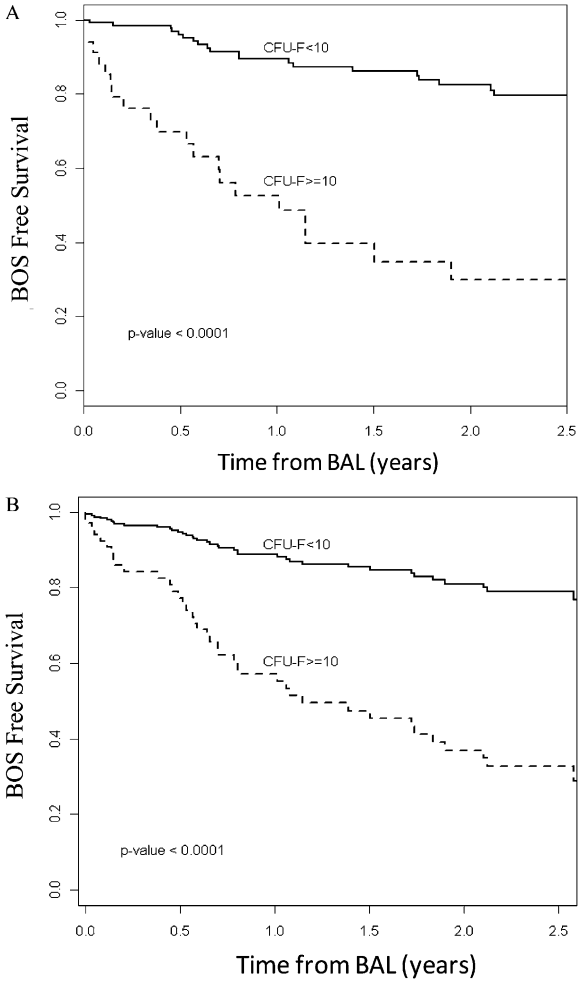

Figure 6. Mesenchymal CFUs in the bronchoalveolar lavage (BAL) as predictors of bronchiolitis obliterans syndrome (BOS) onset in lung transplant recipients. (*A*) Kaplan-Meier curve demonstrating time to BOS in lung transplant recipients grouped by number of mesenchymal CFUs in BAL (CFU ⩾10, *dashed line*; CFU <10, *solid line*). (*B*) CFU-group specific adjusted time to BOS plots based on survival estimates obtained using hazards estimated with Cox models adjusted for average patient profile. Average covariate profile is as follows: time from transplant to BAL = 1.74 year; probability of being male = 52%; probability of histologic diagnosis of acute rejection = 11.9%, lymphocytic bronchitis = 10%; probability of pretransplant diagnosis of idiopathic pulmonary fibrosis = 32.4%, chronic obstructive pulmonary disease = 39.9%, cystic fibrosis = 13.9%; probability of single lung transplantation = 57.8%.

The study population in this investigation was limited to BAL samples from lung transplant recipients in whom CFU assay could be performed. Hence, this study is applicable only to this population and cannot comment on the patients with missing CFU data in the BAL. The present analyses suggest that time to onset of BOS is similar for the group with missing BOS count versus the cohort with known CFU count. However, further follow-up is needed to evaluate definitely whether "missing CFU" is a distinct biologic cohort. An evaluation of this population requires the performance of a prospective study with BAL samples obtained at regular intervals. In such a study, the effect of longitudinal changes in mesenchymal CFUs can be investigated as a predictor of BOS onset and progression.

Although this study provides the first evidence for a dynamic change in MSC numbers in a transplanted lung, the role of endogenous MSCs in adaptive and maladaptive repair responses

TABLE 3. MULTIVARIABLE PROPORTIONAL-HAZARDS ANALYSIS OF THE RISK OF BOS ONSET BY VARIABLES PRESENT AT THE TIME OF BAL

Variable	Hazard Ratio	95% CI (*lower*)	95% CI (*upper*)	*P* Value
Mesenchymal CFU count ⩾10	5.02	2.40	10.51	<0.0001
Time post-transplant, yr	1.04	0.84	1.30	0.71
Acute rejection (⩾A1)	1.22	0.38	3.87	0.74
Lymphocytic bronchitis (⩾B1)	0.72	0.35	1.48	0.37
Cytomegalovirus	0.94	0.31	2.86	0.91
Pretransplant diagnosis				
Idiopathic pulmonary fibrosis	0.45	0.07	2.90	0.40
Emphysema	0.77	0.16	3.81	0.75
Cystic fibrosis	0.80	0.14	4.58	0.80
Others	ref	Ref	ref	NA
Type of transplant, bilateral	1.33	0.54	3.26	0.53
Sex, male	1.54	0.68	3.44	0.30

Definition of abbreviations: BAL = bronchoalveolar lavage; BOS = bronchiolitis obliterans syndrome; CI = confidence interval.

in the lung remains unclear. MSCs have strong immunomodulating properties (34, 35) and exogenous administration of bone marrow–derived MSCs has been shown to ameliorate injury in animal lung injury models (36–38). We have recently demonstrated that MSCs derived from human lung allografts inhibit T cells *in vitro* via secretion of soluble mediators (19). However, MSCs can also play an important role in tissue fibrosis. Bone marrow–derived MSCs can differentiate into myofibroblasts *in vitro* (39, 40) and mesenchymal progenitor cells have been shown to participate in fibrotic responses (41, 42). These observations suggest that MSCs can have divergent effects in acute and chronic injury scenarios and their role in human diseases should be investigated. Our study is the first study to investigate endogenous MSCs in human injury. Increased numbers of lung-resident MSCs were noted early post-transplant in patients with both favorable and unfavorable long-term outcomes demonstrating that these cells are not associated with fibrosis during acute injury. However, increased numbers at later time points post–lung transplantation were associated with development of BOS. On the basis of these observations it can be speculated that lung-resident MSCs, which are recruited in response to injury, can be modulated by the presence of a profibrotic milieu and potentially contribute to fibrogenesis. Further work is needed to elucidate the role of these mesenchymal precursor cells in the pathogenesis of BOS.

In summary, this study demonstrates that an increase in mesenchymal CFU counts in BAL is seen in association with BO/BOS and is a strong predictor of future BOS onset. Because BAL is routinely performed in lung transplant recipients, evaluation of this potential marker of fibroproliferative responses in the lung can provide important prognostic information regarding BOS onset and course. Further research is required to determine the role of serial measurements of mesenchymal CFUs in the follow-up of patients with BOS and whether CFU numbers can be used as biomarkers of disease activity and response.

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