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The role of TGF-β in the association between primary graft dysfunction and bronchiolitis obliterans syndrome

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Abstract

Primary graft dysfunction (PGD) is a possible risk factor for bronchiolitis obliterans syndrome (BOS) following lung transplantation; however, the mechanism for any such association is poorly understood. Based on TGF-β's association with acute and chronic inflammatory disorders, we hypothesized that it may play a role in the continuum between PGD and BOS. Thus, the association between PGD and BOS was assessed in a single-center cohort of lung transplant recipients. Bronchoalveolar lavage fluid concentrations of TGF-β and procollagen collected within 24 hours of transplantation were compared across the spectrum of PGD, and incorporated into Cox models of BOS. Immunohistochemistry localized expression of TGF-β and its receptor in early lung biopsies post-transplant. We found an association between PGD and BOS in both bilateral and single lung recipients with a hazard ratio of 3.07 (95% CI 1.76-5.38) for the most severe form of PGD. TGF-β and procollagen concentrations were elevated during PGD (p<0.01), and

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associated with increased rates of BOS. Expression of TGF-β and its receptor localized to allograft infiltrating mononuclear and stromal cells, and the airway epithelium. These findings validate the association between PGD and the subsequent development of BOS, and suggest that this association may be mediated by receptor/TGF-β biology.

INTRODUCTION

Despite advances in surgical technique and post-operative medical care, survival following lung transplantation continues to lag considerably behind other solid organ transplants, with an estimated median survival of less than 6 years.(1) The primary obstacle to long-term survival is chronic rejection in the form of bronchiolitis obliterans syndrome (BOS), which affects up to 50% of patients within 5 years, and is characterized histopathologically by obliterative bronchiolitis, a progressive fibro-obliterative process affecting the allograft small airways.(2)

A unique feature of lung transplantation appears to be an association between ostensibly non-alloimmune insults to the allograft and the subsequent development of BOS.(3-10) Primary graft dysfunction (PGD) is a form of non-alloimmune acute lung injury that arises within the first 72 hours of transplantation, and is thought to be driven primarily by ischemia-reperfusion injury.(11, 12) PGD is the leading cause of early mortality following transplantation. In addition, recent studies suggest that PGD may also be a risk factor for BOS.(13-15) Little is known about the biomolecular mechanisms that might link these processes. Transforming growth factor-beta (TGF-β) is a critical mediator of extracellular remodeling and fibroproliferation that has also been found to have pleiotropic immunoregulatory properties, and is involved in the pathogenesis of a wide variety of disease states.(16-20) The profibrotic effects of TGF-β are driven by binding and activation of the TGF-β receptor complex, which promotes synthesis of procollagens, the precursors to mature collagen. Among these, procollagen type I (PC) is the propeptide of the most abundant form of collagen in humans. Previous studies have demonstrated that TGF-β concentrations within lungs are also elevated early on in the setting of acute lung injury, the non-transplant correlate of PGD, and TGF-β plays a role in driving the fibroproliferative phase of this condition.(21-24) However the role of TGF-β with respect to PGD specifically following lung transplantation has not been described.

In this study, we characterize the relationship between PGD and BOS in a single-center cohort of bilateral and single lung recipients. Additionally, we hypothesize that the association between PGD and BOS may be mediated, in part, by TGF-β in the early post-operative period, and that bronchoalveolar lavage fluid (BALF) concentrations of TGF-β and PC measured during this critical period may serve as complementary biomarkers for the future development of BOS.

MATERIALS AND METHODS

Study design and patient population

With approval from the institutional review board of the University of California at Los Angeles (protocol number 10-001492), we conducted a retrospective study of lung transplant recipients at our institution between March 1, 2000 and August 31, 2007. To determine the risk of BOS attributable to PGD, basic clinical characteristics, pathology reports from all allograft biopsy specimens, pulmonary function tests, and chest x-rays, arterial blood gases, and inspired oxygen concentrations from within the first 72 hours of transplantation were collected. To assess the association between BALF concentrations of TGF-β and PC and the clinical outcomes of PGD and BOS, we performed a nested study of all prospectively consented subjects with BALF from 24 hours post-transplant available for research use. Subjects were managed and underwent routine follow-up according to our institutional protocols.(25) All clinical protocols remained unchanged over the study period.

Clinical variable classification

BOS, acute cellular rejection (AR), and lymphocytic bronchiolitis (LB) were defined and graded according to International Society for Heart and Lung Transplantation (ISHLT) guidelines.(26-28) A cumulative AR score was derived for each patient, representing the sum of all 'A' grades.(25, 29) PGD was graded immediately after transplantation (T0) and through 72 hours (T72), according to ISHLT criteria.(11) We further classified subjects as follows: "Controls" had PGD grade 0-1 at all time points; subjects with "Transient PGD" had PGD grade 2-3 at T0 with resolution to grade 0-1 at T72; subjects with "Severe PGD" had PGD grade 2-3 at T0 through T72.

In order to ensure adequate spirometric data for the diagnosis of BOS, all study subjects were required to meet the following criteria for inclusion in models analyzing the association between PGD and BOS: (1) at least 6 outpatient pulmonary function tests following hospital discharge; (2) at least 6 months of spirometric follow-up. In order to minimize bias introduced by subjects with ongoing decline in lung function immediately following the development of PGD, these criteria were specifically chosen so that all subjects had stable established peak lung function. In other words, all subjects diagnosed with BOS developed a sustained 20% reduction in FEV1 that had to occur *at least* 6 months after transplantation.

Protein measurement and immunohistochemistry

After centrifugation of BALF specimens, supernatant protein concentrations were measured using standard ELISA for TGF-β_1 (R&D Systems, Minneapolis, MN) and PC (Quidel Corporation, San Diego, CA) according to manufacturers' instructions. Five subjects with hemodynamic instability, anemia and severe PGD underwent surgical re-exploration that included allograft biopsies within the first week of transplantation, and immunohistochemical staining was performed on these biopsy specimens for TGF-β and TGF-β receptor I (TβRI).

Statistical Analysis

Baseline clinical and demographic characteristics were compared between the total and nested cohorts by the Chi-square test for categorical variables and the Mann-Whitney test for continuous variables. Cox proportional hazards (CPH) models of BOS were created for the total cohort, and stratified by transplant type. Single lung transplantation, cumulative AR score, and the presence of LB on biopsies obtained prior to the diagnosis of BOS were then included into multivariable CPH models a priori, and backward elimination was used to screen for additional relevant covariates by Wald testing at a significance level of $p < 0.10$. Interactions were tested in the final models by individually adding multiplicative terms. Freedom from BOS was also estimated using the Kaplan-Meier method. BALF protein concentrations were compared across PGD classes by Kruskal-Wallis testing. Subjects were then dichotomized based on levels above or below the median concentration for each protein, and CPH models of BOS were created as a function of BALF protein concentration with and without adjustment for PGD. Analyses were performed using SAS® software version 9.3 (SAS Institute Inc., Cary, NC), and figures were created using GraphPad Prism® (version 5.0, GraphPad Software, San Diego,CA).

RESULTS

Cohort characteristics

Over the study period, a total of 279 patients underwent transplantation, including 174 bilateral and 105 single lung transplants. Among the bilateral transplants, 5 heart-lung recipients were also included. 148 (53.0%) subjects had PGD grade 0-1 at all time points (T0-T72), and were included in the "Control" group. An additional 124 (44.4%) patients had PGD grade 2-3 at T0 with 76 (27.2%) improving to PGD grade 1 by T72 included in the "Transient PGD" group, and the remaining 48 (17.2%) patients were considered to have "Severe PGD." An additional three patients were excluded from subsequent analyses for delayed onset PGD (e.g. PGD grade 1 at T0-24 progressing to grade 2-3 at T48-72). Four patients were excluded for death due to PGD prior to T72. Forty subjects were excluded from analyses of freedom from BOS due to insufficient spirometric follow-up. Of the remaining 239, all subjects had at least 6 post-transplant pulmonary functions tests over at least 6 months of follow-up. At study completion, there were a total of 684.3 person-years of BOS-free spirometric follow-up and a mean follow-up of 2.86 +/− 1.93 years among those with sufficient pulmonary function tests. A total of 98 subjects developed BOS over the study period with an estimated 50% incidence of BOS at 4.23 years following transplantation based on the Kaplan-Meier method. Seventy-five subjects had BALF collected within 24 hours post-transplant available for measurement of TGF-β and PC concentrations, and were included in the nested protein analyses. Among the nested cohort, the "Control," "Transient PGD," and "Severe PGD" group sample sizes were 44 (58.7%), 18 (24.0) and 13 (17.3%), respectively. Other relevant clinical characteristics for the total and nested cohort are summarized in Table 1, and the characteristics of subjects meeting spirometric criteria for inclusion in BOS analyses are summarized in Table S1. There were no statistically significant differences in any of these characteristics between the total cohort and those meeting spirometric criteria.

PGD is a risk factor for BOS

To characterize the association between PGD and the development of BOS in our cohort, we created crude CPH models of BOS as a function of PGD severity (Table 2). Compared to the "Control" group, subjects in both the "Transient PGD" (hazard ratio [HR] = 2.03, 95% confidence interval [CI] 1.29-3.20; p = 0.002) and "Severe PGD" (HR = 3.96, 95% CI 2.33-6.73; p < 0.001) groups had significantly increased rates of BOS that incrementally increased with PGD severity. After *a priori* adjustment for single lung transplantation, cumulative AR score, and the presence of LB the association between BOS and both "Transient PGD" and "Severe PGD" remained significant. As expected, both cumulative AR and LB were also significant predictors of BOS independent of PGD (Table 2). We explored the utility of adjusting for additional covariates including: recipient age, gender, use of cardiopulmonary bypass, type of induction immunosuppression, ischemia time (time to second lung reperfusion in bilateral recipients), and pre-transplant diagnosis; however, backward selection excluded all of these covariates.

Given conflicting literature regarding the association between PGD and BOS following single lung transplantation,(13-15, 30) we also created similar CPH models of BOS after stratification of our cohort by transplant type (Table 2). In crude models of BOS as well as models adjusted for cumulative AR score and the presence of LB, there continued to be an association between increasingly severe PGD and BOS in both single and bilateral lung recipients. Relative to controls, single lung recipients with either "Transient PGD" or "Severe PGD" had adjusted hazard ratios for BOS of 1.74 (95% CI 0.87-3.48; p = 0.12) and 3.67 (95% CI 1.27-10.58; p = 0.02), respectively. Notably, point estimates of the relative hazards for BOS attributable to PGD were comparable between single and bilateral lung recipients (Table 2). In parallel with these CPH models for BOS, Kaplan-Meier plots of freedom from BOS were constructed for the total cohort and stratified by transplant type (Figure 1).

Augmented TGF-β concentrations in BALF from within 24 hours of transplantation are associated with the development of PGD

Having established an association between PGD and BOS in our cohort, we then explored the role of TGF-β during PGD within a nested cohort of lung transplant recipients (n=75) from whom BALF collected within 24 hours of transplantation was available. We found that compared to controls, subjects with either transient or severe PGD had significantly higher BALF concentrations of TGF-β (p = 0.007 and p = 0.002, respectively) (Figure 2A). Between subjects with transient and severe PGD, the difference in BALF concentrations of TGF-β was not significant.

There is a strong correlation between BALF concentrations of TGF-β and PC

Previous studies have demonstrated that biologically active TGF-β upregulates the production of PC.(31-33) Based on these data we evaluated the BALF samples from the nested cohort of transplant recipients for concentrations of PC. We found a strong correlation between the log-transformed protein concentrations of TGF-β and PC (Pearson's r = 0.64, p < 0.001). Furthermore, we found that compared to controls, subjects with either transient or severe PGD had significantly higher BALF concentrations of PC (p= 0.003 and

p < 0.001, respectively) (Figure 2B). Between subjects with transient PGD and severe PGD, the difference in BALF PC concentration was not significant.

Augmented TGF-β concentrations in BALF from within 24 hours of transplantation are associated with the development of BOS

With previous studies demonstrating that increased expression of TGF-β can be associated with both acute and chronic lung injury,(24) we explored the association between increased TGF-β as well as PC concentrations in the BALF, and the long-term outcome of BOS. Thus, subjects meeting spirometric criteria for BOS analyses in the nested cohort (n=70) were then dichotomized based on median BALF concentrations of either TGF-β or PC (113 pg/ml and 1.34 pg/ml, respectively), and we constructed CPH models of BOS comparing subjects with high BALF concentrations of the proteins to those with concentrations below the medians. In crude CPH models, high levels of either protein were associated with increased rates of BOS (Table 3). These findings were consistent with Kaplan-Meier analyses demonstrating significantly reduced freedom from BOS in recipients with high concentrations of BALF TGF-β or PC (Figure 3A and B). Interestingly, after adjusting for PGD severity in parallel multivariable CPH models, high BALF TGF-β concentration remained a statistically significant independent predictor of BOS with HR = 2.61 (95% CI 1.26-5.39; p = 0.01). Conversely, the association between BALF PC and BOS was considerably dampened and no longer significant in multivariable models after adjustment for PGD severity. Among subjects in the nested cohort with a minimum of 3 years of spirometric follow-up (n=56), receiver operating characteristic analyses for the outcome of BOS at 3 years yielded areas under the curve for BALF TGF-β and PC of 0.77 (95% CI 0.64-0.90; p = 0.002) and 0.72 (95% CI 0.58-0.86; p = 0.008), respectively. There were no statistically significant associations between BALF concentrations of either protein and the subsequent development of AR or LB (data not shown).

Cellular sources of TGF-β and TβRI during severe PGD

With our results demonstrating an association between early BALF TGF-β concentrations and the subsequent development of BOS, we investigated the cellular sources of TGF-β within the allograft during PGD. Immunohistochemical staining was performed on representative surgical lung biopsies from subjects with severe PGD (n=5). We found that TGF-β protein was expressed by allograft bronchial epithelial cells, subepithelial infiltrating mononuclear cells, as well as alveolar macrophages (Figure 4A and B). Importantly, TβRI was predominantly expressed by allograft airway epithelium, peri-airway and interstitial infiltrating mononuclear cells and stromal cells, as well as alveolar macrophages (Figure 4C and D).

DISCUSSION

In this study we examine the association between PGD and BOS in a mixed cohort of single and bilateral lung transplant recipients. We demonstrate that compared to patients with minimal PGD (grade 0-1 at T0-72), patients with PGD grade 2-3 at T0 are at increased risk of subsequently developing BOS, and patients with the most severe form of PGD, persisting through T72 are at the greatest risk. These findings are consistent with prior studies in

bilateral transplant recipients, and corroborate the trend toward increased risk of BOS in subjects with PGD who fail to improve within 72 hours of transplantation suggested by Huang, et al.(13) Indeed, one advantage of our classification of PGD is that it accounts for both the physiological and temporal severity of PGD throughout the early post-transplant period rather than at isolated time points, and delineates the added incremental risk associated with early PGD that fails to improve over time.

In contrast to two prior studies that failed to demonstrate such an association among single lung recipients, the association between PGD and BOS in our cohort persisted after adjustment for transplant type, and was present in stratified analyses of either single or bilateral transplant recipients.(15, 30) The application of the ISHLT PGD grading system to single lung recipients must be approached cautiously. PGD grading presumes that the severity of graft injury correlates with impairment in overall gas exchange. In single lung recipients, the degree of hypoxemia is further modulated by the severity of disease within the native lung and the extent of physiological redistribution of perfusion to functional regions of either lung. Moreover, in subjects transplanted for underlying obstructive lung disease, the presence of native lung hyperinflation and consequent allograft volume loss may create a particular challenge. This anatomical interaction may not only affect gas exchange but could also confound the interpretation of radiographic abnormalities otherwise seen in PGD, potentially biasing studies toward the null due to misclassification. Misclassification bias may also extend to the diagnosis of chronic rejection as progressive native lung hyperinflation may mimic the declining spirometry seen in BOS. Of note, obstructive lung disease was an indication for transplantation in a minority of our subjects, while it accounted for the vast majority of recipients in the prior studies investigating PGD and BOS following single lung transplantation.(15, 30) We acknowledge that the magnitude of any association between PGD and BOS may differ in single lung recipients due to these and other factors. However, if PGD is in fact a risk factor for progressive allograft dysfunction culminating in BOS, we can find no biologically plausible explanation for this association to be entirely unique to bilateral lung recipients.

Elucidating the mechanisms that promote BOS following non-alloimmune insults such as PGD is critical to explaining and potentially improving the relatively poor long-term outcomes compared to other solid organs. As with non-transplant forms of lung injury, PGD may transition to a fibroproliferative phase leading to reduced post-operative lung function and long-term morbidity and mortality. However, our spirometric inclusion criteria for the analyses of BOS included only subjects with stable to improving lung function over the first 6 months post-transplant. Furthermore, the increased rate of spirometric decline among recipients with PGD extends for multiple years following transplantation as suggested by Kaplan-Meier analyses in this and other studies.(13-15) Therefore, we hypothesized that TGF-β would be a potential link between early allograft injury and the later development of fibroplasia of the allograft airways. We found increased concentrations of BALF TGF-β are present during increasingly severe PGD. One of TGF-β's main attributes during fibroproliferative diseases is its interaction with stromal cells, which induces the expression of PC, thereby leading to the deposition of new extracellular matrix.(31-33) To explore the in vivo biological significance of TGF-β during PGD, we evaluated the concentrations of PC in the same BALF. Similar to TGF-β, we found that concentrations of PC in BALF

paralleled the severity of PGD. Moreover, the concentrations of these proteins were strongly correlated. These data imply that TGF-β's augmentation during ischemia reperfusion injury promotes the expression of PC, and may contribute to fibroproliferation in the setting of PGD. This is supported by both human and animal studies involving acute lung injury, which have shown that TGF-β expression correlates with decreases in PaO2/FiO2 ratio as well as fibroplasia of the lung.(22, 24, 34-36) While these studies support the role of TGF-β in the pathogenesis of non-transplant acute lung injury as well as PGD, they do not explain the connection between acute post-transplant injury and the later onset of BOS.

This led us to explore the possibility that TGF-β activity in the early post-operative period may have long-term sequelae such as the subsequent development of BOS. We found that high concentrations of TGF-β in BALF collected within 24 hours of transplantation were associated with increased rates of BOS, and a similar association was seen for BALF PC in crude analyses. Together, this indicates at least two potential mechanisms that could link PGD to BOS. The first involves the self-perpetuation of receptor/TGF-β biology that eventually leads to fibroproliferative changes in the lung.(37-39) The second involves the early influence of this biological axis on stromal cells that can alter their phenotype, allowing for a relentless fibroproliferative process that persists after the resolution of acute injury and inflammation.(40, 41)

Notably, after adjusting for PGD severity, high BALF TGF-β concentrations remained a significant independent predictor of BOS, while the association between PC and BOS was no longer significant. This suggests that receptor/TGF-β biology may promote the development of BOS through mechanisms independent of or in addition to its role in promoting PC during PGD. Thus, we performed immunohistochemical analyses of both TGF-β and TβRI on lung tissue samples obtained during PGD. Both TGF-β and its receptor were expressed by interstitial and peri-airway infiltrating mononuclear cells, as well as allograft airway epithelial cells. Additionally, TβRI was also expressed by interstitial and peri-airway stromal cells. Collectively this suggests that early on, TGF-β is important for the expression of procollagen, in keeping with its known involvement in acute lung injury. (22, 24, 34-36) Prior studies have found increased concentrations of TGF-β in BALF from patients just prior to the development of BOS as well as after its diagnosis.(42-45) Mechanistically, inhibition of TGF-β signaling has also been shown to decrease fibro-obliteration in animal models of obliterative bronchiolitis.(46-48) In light of these findings, the expression of TGF-β and TβRI within and surrounding the allograft airways (the main anatomical targets in BOS) during PGD further supports the contention that receptor/TGF-β biology perpetuates fibroplasia of the allograft bronchioles, thereby implicating this axis in the continuum from PGD to BOS. Expression of both TGF-β and TβRI by the same cell types during PGD also suggests that this may be in part through autocrine induction of TGF-β expression.

In addition to the shortcomings inherent to any retrospective study design, we acknowledge that this study has several limitations. Though we made every effort to adjust for potential confounding in our analyses of the total cohort, data was unavailable to allow further adjustment for several established risk factors of BOS. Sample size limited any further stratification of the cohort to further assess for example the impact of transplant indication

on the association between PGD and BOS in single lung recipients. Furthermore, while the association between PGD and BOS persisted in models stratified by transplant type, small sample size limited the statistical power to adjust for multiple covariates in stratified analyses. In light of the prior publications failing to demonstrate this association in single lung recipients and the relatively small number (n=105) of these subjects in our study, we admit that the association between PGD and BOS specifically among single lung transplant recipients requires further validation. Biopsy specimens for immunohistochemistry were available only from transplant recipients with severe PGD. We acknowledge that comparison of these immunohistochemistry findings with specimens from controls would have complemented the results of BALF protein analyses. Finally, any role for BALF protein concentrations as biomarkers for recipients at greatest risk of early BOS requires validation, and we cannot exclude the possibility that TGF-β signaling may play an active role in the biology underlying PGD, but still be an epiphenomenon of the association between PGD and BOS. Further studies are necessary to clarify and confirm the mechanistic role TGF-β biology may play in the link between early injury and chronic dysfunction of the allograft.

In summary, our results further support the presence of an association between PGD and BOS in a mixed cohort including bilateral as well as single lung recipients. Nested analyses of BALF indicate that receptor/TGF-β biology is upregulated in the setting of PGD. Furthermore, tissue immunohistochemical staining of TGF-β and its receptor localized the activity of this axis to sites of recent injury as well as sites involved in the later development of BOS, suggesting this biology's involvement in the mechanisms linking PGD and BOS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Abbreviations

PGD	Primary graft dysfunction
BOS	Bronchiolitis obliterans syndrome
TGF-β	Transforming growth factor-beta
PC	Procollagen type I
BALF	Bronchoalveolar lavage fluid
AR	Acute (cellular) rejection
LB	Lymphocytic bronchiolitis
ISHLT	International Society for Heart and Lung Transplantation

T0	Time immediately after lung transplantation
T72	72 hours following lung transplantation
TβRI	TGF-β receptor I
CPH	Cox proportional hazards
HR	Hazard ratio

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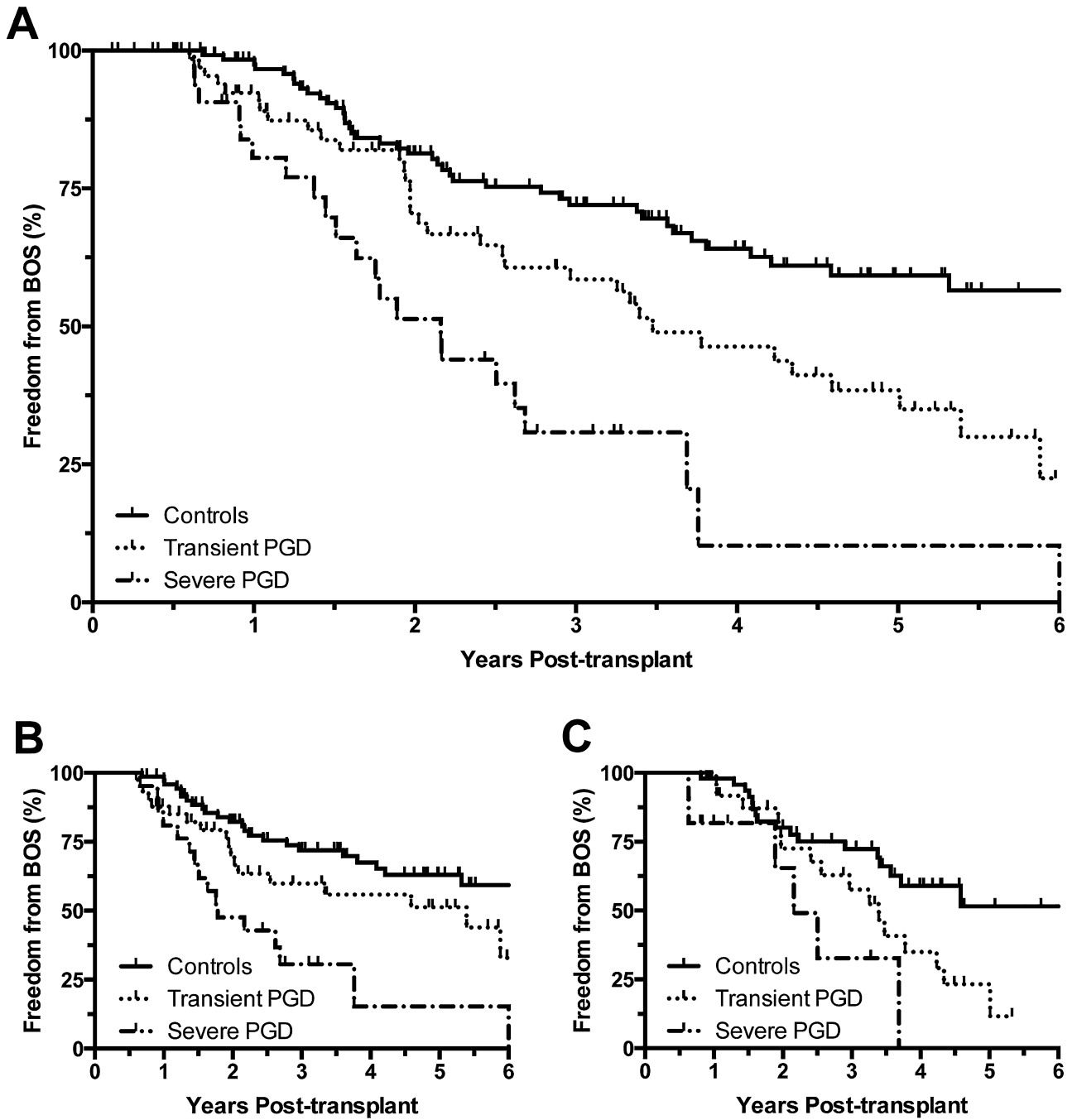
Figure 1.
Kaplan-Meier plots of freedom from BOS by PGD severity for: (A) the total cohort of transplant recipients, demonstrating log-rank hazard ratios of 1.98 (95% CI 1.32-3.52; p = 0.002) and 3.57 (95% CI 3.45-14.95; p < 0.001) for transient and severe PGD, respectively; (B) bilateral lung transplant recipients, demonstrating log-rank hazard ratios of 1.87 (95% CI 1.05-3.89; p = 0.04) and 3.53 (95% CI 2.73-14.87; p < 0.001) for transient and severe PGD, respectively; and (C) single lung transplant recipients, demonstrating log-rank hazard

ratios of 2.36 (95% CI 1.31-4.85; p = 0.04) and 3.44 (95% CI 1.88-31.34; p = 0.01) for transient and severe PGD, respectively.
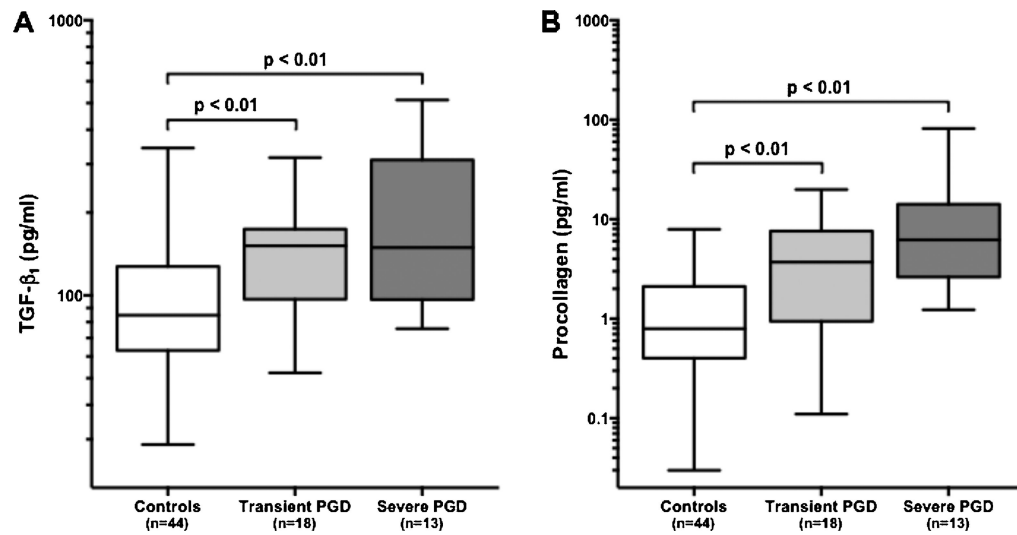
Figure 2.
Concentrations of (A) TGF-β and (B) procollagen in BALF collected within 24 hours of transplantation. Compared to controls, concentrations of TGFb and procollagen were significantly elevated in subjects with either transient and severe PGD. Between the latter groups, differences in BALF concentration for either protein did not meet statistical significance.
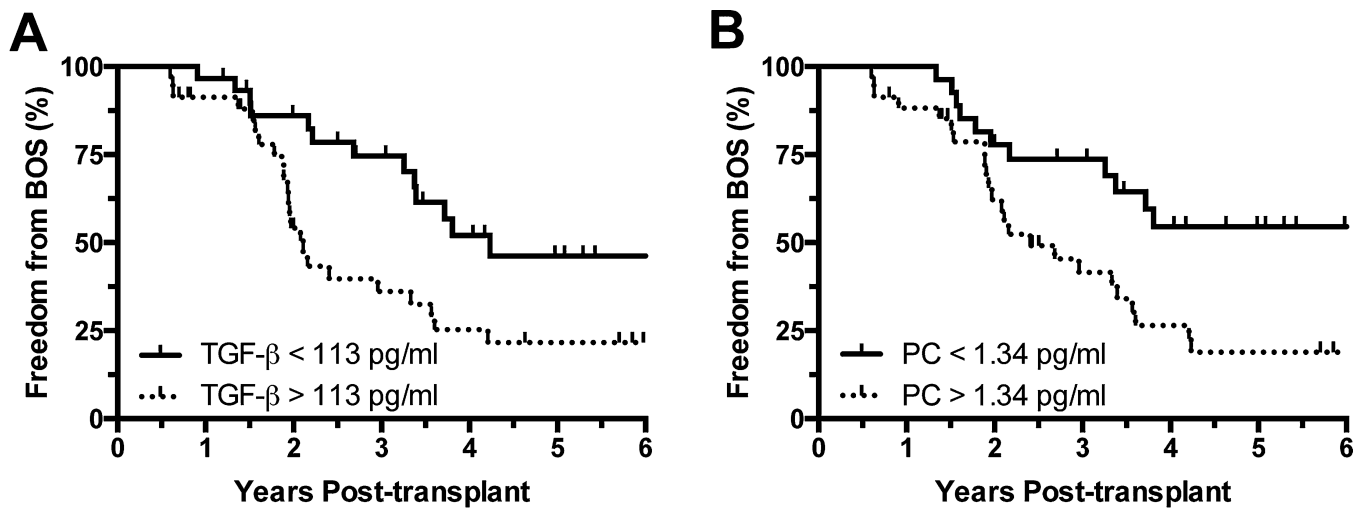
Figure 3.
Kaplan-Meier plots of freedom from BOS dichotomized by BALF protein concentrations above or below median levels for (A) TGF-β, demonstrating a log-rank hazard ratio of 2.43 (95% CI 1.33-4.84; p = 0.01) for [TGF-β] > 113 pg/ml and (B) procollagen, demonstrating a log-rank hazard ratio of 2.26 (95% CI 1.19-4.32; p = 0.01) for [PC] > 1.34 pg/ml.

Figure 4.
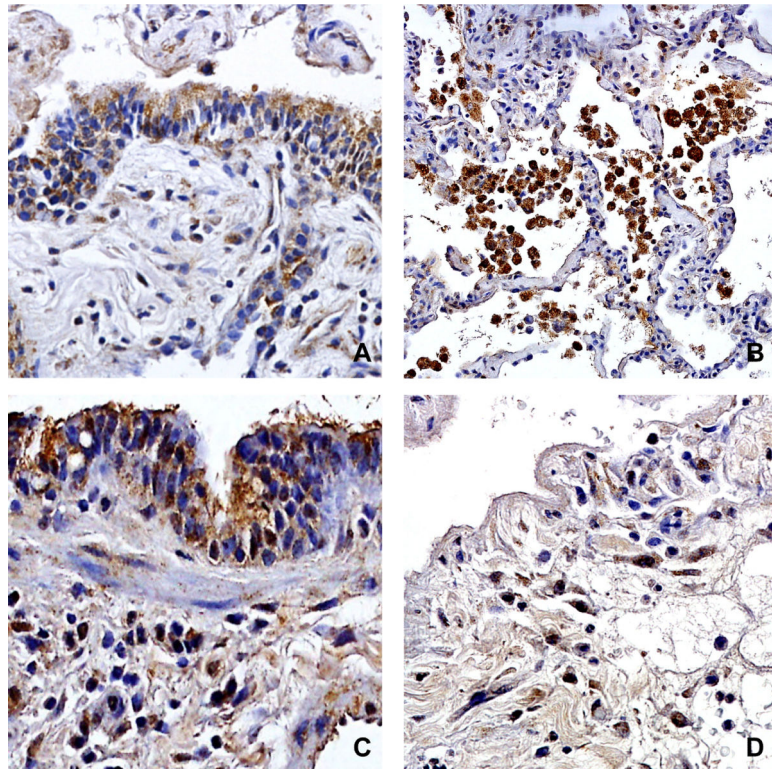Immunohistochemical staining for TGF-β localizing expression to (A) allograft small airway epithelial cells and subepithelial infiltrating mononuclear cells, as well as (B) alveolar macrophages. TβRI expression was noted in (C) small airway epithelial cells, subepithelial stromal cells and infiltrating mononuclear cells, as well as (D) infiltrating stromal cells and mononuclear cells within the interstitium.

Table 1

Characteristics of subjects in the total and nested cohort.

Characteristic	Total Cohort (n=279)	Nested Cohort (n=75)	p-value*
Gender, n (%)			0.4
Male	173 (62%)	37 (49.3%)	
Female	126 (38%)	38 (50.7%)	
Age, yr [IQR])	59 [53-64]	61 [56-65]	0.15
Age 65, n (%)	63 (22.6)	20 (26.7)	0.46
Transplant Type, n (%)			0.56
Single	105 (37.6)	31 (41.3)	
Bilateral	174 (62.4)	44 (58.7)	
Induction Agent, n (%)			0.60
rATG	173 (62.0)	44 (58.7)	
Basiliximab	106 (38.0)	31 (41.3)	
Cardiopulmonary bypass use, n (%)	187 (67.0)	47 (62.7)	0.48
Ischemia time, min [IQR]	321 [267-372]	321 [257-361]	0.44
Pre-transplant diagnosis, n (%)			0.54
Idiopathic pulmonary fibrosis	101 (36.2)	30 (40.0)	
Chronic obstructive pulmonary disease	97 (34.8)	21 (28.0)	
Other	81 (29.0)	24 (32.0)	
Pulmonary arterial hypertension	14 (5.0)	4 (5.3)	
Systemic Sclerosis	14 (5.0)	5 (6.7)	
Other interstitial lung disease	11 (3.9)	4 (5.3)	
Cystic fibrosis	10 (3.6)	1 (1.3)	
Sarcoidosis	9 (3.2)	3 (4.0)	
Bronchiolitis obliterans syndrome	9 (3.2)	3 (4.0)	
Non-CF bronchiectasis	7 (2.5)	1 (1.3)	
α1-antitrypsin deficiency	6 (2.2)	2 (2.7)	
Lymphangioleiomyomatosis	1 (0.4)	1 (1.3)	
PGD Class, n (%)			0.77
Controls	148 (53.0)	44 (58.7)	
Transient PGD	76 (27.2)	18 (24.0)	
Severe PGD	48 (17.2)	13 (17.3)	

IQR = Interquartile range; PGD = primary graft dysfunction.

*Categorical variables were compared by the Chi-square test; continuous variables were compared by the Mann-Whitney test

Table 2

Crude and adjusted Cox proportional hazards models for freedom from BOS by PGD severity for subjects from the cohort meeting spirometric criteria for BOS analysis (n=239), further stratified by transplant type.

Variable	Crude[1] HR [95% CI]	p-value	Adjusted[2] HR [95% CI]	p-value
Total cohort				
PGD severity				
Controls	1.00		1.00	
Transient PGD	2.03 [1.29-3.20]	0.002	2.00 [1.27-3.16]	0.003
Severe PGD	3.96 [2.33-6.73]	< 0.001	3.07 [1.76-5.38]	< 0.001
Cumulative AR score	--		1.12 [1.02-1.22]	0.02
Lymphocytic bronchiolitis	--		2.10 [1.20-3.68]	0.01
Single lung transplant	--		1.30 [0.86-1.98]	0.21
Bilateral lung transplants				
PGD severity				
Controls	1.00		1.00	
Transient PGD	1.91 [1.04-3.50]	0.04	2.08 [1.13-3.84]	0.02
Severe PGD	3.70 [1.94-7.05]	< 0.001	2.82 [1.45-5.50]	0.002
Cumulative AR score	--		1.13 [1.01-1.27]	0.04
Lymphocytic bronchiolitis	--		1.69 [0.82-3.51]	0.16
Single lung transplants				
PGD severity				
Controls	1.00		1.00	
Transient PGD	2.13 [1.07-4.21]	0.03	1.74 [0.87-3.48]	0.12
Severe PGD	4.25 [1.64-11.04]	0.003	3.67 [1.27-10.58]	0.02
Cumulative AR score	--		1.13 [0.99-1.30]	0.10
Lymphocytic bronchiolitis	--		2.82 [1.13-7.00]	0.03

HR = hazard ratio; CI = confidence interval; BOS = bronchiolitis obliterans syndrome; PGD = primary graft dysfunction; AR = acute rejection.

[1] Crude models included only Transient PGD and Severe PGD as independent categorical variables with Controls as reference.

[2] All adjusted models included PGD severity, cumulative AR score, and the presence of LB on transbronchial biopsies. The adjusted model for the total cohort was also further adjusted for transplant type.

Table 3

Crude and adjusted Cox proportional hazards models for freedom from BOS by BALF protein concentration within the nested cohort of subjects meeting spirometric criteria for BOS analysis (n=70).

Variable	Crude[1] HR [95% CI]	p-value	Adjusted[2] HR [95% CI]	p-value
BALF TGF-β concentration				
[TGF-β] < 113 pg/ml	1.00		1.00	
[TGF-β] > 113 pg/ml	2.54 [1.28-5.01]	0.007	2.61 [1.26-5.39]	0.01
Transient PGD	--		1.54 [0.71-3.32]	0.27
Severe PGD	--		4.47 [1.87-10.69]	0.001
BALF PC concentration				
[PC] < 1.34 pg/ml	1.00		1.00	
[PC] > 1.34 pg/ml	2.32 [1.16-4.65]	0.02	1.45 [0.63-3.37]	0.39
Transient PGD	--		1.86 [0.79-4.34]	0.15
Severe PGD	--		3.39 [1.24-9.29]	0.02

HR = hazard ratio; CI = confidence interval; BOS = bronchiolitis obliterans syndrome; PGD = primary graft dysfunction; BALF = bronchoalveolar lavage fluid; TGFβ = transforming growth factor-beta; PC = procollagen.

[1] Crude models included only the respective protein BALF concentration dichotomized by values above or below median concentration.

[2] All adjusted models included the respective protein concentration as in crude analyses, along with further adjustment for PGD severity as covariates.