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Aspiration, Localized Pulmonary Inflammation, and Predictors of Early-Onset Bronchiolitis Obliterans Syndrome after Lung Transplantation

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Abstract

BACKGROUND—We hypothesized that immune mediator concentrations in the bronchoalveolar fluid (BALF) are predictive of bronchiolitis obliterans syndrome (BOS) and demonstrate specific patterns of dysregulation, depending on the presence of acute cellular rejection, BOS, aspiration, and timing of lung transplantation.

STUDY DESIGN—We prospectively collected 257 BALF samples from 105 lung transplant recipients. The BALF samples were assessed for absolute and differential white blood cell counts and 34 proteins implicated in pulmonary immunity, inflammation, fibrosis, and aspiration.

RESULTS—There were elevated BALF concentrations of interleukin (IL)-15, IL-17, basic fibroblast growth factor, tumor necrosis factor–α, and myeloperoxidase, and reduced concentrations of α_1-antitrypsin, which were predictive of early-onset BOS. Patients with BOS had an increased percentage of BALF lymphocytes and neutrophils, with a reduced percentage of macrophages ($p < 0.05$). The BALF concentrations of IL-1β; IL-8; interferon-γ–induced protein 10; regulated upon activation, normal T-cell expressed and secreted; neutrophil elastase; and pepsin were higher in patients with BOS ($p < 0.05$). Among those with BOS, BALF concentrations of IL-1RA; IL-8; eotaxin; interferon-γ–induced protein 10; regulated upon activation, normal T-cell expressed and secreted; myeloperoxidase; and neutrophil elastase were positively correlated with time since transplantation ($p < 0.01$). Those with worse grades of acute cellular rejection had an increased percentage of lymphocytes in their BALF ($p < 0.0001$) and reduced BALF concentrations of IL-1β, IL-7, IL-9, IL-12, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, interferon-γ, and vascular endothelial growth

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Study conception and design: Fisichella, Davis
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factor ($p \leq 0.001$). Patients with aspiration based on detectable pepsin had increased percentage of neutrophils ($p < 0.001$) and reduced BALF concentrations of IL-12 ($p < 0.001$).

CONCLUSIONS—The BALF levels of IL-15, IL-17, basic fibroblast growth factor, tumor necrosis factor–α, myeloperoxidase, and α_1-antitrypsin at 6 to 12 months after lung transplantation are predictive of early-onset BOS, and those with BOS and aspiration have an augmented chemotactic and inflammatory balance of pulmonary leukocytes and immune mediators. These data justify the surgical prevention of aspiration and argue for the refinement of antirejection regimens.

Lung transplantation patients continue to have the worst survival of all solid organ transplant recipients, despite attempts at refining surgical technique and antirejection regimens.[1] The reduced survivability after lung transplantation is multifactorial and involves donor-related factors, primary graft dysfunction, allorecognition, and bronchiolitis obliterans syndrome (BOS), which is characterized by progressive fibrous obliteration of the small airways.[2,3] Affecting half of lung transplant recipients by 5 years,[1] BOS is also a multidimensional process that appears to involve both alloimmune and non-alloimmune factors, such as ischemia/reperfusion, infection, and gastroesophageal reflux disease (GERD)–related aspiration.[4]

Our work and that of others has identified GERD as exceedingly common among lung transplant recipients.[5–9] In addition, we have affirmed that the surgical correction of GERD is not only safe after lung transplantation,[10–12] but that it can also stabilize, if not prolong, pulmonary function.[7,8,13,14] Our most recent findings have demonstrated prevention of aspiration by way of reduced pepsin levels in the bronchoalveolar lavage fluid (BALF) after laparoscopic antireflux surgery (LARS),[14] which appears to parallel a less proinflammatory and fibrogenic environment within the pulmonary allograft.[15,16]

The aim of our current study was to characterize the biologic changes that occur with BOS, acute cellular rejection (ACR), and aspiration. In addition, we hoped to identify a unique pattern of immune mediators within the BALF that would be predictive of early-onset BOS when measured within the first year after lung transplantation. We hypothesized that a proinflammatory and fibrogenic pulmonary microenvironment is characteristic of ACR, aspiration, development of BOS, and timing of lung transplantation.

METHODS

Patients and parameters

From September 2009 to January 2012, there were 105 lung transplantation patients prospectively enrolled, on whom transbronchial biopsy and bronchoalveolar lavage were performed during routine surveillance or when clinically indicated by reduced pulmonary function on spirometry. At our institution, surveillance bronchoscopy is performed 1, 3, 6, 9, and 12 months after transplantation. Clinical variables and outcomes of interest were recorded, including age, sex, indication for transplantation, time since transplantation, identification of ACR by transbronchial biopsy, diagnosis of BOS, presence of GERD, and evidence of aspiration as determined by measureable pepsin in the BALF.

All study subjects provided informed consent. Participants were excluded for the following: age younger than 18 years, combined heart and lung transplantation, malignancy, current smoking, and pregnancy. This study was approved by the Loyola University Medical Center Institutional Review Board (LU202400).

Pulmonary function testing

All lung transplantation patients underwent serial pulmonary function testing according to institutional protocol, with spirometry and flow volume assessments performed at each clinic appointment and with any substantial change in respiratory symptoms. This generates a schedule of post-transplantation documentation of the forced expiratory volume in 1 second once per week for the first month, twice monthly for the next 2 months, then every third month, or more frequently depending on clinical indication. Additionally, full pulmonary function testing with and without bronchodilators is performed 6 months post transplantation, and annually thereafter. All forced expiratory volume in 1 second data consist of pulmonary function assessment without bronchodilators.

Immunosuppression

The standard maintenance immunosuppression regimen at our institution includes a calcineurin inhibitor (tacrolimus), an anti-metabolite (azathioprine or mycophenolate mofetil), and steroids. Patients routinely received induction immunosuppression with either basiliximab or daclizumab, with the exception of those patients sero-negative for cytomegalovirus receiving an allograft from a cytomegalovirus-seropositive donor.

Esophageal function testing

Esophageal function testing was undertaken as we have described previously.[5] Briefly, proton pump inhibitors were stopped for 14 days and histamine H_2-receptor antagonists were stopped for 3 days before pH monitoring. A pH catheter (Sleuth system with BioVIEW software; Sandhill Scientific Inc.) was placed with the distal pH sensor positioned 5 cm superior to the manometrically determined upper border of the lower esophageal sphincter. The DeMeester score was calculated for the distal pH recordings, and a score >14.7 was considered diagnostic of GERD.

Bronchoalveolar fluid collection, storage, and sample processing

Bronchoalveolar fluid was routinely collected from the right middle lobe for unilateral right and bilateral lung transplants, and from the lingula for unilateral left lung transplants.[14] The BALF was stored on ice and taken to the laboratory for processing within 4 hours. The BALF was then centrifuged at 1500 rpm for 10 minutes, aliquoted, and snap frozen at −80°C for analysis of pulmonary immune mediators.[14] In addition to absolute and differential WBC counts, the BALF samples were assessed for 34 proteins implicated in pulmonary immunity, inflammation, fibrosis, and aspiration. Specifically, pulmonary cytokines, chemokines, and growth factors in the BALF samples were measured by multiplex bead array (Bio-Rad Laboratories) according to manufacturer instructions and as previously described by our own laboratories.[17] Similarly, enzyme-linked immunosorbent assay was undertaken to measure the BALF concentrations of α_1-antitrypsin (A1AT;

Immunology Consultants Laboratories, Inc.), neutrophil elastase (NE; Cell Sciences), activated transforming growth factor–β_1 (R&D Systems), and pepsin according to manufacturer instructions and as we have described previously.[14] Finally, the concentration of myeloperoxidase (MPO) in the BALF was measured with the O-dianisidine MPO assay.[18] All data were normalized per milligram of protein.

Determination of acute cellular rejection, aspiration, and bronchiolitis obliterans syndrome

The transbronchial biopsy were assessed for ACR, the severity of which was graded according to the Revision of the 1996 Working Formulation for the Standardization of Nomenclature in the Diagnosis of Lung Rejection.[19] Comparisons were made between those without ACR (A0), those with minimal-to-mild ACR (A1/A2), and those with moderate-to-severe ACR (A3/A4). Patients were grouped as such given that few BALF samples were collected at the time of ACR grades 3 and 4. Comparisons of the grades of ACR were also performed after excluding those with concurrent bacterial and viral infections.

Evidence of aspiration was confirmed with any BALF pepsin level greater than the lower limit of detection of the assay (1 ng/mL).

Presence and severity of BOS (grades 0 to 3) of the lung transplant recipients were established according to the guidelines of the International Society of Heart and Lung Transplantation.[20] However, comparisons were made between those with and without BOS because very few samples were collected from patients with BOS grades 2 (n = 4) or 3 (n = 6). This is a reflection of our study protocol in that the majority of samples are collected during the first year post lung transplantation within the period of routine surveillance for ACR and when few patients would have already had BOS develop. Also, few samples of BOS grades 2 and 3 were collected because in our experience patients who have BOS develop have a rapid decline toward mortality.

Statistical analysis and mediators of interest

All data were assessed for normality by the D'Agostino and Pearson omnibus normality test and parametric or nonparametric tests were applied where appropriate. Dichotomous variables are reported as a percent and number, nonparametric variables are reported as median with the interquartile range (IQR), and continuous variables of parametric tests are reported as mean with SD. Correlations were performed with Spearman's rank correlation coefficient. Logistic regression was performed based on log-transformed data to identify factors predictive of early-onset BOS. Statistical analyses were calculated with SAS software (version 9.1; SAS Institute Inc) and GraphPad Prism 5 for Windows (GraphPad Software). A difference between observed variables was considered statistically significant either when the 95% CI did not include 1.000, or when $p < 0.05$.

Specific mediators of interest were those consistently implicated by the literature in lung transplant dysfunction, those at physiologically relevant levels (ie, consistently above the lower limit of assay detection), and those passing the multiple comparisons test of α/n.

RESULTS

Demographics and clinical characteristics

Median age of the 105 lung transplant patients enrolled was 59 years (IQR 50 to 62 years), 48 (46%) were female. The distribution of indications for lung transplantation was as follows: chronic obstructive pulmonary disease (n = 40), idiopathic pulmonary fibrosis (n = 24), cystic fibrosis (n = 16), α_1-anti-trypsin deficiency (n = 8), sarcoidosis (n = 4), pulmonary artery hypertension (n = 2), bronchiolitis obliterans organizing pneumonia (n = 1), Jo-1 syndrome (n = 1), lymphangioleiomyomatosis (n = 1), pulmonary veno-occlusive disease (n = 1), scleroderma (n = 1), and pulmonary fibrosis from work exposure (n = 1), rheumatoid arthritis (n = 1), dermatomyositis (n = 1), polymyositis (n = 2), and pneumoconiosis (n = 1). Median duration of post-transplantation follow-up was 33 months (IQR 21 to 42 months). After excluding 4 patients transferring to our center with incomplete forced expiratory volume in 1 second data, we found that 29 (29%) were diagnosed with BOS, with a median time to BOS of 26 months (IQR 15 to 45 months).

Predictors of early-onset bronchiolitis obliterans syndrome

Logistic regression analysis of the BALF samples collected 6 to 12 months post lung transplantation was performed to identify pulmonary immune mediators predictive of BOS developing by 30 months (Table 1). We found that elevated concentrations of interleukin (IL)-15, IL-17, basic fibroblast growth factor, tumor necrosis factor–α, and MPO were predictive of BOS by 30-months post lung transplantation. Likewise, onset of BOS by 30 months after lung transplantation was also predicted by reduced BALF concentrations of A1AT.

After adjusting for the presence of pulmonary infection (bacterial or viral) and severity of ACR, we found that several BALF immune mediators when measured from 6 to 12 months post transplantation remained predictive of early-onset BOS. These included elevated concentrations of IL-15 (odds ratio [OR] = 1.78; 95% CI 1.02–3.15), IL-17 (OR = 1.61; 95% CI 1.02–2.54), tumor necrosis factor–α (OR = 1.98; 95% CI 1.22–3.23), and MPO (OR = 3.94; 95% CI 1.16–13.35), and lower levels of A1AT (OR = 0.24; 95% CI 0.07–0.91). We did not adjust for primary graft dysfunction as primary graft dysfunction developed in very few patients. We did not adjust for the effects of azithromycin because, per institutional protocol, azithromycin is only added to a patient's regimen once BOS has been confirmed.

Given the profound immunological changes that occur immediately after lung transplantation (eg, as the consequence of ischemia-reperfusion, mechanical ventilation, and considerable immunosuppression), we assumed identification of a BALF immune mediator just 1 to 6 months after lung transplantation that predicts BOS by 30 months would be unlikely. This assumption was generally verified, as of all the mediators that we measured in the samples collected between 1 and 6 months after the time of transplantation, only the BALF concentration of A1AT was predictive of BOS by 30 months (OR = 0.20; 95% CI 0.04–0.95). Lastly, none of the absolute and differential WBC counts in the BALF were predictive of BOS by 30 months, regardless of measurement from 1 to 6 months or 6 to 12 months after lung transplantation (data not shown).

Bronchoalveolar fluid immune mediator profile, bronchiolitis obliterans syndrome, and time since lung transplantation

Bronchoalveolar fluid from patients with BOS was compared with that from patients without BOS to characterize the pulmonary immune mediator profile of chronic lung transplant rejection. Patients with BOS have an obvious shift of the absolute and differential WBC counts in the BALF that differs greatly from normal physiology. Specifically, compared with those free from BOS, patients with BOS have a significantly reduced percentage of macrophages in their BALF (median 50% vs 85%; $p < 0.0001$) with a concurrently increased percentage of lymphocytes (median 6% vs 4%; $p = 0.043$) and neutrophils (median 11% vs 5%; $p = 0.013$). Not only does the differential of WBCs vary markedly between those with and without BOS, so does the absolute number of WBCs ($p = 0.008$), lymphocytes ($p = 0.002$), and neutrophils ($p = 0.006$).

Table 2 shows that the neutrophilic composition of leukocytes in the BALF is paralleled by a proinflammatory, fibrogenic, and chemotactic balance of pulmonary immune mediators among those with BOS. For instance, among mediators measured at physiologically relevant concentrations (ie, consistently above the lower limit of assay detection), patients with BOS had elevated BALF concentrations of IL-1β ($p = 0.003$); IL-8 ($p = 0.002$); interferon-γ–induced protein 10 (IP-10; $p = 0.003$); regulated upon activation, normal T-cell expressed and secreted ($p = 0.003$); and NE ($p < 0.001$). Finally, those with BOS also had elevated BALF concentrations of pepsin ($p = 0.001$), supporting an association between aspiration and chronic lung transplant rejection.

The shift in balance to a reduced percentage of macrophages and an elevated percentage of lymphocytes and neutrophils was also reflected in the time since lung transplantation. For example, among the entire cohort, the percentage of BALF lymphocytes correlated positively with time since transplantation ($r = 0.24$; $p < 0.001$), and the percentage of macrophages demonstrated a negative relationship ($r = -0.15$; $p < 0.05$). The changes over time since transplantation of the absolute and differential WBC counts was most pronounced among those with BOS, as in the BALF of these patients, the percentage of macrophages correlated negatively with time since transplantation ($r = -0.42$; $p < 0.001$), and the percentage of neutrophils correlated positively ($r = 0.33$; $p < 0.05$).

Similar to the BALF leukocyte count and differential, correlations with time since lung transplantation were also noted on evaluation of the pulmonary immune profile. Among those with BOS, time since lung transplantation was significantly correlated with BALF concentrations of IP-10 ($r = 0.35$; $p < 0.01$), NE ($r = 0.54$; $p < 0.001$), and pepsin ($r = 0.25$; $p < 0.05$).

Bronchoalveolar fluid immune mediator profile and acute cellular rejection

As among patients with BOS, notable findings were identified on analysis of the BALF of those with ACR at the time of surveillance or diagnostic bronchoscopy. First, compared with those free of ACR, those with worse grades of ACR had a greater percentage and total number of lymphocytes ($p < 0.001$). Second, compared with those without ACR, those with ACR grades A3/A4 had lower BALF concentrations of numerous cytokines, chemokines,

and growth factors, such as IL-1β, IL-7, IL-9, IL-12, IL-13, IL-15, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ, macrophage inflammatory protein-1β, and vascular endothelial growth factor ($p \leq 0.05$, after post-hoc analysis; Table 3). Conversely, BALF concentrations of transforming growth factor–β were higher in those with ACR grades A3/4 ($p < 0.05$, after post-hoc analysis). Differences in the BALF concentrations between the grades of ACR were also noted for IL-1RA ($p = 0.005$), IL-8 ($p = 0.009$), IL-10 ($p = 0.017$), and platelet-derived growth factor ($p = 0.004$), which were all lower among those with worse grades of ACR (Table 3). These data represent 152 samples from 85 patients with grade A0 ACR, 62 samples from 48 patients with grades A1/A2 ACR, and 9 samples from 9 patients with grades A3/4 ACR. Inherently, this does include some patients with recurrent episodes of ACR.

Bronchoalveolar fluid immune mediator profile and aspiration

Compared with nonaspirators, lung transplantation patients identified with aspiration based on detectable pepsin in their BALF had a reduced percentage of macrophages (90% vs 74%; $p < 0.0001$) and an increased percentage (3% vs 9%; $p < 0.001$) and total number (5 vs 11 cells/μL) of neutrophils. Likewise, the BALF profile of lung transplantation patients with evidence of aspiration was markedly different than that of nonaspirators, in that approximately half of the pulmonary immune mediators that were measured were different between the groups (Table 4). Of these mediators, those that varied the greatest between aspirators and nonaspirators were IL-8 (median 135 vs 49 pg/mL; $p < 0.001$) and NE (median 68 vs 0 ng/mL; $p < 0.001$), although the concentrations of IL-1β, IL-1RA, and IP-10 all reached a significance level of $p < 0.001$. We also identified that the BALF concentrations of pepsin correlated positively ($p < 0.05$) with those of complement component 5a, IL-1β, IL-1RA, IL-8, IP-10, tumor necrosis factor-α, and NE. On the contrary, BALF concentrations of pepsin correlated negatively ($p < 0.05$) with those of IL-12 and the ratio of A1AT to NE. These data represent 119 samples from 67 patients without aspiration and 133 samples from 69 patients with aspiration.

Finally, only the concentration of GM-CSF was different between those with and without GERD on 24-hour ambulatory pH monitoring (48 vs 95 pg/mL; $p < 0.001$; data not shown). Along these lines, only the BALF concentrations of GM-CSF and IL-10 seemed to correlate with the severity of reflux. For example, BALF concentrations of GM-CSF correlated inversely with percent total time that the pH was <4 at both the proximal and distal esophageal sensors, the total episodes of reflux, the DeMeester score, and the mean acid time in the supine position ($p < 0.05$), and the BALF concentrations of IL-10 correlated inversely with percent total time that the pH was <4 at both the proximal and distal esophageal sensors, the total episodes of reflux, and the mean acid time in the supine position ($p < 0.05$).

DISCUSSION

The primary results of our study are 3-fold. First, we have identified several mediators that are predictive of early-onset BOS when measured in the BALF 6 to 12 months post lung

transplantation. Second, we have shown that the BALF, among lung transplantation patients with BOS, is shifted in immune composition to one marked by decreased macrophages, increased neutrophils, and a significant level of proinflammatory and fibrogenic mediators that is particularly characterized by injurious and chemotactic factors. Third, we have clarified for the first time in a large sample size, the profound immunological changes evoked by aspiration after lung transplantation, which suggests the biological mechanism by which LARS can stabilize and prolong pulmonary function in these patients.

Many studies in both animal models and humans have characterized BOS as a process invoked by chronic injurious stimuli, implicating numerous immune mediators that are pathophysiologically relevant. Yet, few have identified those as actually predictive of BOS development. Our study has shown that when measured 6 to 12 months post transplantation, elevated BALF levels of IL-15, IL-17, basic fibroblast growth factor, tumor necrosis factor–α, and MPO, and reduced levels of A1AT, are predictive of BOS by 30 months. These findings also appear to confirm a central tenet of development of BOS, which is a shift in the pulmonary allograft environment to one characterized by increased neutrophilia that is demonstrated by elevated BALF levels of NE and MPO.[21–28] Interestingly, the microenvironment of those with BOS has been noted to involve NE activity that is unopposed by protective factors, such as A1AT, which our study also confirms[21]; however, more research is required to evaluate our findings, as well as the seemingly protective effect of A1AT against development of BOS. Although it has been shown in lung transplantation that A1AT can protect against the effects of NE,[21] A1AT can be protective through other mechanisms. For example, A1AT can promote anti-inflammatory conditions that favor the development of regulatory T cells.[22] In addition, it has been shown that A1AT can block the production and activity of IL-8 in human cells. This is intriguing, given the seemingly ubiquitous role of IL-8 in BOS after lung transplantation, and that A1AT infusion has a very favorable safety profile.[23–27] Most importantly, BOS is likely induced by increased proinflammatory signaling and chemotaxis. In particular, IL-8 has routinely been identified as a central player in the development of BOS[28–32] and, in our study, BALF levels of IL-8 were 5-fold greater among those with chronic lung transplant rejection. Many studies additionally suggest that IL-17 is also a crucial proinflammatory mediator that is implicated in BOS and that might be responsible for the elevated pulmonary IL-8 levels among lung transplantation patients with chronic rejection.[32–36] Although we failed to identify IL-17 as elevated in those with BOS, we did find that higher BALF IL-17 concentrations just 6 to 12 months post lung transplantation were predictive of the onset of BOS by 30 months. Taken together, our data suggest the balance between a pro-Th17 milieu (signified by increased IL-17, IL-8, and neutrophils) and negative regulators of Th17, such as Th1 cells (ie, decreased IL-12) or IL-10, can influence early-onset of BOS. Of note, we have intentionally chosen not to pursue specific cutoff values for parameters at 6 to 12 months that could be used to predict the development of early BOS for 2 reasons. First, lung transplantation patients are extraordinarily heterogenous; a cutoff point for one patient might not be the same for another one. With data limited to just one center, we are not yet comfortable suggesting such values. Second, even if we had suggested specific cutoff values, these could end up being not very useful clinically, given that the methods for analyzing protein content

in the BALF are far from standardized among lung transplantation centers. As such work goes forward, this point will need to be addressed.

Although our findings related to BOS after lung transplantation appear relatively straightforward, at first glance those related to ACR are not. Given that multiple acute rejection episodes and proinflammation have been linked to BOS, we had expected a proinflammatory balance to be evident in our panel of pulmonary immune mediators. In fact, this was not the case, as nearly every immune mediator aside from transforming growth factor–β was lower among those with ACR. These findings might not actually be surprising, as difficulties with characterizing the immune profile during ACR have typically been met with muted success, and are likely related to a wide variety of factors, such as time since transplantation, current immunosuppression, infections, severity of ACR, and sampling techniques.[33,36] The lymphocytosis that we have found during ACR is intuitive and has been described previously.[33,36] Together with an influx of neutrophils that has been noted during ACR, a chronic proinflammatory and fibrogenic environment can be generated by repeated ACR insults. The significance of the BALF composition of immune mediators immediately during ACR remains unclear.

Results of our study also imply that not all patients with GERD aspirate, because few local pulmonary immune mediators were different among those with and without GERD. This finding might indicate that GERD alone does not evoke the profound pulmonary immune changes that are associated with actual evidence of aspiration. In addition, these results suggest that the detection of markers of aspiration in the BALF should be used in addition to esophageal function testing to identify lung transplantation patients most likely to benefit from LARS. Taken together, our experience suggests that the identification of lung transplantation patients for LARS should be multidimensional, and that analyzing the BALF for markers of aspiration is a useful adjunct to esophageal function testing for this purpose, particularly because 24-hour ambulatory testing is expensive, invasive, uncomfortable, and rarely tolerated when repeat testing is indicated.

Finally, our results, which delineate the biological effects of aspiration on the lung transplant microenvironment, provide a greater depth of understanding into a modifiable cause of BOS. First, our work and that of others has found BALF neutrophilia and IL-8 levels to be closely linked with aspiration after lung transplantation.[15,37,38] This is interesting, as these are both consistently associated with the onset of BOS. Second, we and others have also identified that lung transplantation patients with objective evidence of aspiration have a quicker progression to BOS.[7,8,13,14] Not only can the progression to BOS be delayed with antireflux surgery, but the proinflammatory and fibrogenic environment induced by aspiration can be improved shortly after LARS.[15] These previous findings lend considerable weight to the profound alterations in cytokines, chemokines, growth factors, and pulmonary leukocyte composition in the BALF, which our current study delineates among lung transplantation patients with aspiration. Although the best way in which to identify aspiration in lung transplantation patients remains to be defined, we have previously demonstrated that LARS effectively reduces the amount of pepsin found in the BALF of these patients and recommend that LARS be performed as soon as it is safe in properly identified candidates.[14]

Our study differs from many previous studies for a variety of reasons. First, the size of the patient population in addition to the overall sample size is rarely achieved. This makes our dataset particularly robust, which is essential for identifying mediators that might be predictive of early-onset BOS. No other study of which we are aware has attempted to identify predictors of BOS before its onset. Second, we have used multiplex analysis to investigate a wide breadth of mediators for their relationship to ACR, BOS, and aspiration. From this approach, we confirmed the previous work of others, at the same time opening the door for other avenues of exploration, particularly along the lines of biological effects of aspiration. This latter point advances the field because it provides an even stronger argument for identifying lung transplantation patients at risk for aspiration and a basis for means to prevent aspiration, such as through LARS.

The practical implications of our study are mainly that our results try to shed light on the biological link between aspiration, inflammation, and BOS, as the latter is the leading cause of mortality after lung transplantation. As such, the identification of predictors of early-onset BOS could allow for earlier treatment and hopefully prevention of BOS in lung transplantation patients. Means to accomplish this could be through earlier implementation of azithromycin or adjustment/augmentation of immunosuppressive therapy to delay or prevent the onset of BOS.

We acknowledge some important limitations. First, there is considerable heterogeneity among the data, particularly in terms of the indications for lung transplantation, donor and recipient immunological variability, age, time since transplantation of sample acquisition, GERD status, and risk factors for aspiration. Our data are, by and large, generalizable, despite the heterogeneity between lung transplant recipients. Second, as a consequence of the prospective nature of the study, our median follow-up duration was limited to 33 months. Additional follow-up is still required to assess the long-term implications of imbalances of pro-inflammatory, fibrogenic, and chemotactic signaling among our study population. Third, although the immunosuppressive regimen at our institution is fairly well defined, we could not account for differences in immunosuppressive regimens, as our study protocol was not designed to do so.

CONCLUSIONS

We conclude that lung transplantation patients at greatest risk for BOS developing can be identified by analysis of BALF proteins within the first year post lung transplantation. In addition, our results confirm that the development of BOS hinges on a shift in balance to a proinflammatory and damaging microenvironment within the pulmonary allograft, and that this shift in balance is a multifactorial process involving both alloimmune and nonalloimmune factors. These results affirm that ongoing emphasis must be placed on novel means to modulate the immune system post lung transplantation, and recently discovered avenues, such as azithromycin and LARS, should continue to be aggressively pursued.

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Abbreviations and Acronyms

ACR	acute cellular rejection
A1AT	α1-antitrypsin
BALF	bronchoalveolar fluid
BOS	bronchiolitis obliterans syndrome
GERD	gastroesophageal reflux disease
GM-CSF	granulocyte-macrophage colony-stimulating factor
IPO-10	interferon-γ–induced protein 10
IQR	interquartile range
LARS	laparoscopic antireflux surgery
MPO	myeloperoxidase
NE	neutrophil elastase
OR	odds ratio

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Table 1

Odds Ratios for the Development of Bronchiolitis Obliterans Syndrome by 30 Months Post Lung Transplantation as Predicted by Immune Mediators Measured in Bronchoalveolar Fluid at 6 to 12 Months after Lung Transplantation

	Concentration, per mg protein	
Protein	**Odds ratio**	**95% CI**
C5a	1.26	0.31–5.10
IL-1β	0.94	0.66–1.35
IL-1RA	1.48	0.88–2.48
IL-2	1.05	0.66–1.67
IL-4	0.80	0.39–1.63
IL-5	0.84	0.34–2.08
IL-6	1.12	0.64–1.96
IL-7	1.06	0.68–1.64
IL-8	1.04	0.67–1.60
IL-9	1.38	0.83–2.28
IL-10	0.75	0.49–1.15
IL-12	0.86	0.51–1.45
IL-13	1.20	0.80–1.80
IL-15	1.57	1.01–2.44
IL-17	1.50	1.00–2.24
Eotaxin	1.06	0.82–1.36
bFGF	1.41	1.02–1.93
G-CSF	0.93	0.58–1.49
GM-CSF	1.26	0.71–2.22
IFN-γ	0.85	0.64–1.13
IP-10	1.28	0.72–2.27
MCP-1	1.17	0.68–2.01
MIP-1α	1.31	0.73–2.36
MIP-1β	1.64	0.85–3.19
PDGF	1.17	0.82–1.67
RANTES	0.89	0.67–1.19
TGF-β	0.71	0.24–2.08
TNF-α	2.03	1.27–3.23
VEGF	1.09	0.71–1.68
MPO	3.22	1.22–8.50
A1AT	0.24	0.08–0.78
NE	1.07	0.87–1.30
A1AT/NE	0.87	0.55–1.38
Pepsin	1.08	0.75–1.55

A1AT, α_1-antitrypsin; bFGF, basic fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon-γ–induced protein 10; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory factor; MPO, myeloperoxidase; NE, neutrophil elastase; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T-cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Table 2

Bronchoalveolar Fluid Cytokines, Chemokines, and Growth Factors as Related to the Diagnosis of Bronchiolitis Obliterans Syndrome

	Concentration, per mg protein, median (IQR)		
Protein	**BOS (−)**	**BOS (+)**	**p Value**
C5a, pg	2,562 (1,945–3,492)	2,914 (2,302–3,433)	0.588
IL-1β, pg	4 (0–14)	11 (3–65)	0.003
IL-1RA, pg	403 (189–1,007)	582 (260–2,486)	0.115
IL-2, pg	0 (0–6)	0 (0–0)	0.021
IL-4, pg	3 (1–6)	2 (0–4)	0.175
IL-5, pg	2 (0–4)	2 (1–4)	0.200
IL-6, pg	20 (9–39)	22 (8–56)	0.810
IL-7, pg	23 (9–50)	7 (2–54)	0.059
IL-8, pg	348 (154–1,267)	1,085 (244–4,657)	0.012
IL-9, pg	19 (7–43)	11 (1–20)	0.007
IL-10, pg	27 (9–72)	16 (2–58)	0.246
IL-12, pg	182 (64–346)	147 (21–243)	0.053
IL-13, pg	17 (7–37)	13 (1–22)	0.058
IL-15, pg	22 (4–43)	11 (1–30)	0.098
IL-17, pg	6 (0–30)	11 (0–25)	0.680
Eotaxin, pg	0 (0–80)	34 (0–84)	0.319
bFGF, pg	14 (0–47)	26 (3–85)	0.182
G-CSF, pg	202 (118–367)	145 (51–247)	0.042
GM-CSF, pg	370 (190–673)	347 (132–803)	0.633
IFN-γ, pg	101 (22–191)	74 (18–111)	0.228
IP-10, pg	5,583 (2,907–14,992)	10,742 (6,253–22,682)	0.003
MCP-1, pg	333 (176–764)	211 (138–278)	0.020
MIP-1α, pg	2 (0–6)	1 (0–6)	0.921
MIP-1β, pg	76 (44–136)	81 (57–156)	0.484
PDGF, pg	34 (3–83)	48 (6–80)	0.901
RANTES, pg	34 (0–92)	60 (40–110)	0.020
TGF-β, pg	200 (136–268)	202 (136–253)	0.780
TNF-α, pg	0 (0–12)	2 (0–13)	0.347
VEGF, pg	2,182 (931–3,901)	2,128 (646–3,106)	0.263
MPO, U	1,007 (674–1,892)	1,171 (551–1,533)	0.998
A1AT, μg	14 (9–26)	13 (10–23)	0.906
NE, ng	26 (0–602)	802 (242–1,385)	<0.001
A1AT/NE	0.5 (0.0–14.1)	0.0 (0.0–0.1)	<0.001
Pepsin, ng	2 (0–24)	31 (0–105)	0.001

A1AT, α_1-antitrypsin; bFGF, basic fibroblast growth factor; BOS, bronchiolitis obliterans syndrome; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon-γ–induced protein 10; IQR, interquartile range; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory factor; MPO, myeloperoxidase; NE, neutrophil elastase; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T-cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Table 3

Bronchoalveolar Fluid Cytokine, Chemokine, and Growth Factors as Related to Acute Cellular Rejection

	Concentration, per mg protein, median (IQR)			
Protein	**A0**	**A1/A2**	**A3/A4**	**p Value**
C5a, pg/mg	2,773 (1,968–3,874)	2,307 (1,848–3,320)	667.3	0.141
IL-1β, pg/mg	4.8 (0.8–11.8)	0.2 (0.0–5.0)*	0.0 (0.0–1.1)*	<0.001
IL-1RA, pg/mg	461.3 (206.3–1,128)	249.5 (96.1–544.1)*	157.2 (63.9–650.0)	0.005
IL-2, pg/mg	0.0 (0.0–6.7)	0.0 (0.0–2.7)	0.0 (0.0–2.7)	0.707
IL-4, pg/mg	2.7 (0.4–6.0)	1.7 (0.6–4.0)	0.6 (0.2–3.2)	0.339
IL-5, pg/mg	1.6 (0.0–4.2)	1.9 (0.0–3.8)	0.7 (0.0–1.3)	0.453
IL-6, pg/mg	20.1 (9.4–38.2)	15.3 (5.0–25.0)	16.6 (5.4–210.2)	0.080
IL-7, pg/mg	28.2 (10.0–63.3)	16.4 (5.0–46.9)	3.2 (0.3–12.0)*	0.001
IL-8, pg/mg	345.8 (176.1–1,109)	207.4 (82.6–484.0)*	232.1 (37.0–2386)	0.009
IL-9, pg/mg	21.4 (8.4–47.1)	12.5 (2.7–24.3)*	3.9 (2.1–5.8)*	<0.001
IL-10, pg/mg	34.7 (8.9–80.5)	22.6 (4.2–42.7)	10.0 (4.0–12.2)	0.017
IL-12, pg/mg	222.5 (93.2–411.9)	111.2 (41.3–255.8)*	27.5 (5.1–120.3)*	<0.0001
IL-13, pg/mg	17.6 (8.4–36.6)	14.2 (4.1–40.8)	4.8 (0.0–8.4)*	0.016
IL-15, pg/mg	25.7 (5.2–48.7)	21.0 (2.9–43.0)	1.6 (0.3–21.7)*	0.031
IL-17, pg/mg	9.4 (0.0–34.4)	1.5 (0.0–21.7)	0.1 (0.0–8.3)	0.053
Eotaxin, pg/mg	0.0 (0.0–80.4)	0.0 (0.0–91.2)	0.0 (0.0–64.9)	0.648
bFGF, pg/mg	15.1 (0.0–34.4)	9.8 (0.0–36.6)	12.5 (2.8–35.0)	0.818
G-CSF, pg/mg	231.2 (127.3–372.9)	147.8 (69.2–234.8)*	18.1 (46.5–130.4)*	<0.0001
GM-CSF, pg/mg	403.5 (226.4–749.8)	233.3 (137.3–544.2)*	125.8 (6.4–220.5)*	<0.001
IFN-γ, pg/mg	112.8 (33.8–213.9)	56.1 (5.9–112.9)*	21.3 (0.0–42.5)*	<0.001
IP-10, pg/mg	4,565 (2,643–11,752)	5,526 (3,381–11,894)	3939 (1127–16161)	0.550
MCP-1, pg/mg	309.1 (177.3–654.8)	223.3 (125.7–452.0)	263.3 (61.8–325.4)	0.168
MIP-1α, pg/mg	1.1 (0.0–5.6)	0.3 (0.0–2.8)	0.6 (0.0–1.8)	0.259
MIP-1β, pg/mg	78.9 (44.3–137.0)	62.4 (38.0–107.5)	30.0 (12.4–100.9)*	0.019
PDGF, pg/mg	44.4 (10.1–94.0)	24.6 (0.0–49.7)*	2.0 (0.0–24.8)	0.004
RANTES, pg/mg	40.5 (3.3–103.0)	26.9 (0.0–70.6)	10.4 (2.1–138.7)	0.452
TGF-β, pg/mg	34.1 (22.8–52.4)	29.1 (17.6–48.5)	74.9 (59.6–320.7)*	0.024
TNF-α, pg/mg	0.0 (0.0–9.1)	0.0 (0.0–8.7)	1.2 (0.0–15.7)	0.849
VEGF, pg/mg	2,713 (1,337–4,320)	1,308 (552.9–2,265)*	176.8 (71.6–972.7)*	<0.0001
MPO, U/mg	1,064 (681.7–2,396)	783.7 (544.7–1,264)	5,259 (110.2–10,409)	0.281
A1AT, μg/mg	14,971 (9,988–26,500)	10,060 (7,800–23,357)	4,405 (1,096–7,713)	0.063
NE, ng/mg	0.0 (0.0–557.3)	124.2 (0.0–479.7)	1,120 (16.2–2,224)	0.492
A1AT/NE	2,553 (26.6–16,249)	102.9 (27.3–8137)	238.2 (0.5–475.8)	0.173

* $p < 0.05$, vs. A0.

A1AT, α_1-antitrypsin; bFGF, basic fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon-γ–induced protein 10; IQR, interquartile range; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory factor; MPO, myeloperoxidase; NE, neutrophil elastase; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T-cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Table 4

Bronchoalveolar Fluid Cytokines, Chemokines, and Growth Factors as Related to Aspiration as Defined by the Detection of Pepsin

	Concentration, per mg protein, median (IQR)		
Protein	**Aspiration (−)**	**Aspiration (+)**	**p Value**
C5a, pg	2,865 (2,250–3,551)	2,334 (1,758–3,362)	0.040
IL-1β, pg	3 (0–12)	6 (1–35)	0.002
IL-1RA, pg	352 (186–752)	538 (206–1,523)	0.018
IL-2, pg	0 (0–8)	0 (0–4)	0.045
IL-4, pg	4 (1–8)	2 (0–5)	0.003
IL-5, pg	2 (0–5)	2 (0–3)	0.377
IL-6, pg	22 (10–36)	21 (9–44)	0.774
IL-7, pg	25 (10–57)	22 (6–45)	0.193
IL-8, pg	288 (153–854)	653 (177–2,707)	0.009
IL-9, pg	22 (8–47)	15 (4–29)	0.011
IL-10, pg	36 (12–87)	18 (6–58)	0.002
IL-12, pg	245 (85–401)	131 (45–270)	<0.001
IL-13, pg	23 (7–42)	14 (6–28)	0.018
IL-15, pg	30 (6–48)	15 (4–38)	0.019
IL-17, pg	5 (0–37)	8 (0–27)	0.815
Eotaxin, pg	0 (0–72)	11 (0–91)	0.064
bFGF, pg	10 (0–42)	18 (0–39)	0.273
G-CSF, pg	221 (125–350)	188 (81–392)	0.305
GM-CSF, pg	423 (241–806)	318 (146–585)	0.004
IFN-γ, pg	114 (31–233)	73 (16–168)	0.016
IP-10, pg	5,327 (2,674–12,847)	7,425 (3,713–19,713)	0.014
MCP-1, pg	350 (192–757)	269 (164–636)	0.232
MIP-1α, pg	1 (0–5)	2 (0–8)	0.224
MIP-1β, pg	74 (41–134)	80 (50–151)	0.081
PDGF, pg	34 (0–79)	39 (11–89)	0.376
RANTES, pg	36 (0–102)	41 (12–93)	0.252
TGF-β, pg	194 (131–270)	204 (139–262)	0.674
TNF-α, pg	0 (0–9)	2 (0–16)	0.017
VEGF, pg	2,325 (1,034–4,134)	2,067 (722–3,612)	0.211
MPO, U	1,119 (751–1,975)	902 (593–1,578)	0.090
A1AT, μg	15 (9–29)	13 (8–23)	0.169
NE, ng	0 (0–454)	203 (0–1,139)	0.011
A1AT/NE	3.1 (0.0–14.2)	0.1 (0.0–9.3)	0.009

A1AT, α1-antitrypsin; bFGF, basic fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon-γ–induced protein 10; IQR, interquartile range; MCP, monocyte

chemotactic protein; MIP, macrophage inflammatory factor; MPO, myeloperoxidase; NE, neutrophil elastase; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T-cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.