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Bronchoalveolar Lavage Cell Immunophenotyping Facilitates Diagnosis of Lung Allograft Rejection

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Abstract

Supplementary methods to identify acute rejection and to distinguish rejection from infection may improve clinical outcomes for lung allograft recipients. We hypothesized that distinct bronchoalveolar lavage (BAL) cell profiles are associated with rejection and infection.

We retrospectively compared 2,939 BAL cell counts and immunophenotypes against concomitantly obtained transbronchial biopsies and microbiologic studies. We randomly assigned 317 subjects to a derivation or validation cohort. BAL samples were classified into four groups: infection, rejection grade ≥A1, both, or neither. We employed generalized estimating equation and survival modeling to identify clinical predictors of rejection and infection.

We found that CD25+ and NK cell percentages identified a two-fold increased odds of rejection compared to either the infection or the neither infection nor rejection groups. Also, monocytes, lymphocytes, and eosinophil percentages were independently associated with rejection. A four-predictor scoring system had high negative predictive value (96–98%) for grade ≥A2 rejection, predicted future rejection in the validation cohort, and predicted increased risk of bronchiolitis obliterans syndrome in otherwise benign samples.

In conclusion, BAL cell immunophenotyping discriminates between infection and acute rejection and predicts future outcomes in lung transplant recipients. Although it cannot replace histopathology, immunophenotyping may be a clinically useful adjunct.

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None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

Keywords

Lung; Rejection; Lymphocyte; CD25+ T cell; NK cell

Introduction

Lung transplantation is a potentially life-saving therapy for otherwise incurable lung diseases. Acute allograft rejection, however, is a major cause of post-transplantation morbidity. Within the first post-operative year, it affects at least 35% of lung transplant recipients, prompting treatment with augmented immunosuppressive medications in 89% of cases, and it causes 2% of deaths (1). Additionally, some (but not all) studies identify acute rejection as a risk factor for chronic allograft dysfunction in the form of bronchiolitis obliterans syndrome (BOS) (2–4).

Clinically, acute rejection can present as a syndrome indistinguishable from pulmonary infection (5) or can be asymptomatic. As a result, a number of transplant centers, including ours, perform surveillance transbronchial biopsies via flexible bronchoscopes to identify and treat clinically occult acute rejection, with the goal of decreasing the long-term risk for BOS (6). Other centers reserve transbronchial biopsies for patients with findings consistent with acute rejection, such as dyspnea, cough or decline in forced expiratory volume in one second (FEV_1) (7).

Pathological interpretation of transbronchial biopsies remains the gold standard for diagnosis of acute rejection. As a diagnostic test, however, it has limitations. These biopsies are invasive, carrying a risk of bleeding and pneumothorax (6, 8), and are hampered by sampling error and low inter-observer reliability (9–12). Further, the clinical significance of low grade (A1) rejection is unclear. While a single episode of A1 rejection may be a risk factor for subsequent BOS (13), clinical approaches to treatment vary. A finding of grade A1 acute rejection on transbronchial biopsy may be ignored, prompt repeat biopsy, or lead to augmentation of immunosuppression depending on practice patterns and clinical context (6, 11).

For these reasons, investigators have sought additional diagnostic information to augment histopathological findings in the diagnosis of acute rejection. A number of studies evaluated characteristics of BAL fluid for connections to pathology observed on transbronchial biopsy. These studies demonstrated associations between acute rejection and increased neutrophils (14, 15), lymphocytes (16), basophils, eosinophils (17, 18), terminally differentiated T cells (19), decreased regulatory T cells (20), and CD4 to CD8 T cell ratios (21).

Based on these data and given the variety of putative predictors for acute rejection, we evaluated relationships between BAL fluid immunophenotypes and acute rejection or infection. We further investigated whether a combination of immunophenotypes could provide a clinically useful scoring system to help identify the presence of acute rejection, discriminate acute rejection from infection, and predict future acute rejection. In a large cohort of lung transplant recipients from a single center, our results show that BAL

immunophenotyping can distinguish between acute rejection and infection, predict rejection on future biopsies, and identify patients at increased risk for BOS.

Methods

Study population

We performed a retrospective cohort study of all patients who received single-, double-, or heart-lung allografts at University of California San Francisco (UCSF) between March of 1997 and November of 2011. Clinical data from medical records were abstracted and coded as previously described (4). This study took place under a protocol approved by the UCSF Committee on Human Research (10–00721).

Predictors

BAL fluid was collected from subjects as part of routine surveillance bronchoscopy, which was performed 0.5, 1, 2, 3, and 6 months after transplantation and then annually. In 2009, surveillance bronchoscopy was terminated after two years following transplantation. BAL fluid also was collected for clinical indications, such as a decline in FEV_1. Lavage was generally performed in right middle lobe or lingula using five 20-ml aliquots of saline solution. Sequential samples of BAL fluid were allocated, in order of collection, for microbiologic analysis, cytology, automated cell counts and leukocyte differential, and flow cytometry. Assays were performed by UCSF clinical labs and were part of patients' medical records.

Lymphocyte phenotypes were determined by flow cytometry, which identified NK cells, CD4+ and CD8+ T cells, CD25+ cells and CD8+CD57+ cells. CD25+ cells are typically regulatory or activated T cells, while CD8+CD57+ cells are thought to be effector T cells terminally differentiated by chronic antigen exposure (22). Samples were processed on the day of collection. BAL fluid was passed through sterile gauze into a 50-ml conical vial, centrifuged and washed twice with PBS, and then resuspended in 200 to 400 μl of buffer containing PBS, 10% acid-citrate-dextrose, fetal calf serum, and penicillin/streptomycin. CD4 to CD8 cell ratios and NK cell percentage were determined using antibody panels (BD340499 and BD340500, respectively; BD Biosciences, San Jose, CA). CD8+CD57+, CD8+CD25+, and CD25+ cells were detected and counted using a mixture of Simultest anti-CD57 FITC/anti-CD8 PE (BD349510), anti-CD45 PerCP-Cy5.5 (BD340952), and anti-CD25-APC (BD555434). Fifty microliters of BAL-derived cells were incubated with 10 μl of antibody mixture for 20 min. RBCs were lysed with FACS lysis buffer (BD Biosciences). One or more runs of 50,000 cells were acquired with a target of 200 CD45+ lymphocytes. Samples with fewer than 200 lymphocytes were manually reviewed. Representative data are shown in Figure 1.

Outcomes

Acute cellular rejection was assessed and graded in transbronchial biopsies by experienced thoracic pathologists using standard nomenclature (23).

Patients were classified as infected if BAL fluid microbiologic studies identified viruses, bacteria, fungi, or mycobacteria. Bacterial cultures were considered positive if cultures showed at least moderate growth or grew $\geq 10^4$ colony-forming units/ml of a bacterial species, excluding oral flora. The presence of community-acquired viral infection, including influenza A and B, parainfluenza, adenovirus, and respiratory syncytial virus, was tested by direct fluorescence assay. Cytomegalovirus culture results were not included in the analysis.

Bronchiolitis obliterans syndrome (BOS) was defined as a sustained fall of ≥20% from the baseline post-transplant FEV1, as previously described (4).

Analytical approach

Statistical analysis was performed in R (version 2.14.1, R Foundation for Statistical Computing, Vienna, Austria) using “GEE”, “ROCR,” and “survival” libraries. Subjects were randomly assigned 2:1 to derivation and validation cohorts. Boxplots were generated using default settings in the R graphics library, with whiskers at the default of 1.5 times the interquartile range, and notches indicating confidence intervals for the median as previously described (24). To allow for comparison between predictors with variable distributions, initial univariate regression was performed on *t*-statistics that were derived from predictors by subtracting the sample mean and dividing by the sample standard deviation for each value. To account for repeated observations in linear and logistic regression models, we used generalized estimating equations (GEE) with an exchangeable covariance matrix. Robust variance estimates were used for inference (25).

To create a clinical scoring system using BAL fluid profiles, we generated cutoffs for dichotomous variables. Using the ROCR library “performance” function, we maximized a χ^2-statistic for identifying rejection scores ≥A1 or rejection rather than infection, examining cutoffs that range over the 25^{th} to 75^{th} percentiles of predictor values. We then evaluated putative predictors of minimal or greater degrees of acute rejection (score ≥A1) using multivariate, logistic GEE in the derivation cohort. The top four predictors were selected according to odds ratios in multivariate analysis. *K*-fold cross-validation was performed as described in the supplement.

For multivariate analysis, we controlled for patient characteristics frequently associated with lung allograft rejection. These included age and CMV status at time of transplantation, gender, transplant type, and lung disease category necessitating transplantation. Because the frequency of rejection decreases with time, we adjusted for time since transplantation in multivariate models. Also, since patients with more infection or rejection episodes were likely to receive more biopsies, we included the number of biopsies each subject received during the study period as an adjustment term. Further, some changes in the care of lung transplant recipients, including immunosuppressive regimens, occurred during the study period. Specifically, patients were transitioned from cyclosporine to tacrolimus in 2000 and from azathioprine to mycophenolate mofetil in 2002. Prednisone was used as a third immunosuppressive agent throughout the study period. To explore the potential impact of these changes in immunosuppressive practice patterns, we performed a sensitivity analysis by stratifying subjects transplanted before or after 2002.

Finally, we assessed whether BAL-based rejection scores could predict rejection on subsequent samples and the future development of BOS. We selected all pairs of samples for a given subject where the second sample was acquired within 45 days of the first sample. We then used GEE to assess whether the BAL-based score applied to the first sample predicted rejection in the second sample. To assess scores as predictors of BOS, we calculated the maximum value of the given score over the first 90 days, excluding samples with infection or ≥A1 rejection, for subjects in the pooled derivation and validation cohorts. We used a Cox proportional hazards model of time to development of BOS that was left-truncated at 90 days and adjusted for patient characteristics and the number of biopsies a subject received. Kaplan-Meier curves were right-censored at 5 years.

Results

Study population

During the study period, 356 subjects underwent lung transplantation. Nineteen died within the first 90 days and 15 sought follow-up care at a different institution. An additional 5 subjects were excluded because infection was detected at every surveillance bronchoscopy. The remaining 317 subjects were randomly assigned to derivation cohort (212 subjects) or validation cohorts (105 subjects). In the randomized subjects, there were 2,939 total biopsy and BAL procedures, with 1,993 in the derivation group and 946 in the validation group.

Characteristics of the derivation and validation cohorts are shown in Table 1. UCSF is a referral center for interstitial lung diseases. As a result, compared with International Society for Heart and Lung Transplantation (ISHLT) registry data (1), our study population included a greater proportion of patients receiving bilateral lung transplantation (80% vs. 60%), and transplantation for pulmonary fibrosis (40% vs. 26%) and pulmonary hypertension (6% vs. 3%). Relative to programs included in registry data, we performed fewer transplants for cystic fibrosis (10% vs. 17%) and COPD (18% vs. 35%). However, the incidence of acute ≥A2 rejection in the first post-operative year of 35.2% and 28.8% in the derivation and validation cohorts, respectively ($P = 0.32$), was similar to ISHLT registry data, where 35% of transplant recipients had acute rejection in the first year (1).

BAL fluid characteristics

We stratified BAL fluid profiles into four groups based on the concurrent results of microbiological and pathological analyses. In the derivation cohort, 1,145 samples had neither infection nor ≥A1 rejection. Of the remaining samples, 629 were obtained in the setting of infection, 283 in the setting of ≥A1 rejection, and 132 in the setting of combined infection and rejection. Figures 2 and S1 show distributions of the BAL immunophenotyping parameters. Relative to samples in the derivation cohort with neither infection nor rejection, NK cells were increased in the infection group ($P < 0.05$) but decreased in the rejection and combined infection/rejection groups ($P < 0.01$ for each). CD25+ cells were increased in rejection and decreased in infection ($P < 0.01$ for each) and CD8+CD25+ cells were decreased in infection ($P < 0.001$). T cell percentages were increased in infection, rejection, and both (all $P < 0.01$). Monocytes were decreased in rejection ($P < 0.001$) and combined infection and rejection ($P < 0.01$), while neutrophils

were increased in infection (P <0.05) and both (P <0.01). Lymphocytes were increased in rejection (P <0.001), as were eosinophils (P <0.01); however >90% of samples lacked eosinophils.

Using GEE, we found that white blood cell counts were associated with rejection with an A-score increase of 0.034 per one standard deviation (SD) increase in white blood cell count (95% CI 0.007–0.061, P = 0.01), while red blood cell counts were not (P = 0.07). Both cell counts had a wide range of values. Unlike a previous study (26), ours did not detect an association between rejection or infection and CD4+ T cells, CD8+ T cells, or CD4+/CD8+ ratio. While B cells were associated with rejection (A-score increase 0.06, 95% CI 0.02–0.09 per SD increase, P = 0.001), these cells were uncommonly seen, with 90% of samples having ≤2% B cells. Similarly, basophils also were associated with rejection (A-score increase 0.54, 95% CI 0.24–0.85 per SD increase, P <0.001) but were very rare, as none were detected in 99% of samples.

Identification of acute rejection

The above analyses demonstrated several BAL immunophenotypes specific for acute rejection. To develop a scoring system, we sought to select the parameters most strongly and independently associated with acute rejection. First, we used the derivation cohort to assess each parameter's ability to predict rejection in a univariate, linear, GEE regression model. Figure 3A shows the change in mean A-score per standard deviation increase in a given variable. Using a P <0.05 cutoff, increasing white blood cell counts and percentages of T cells, B cells, CD8+ cells, neutrophils, lymphocytes, eosinophils, basophils, CD25+ cells, and CD8+CD57+ cells were associated with increasing A-score on pathological specimens obtained during the same bronchoscopy. By contrast, increasing NK cells and monocytes were negatively associated with acute rejection. Although the largest changes were seen with increasing eosinophils and basophils, these cell types were rarely observed. Similar associations were seen with B-grade rejection (Figure S2).

To identity the factors independently predicting acute rejection, we applied a multivariate, linear regression GEE model, using dichotomized predictors statistically associated with acute rejection (Figure 3B). We found that monocytes <75%, CD25+ cells >8%, NK cells <5%, and eosinophils >0% were independently associated with rejection scores ≥A1, with odds ratios ranging from 1.8 to 2.4. Because the log-odds ratios for these predictors were similar, we used simple summation to derive a predictive scoring system for acute rejection (R-score). As shown in Figure 3C, the R-score predicted acute rejection at both the ≥A1 and ≥A2 cutoffs, with area under the receiver-operating curve (AUC) values of approximately 0.6 in the validation cohort. Further, we identified an approximately 2-fold increase in the odds of observing acute rejection per increase in R-score, even after adjusting for patient characteristics. Scoring systems derived from cross-validation were similar (Supplementary Table 1). Stratified sensitivity analysis showed that this association remained significant (P <0.01) in the pre- and post-2002 eras (Supplementary Table 2). Over one-third of patients had an R-score of 0, corresponding to a 96% negative predictive value for biopsy-based rejection scores ≥A2 in the validation group.

Distinguishing between infection and rejection

Because results of BAL immunophenotyping may be available before pathological interpretation of transbronchial biopsies and microbiologic studies are complete, these predictors could be useful in cases when patients present with a clinical syndrome consistent with either infection or rejection. We therefore assessed their ability to classify samples taken at the time either of infection or rejection, excluding samples with both together or with neither. For each BAL fluid parameter the odds of being in the rejection group rather than the infection group per standard deviation increase in that characteristic were calculated (Figure 4A). In a univariate, logistic GEE model applied to the derivation cohort, increasing percentages of lymphocytes, eosinophils, CD25+ cells, and CD8+CD25+ cells were associated with greater odds of rejection, while increasing percentages of NK cells, CD4+ cells and monocytes were associated with greater odds of infection.

In the derivation cohort, CD25+ cells >9%, NK cells <5%, lymphocytes >12%, and monocytes <71% each were associated with approximately 2-fold higher odds of being in the rejection group than in the infection group (Figure 4B). As shown in Figure 4C, the AUC for this infection vs. rejection (IR) score was 0.61, with a 1.7-fold increase in the odds of rejection per unit increase in IR score, in the validation group. Sensitivity analysis showed that this association remained significant (P <0.01) in the pre- and post-2002 eras. Odds ratios remained significant after adjusting for the time since transplantation, number of biopsies a subject received, and subject characteristics. Despite overlap between the infection and rejection groups, these immunophenotypes facilitated discrimination between infection and rejection.

Prediction of rejection on future biopsies

Given the known limitations in inter-observer reliability and potential for sampling error in transbronchial biopsies, it is possible that some episodes of acute rejection are misclassified. Additionally, BAL fluid characteristics might identify early acute rejection below the resolution provided by histopathological criteria. For these reasons, we hypothesized that BAL fluid characteristics associated with current rejection might predict future rejection, which could ensue if rejection was unrecognized and therefore untreated. Therefore, we investigated whether our clinical BAL scores could predict future rejection as defined by transbronchial biopsy score ≥A1 on the next biopsy performed within the subsequent 45 days. Using a logistic GEE model, we determined that both the R- and IR-scores predicted ≥A1 rejection in the subsequent biopsy (Table 2). These findings were consistent across the derivation and validation cohorts, with odds ratios ranging from 1.5 to 1.8 per unit increase in score (all $P \leq 0.001$). These findings remained significant ($P \leq 0.001$) even after adjusting for current A-score, time since transplantation, number of biopsies received, and patient characteristics. Interestingly, the odds ratios estimated by these BAL-based scores were higher than those estimated by the biopsy-based A-score for predicting acute rejection on the next biopsy. Similarly, even when limited to samples collected when the transbronchial biopsy score was A0, a one-point increase in either BAL-based score predicted identification of ≥A1 rejection at the next bronchoscopic biopsy for both cohorts (P <0.01 for each).

Prediction of BOS

We previously reported that the maximum A-score within 90 days following was not associated with an increased risk of BOS (4), which was consistent with prior studies demonstrating an “uncoupling” of acute cellular rejection episodes and BOS related to early aggressive treatment (27). By contrast, an increasing maximum value of the IR-score within the first 90 days was associated with an increased risk of BOS (Figure 5) with a hazard ratio (HR) per unit increase in score of 1.46 (95% CI 1.07–2.00, $P = 0.02$). As shown in Figure S3, we did not find a statistically significant association between the R-score and BOS (HR 1.23, 95% CI 0.89–1.69, $P = 0.21$). However, subjects with an R-score >2 were at increased risk for BOS (HR 3.37, 95% CI 1.04–10.9, $P = 0.04$).

Discussion

We found that immunophenotyping of BAL cells can be used to assess the likelihood of acute rejection in lung transplant recipients. A scoring system based on BAL immunophenotyping also distinguished infection from rejection. Moreover, BAL-based scoring systems predicted rejection on subsequent biopsy within 45 days, even when rejection was not identified in the biopsy associated with the prediction sample. Further, when episodes of infection and ≥A1 rejection were excluded, these scoring systems identified patients at increased risk for BOS. This ability of immunophenotyping to predict future rejection and BOS may be particularly useful. These findings suggest that BAL fluid characteristics can identify cases of acute rejection that are not detected by transbronchial biopsies. While information from BAL fluid cannot replace pathological or microbiological analyses, immunophenotyping may provide information that aids decision-making for clinicians weighing treatment options in cases of borderline rejection (e.g., grade A1) or of rejection in the setting of concurrent infection.

For the assessment of rejection in comparison with normal samples, we identified four high-risk features: monocytes <75%, CD25+ cells >8%, NK cells <5%, and eosinophils >0%. The absence of these features rendered acute rejection unlikely, as detected by results of transbronchial biopsies, with a negative predictive value of >96%. On the other hand, the increasing R-scores were only modestly predictive of clinical acute rejection. We observed a slightly different set of predictors for distinguishing between infection and rejection: CD25+ cells >9%, NK cells <5%, lymphocytes >12% and monocytes <71%. Despite a robust statistical association with rejection, even with an IR-score ≥3, rejection scores ≥A2 were found on transbronchial biopsy in only about half of the subjects. Of these associations, NK cell and CD25+ cell percentages may be the most clinically useful because their classifying ability is enhanced when distinguishing between rejection and infection, whereas the standard leukocyte differential count may be most useful for distinguishing rejection from normal.

Our finding of an independent association between NK cells and acute rejection is intriguing and consistent with emerging literature. NK cells are implicated in allograft tolerance through destruction of donor antigen-presenting cells (28–30), and in rejection through activation in response to missing host MHC ligands (31, 32). We found that a low BAL NK cell frequency was associated with acute rejection, which is consistent with recent mouse

models showing essential roles for NK cells in tolerance of lung allografts and with the reported association between an activating KIR haplotype and protection from BOS (30, 33).

CD25 positivity identified another important cell type in BAL fluid associated with acute rejection. CD25 is the key constituent of the high-affinity IL-2 receptor and is a marker for regulatory and activated T cells (34). It is unclear which type of T cell underlies the observed association between CD25 positivity and acute rejection. The substantial covariance between CD8+CD25+ cells and CD25 positive cells (data not shown) might suggest that these are activated T cells. However, an association between BAL FOXP3+ regulatory T cells and acute rejection has been reported (35). As in other organs (36), these regulatory T cells may be recruited to a site of ongoing inflammation.

Our study has notable strengths. The large sample size, including nearly 3,000 biopsy and BAL procedures over 15 years, provided power to conduct multivariate analyses that identified biologically plausible predictors of rejection and infection after controlling for potential confounders. This sample size also allowed us to validate our principal findings, including novel BAL immunophenotyping-based rejection scores, in a validation cohort distinct from the cohort in which they were derived. Despite these strengths, the study has limitations. First, the data are from a single center. Results generated from other centers might vary due to differences in BAL acquisition practices, laboratory analysis, or pathological interpretation. Also, it is possible that immunosuppression regimens, which changed over time, could affect the utility of these scoring systems. However, sensitivity analysis did not identify a “treatment era” effect. The study highlights the challenges of developing clinical prediction scores, the performance of which is based on comparison with transbronchial biopsy-based histopathological grading. While histopathology is the gold standard for diagnosing acute rejection, it is prone to misclassification. An important source of misclassification is the lack of inter-observer agreement, which has ranged from substantial to slight as assessed by Cohen kappa coefficients (9–12). Similarly, because we defined infection by microbiological culture and DFA, there is likely to be misclassification in this group, including false positives from asymptomatic colonization and false negatives from inadequate sampling or culturing techniques. Sampling error provides additional potential for misclassification of rejection and infection. However, inter-observer variability, infection misclassification, and sampling error, if present, would bias our results towards the null.

In summary, we used BAL fluid cell phenotypes to develop clinical scoring systems to identify acute rejection in lung transplant recipients and to identify patients at risk of future rejection and BOS. These predictors highlight the importance of CD25+ and NK cells, and may be useful in identifying acute rejection when pathology is unavailable or ambiguous. In practice, the clinical utility of BAL immunophenotyping will need to be assessed by prospective multi-center studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	Allophycocyanin
AUC	Area under the receiver-operating curve
BAL	bronchoalveolar lavage
BOS	Bronchiolitis obliterans syndrome
CI	Confidence Interval
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
FACS	Fluorescence-activated cell sorting
FEV1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
GEE	Generalized Estimating Equation
ISHLT	International Society for Heart and Lung Transplantation
KIR	Killer-cell immunoglobulin-like receptors
NK	natural killer
PBS	Phosphate buffered saline
PerCP	Peridinin chlorophyll protein
RBC	Red blood cell
UCSF	University of California, San Francisco
WBC	White blood cell

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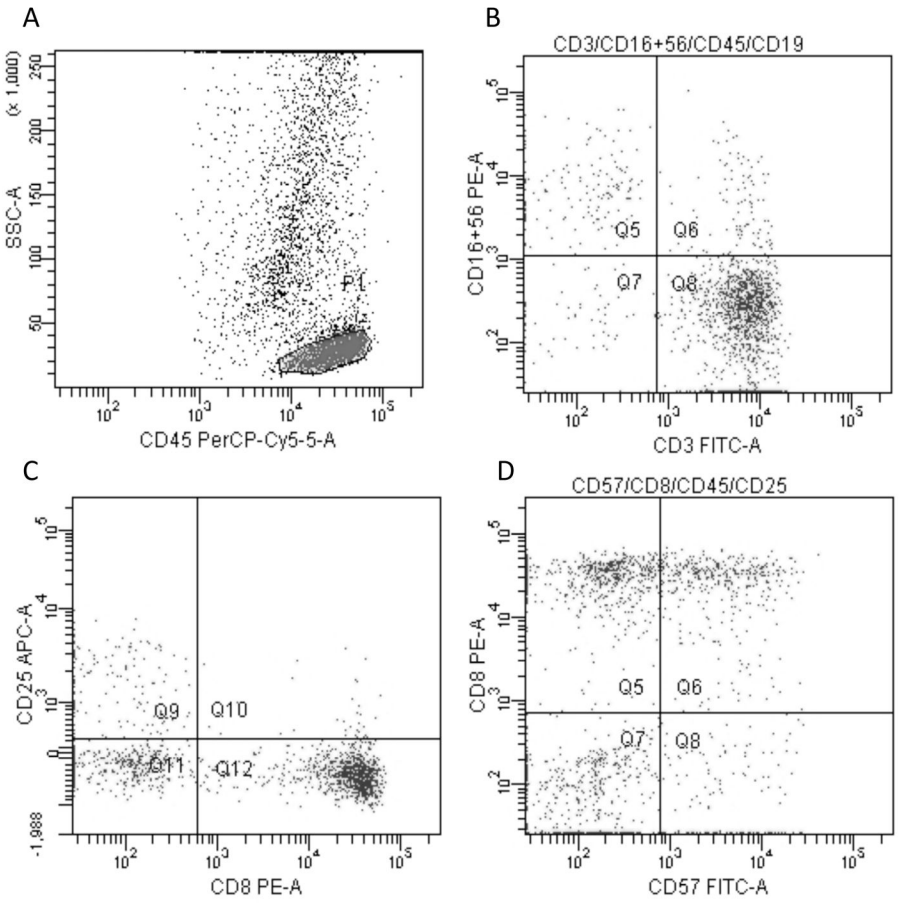


Figure 1. Immunophenotyping of lymphocytes in BAL fluid

Lymphocytes were identified by side scatter and CD45 positivity, shown in grey (A). NK cells were defined as CD3 and CD16+ and/or CD56+, which is Q5 in panel B. In panel C, CD25+ cells were calculated as Q9+Q10, while CD8+CD25+ cells were Q10 alone. CD8+CD57+ cells were Q6 in panel D.

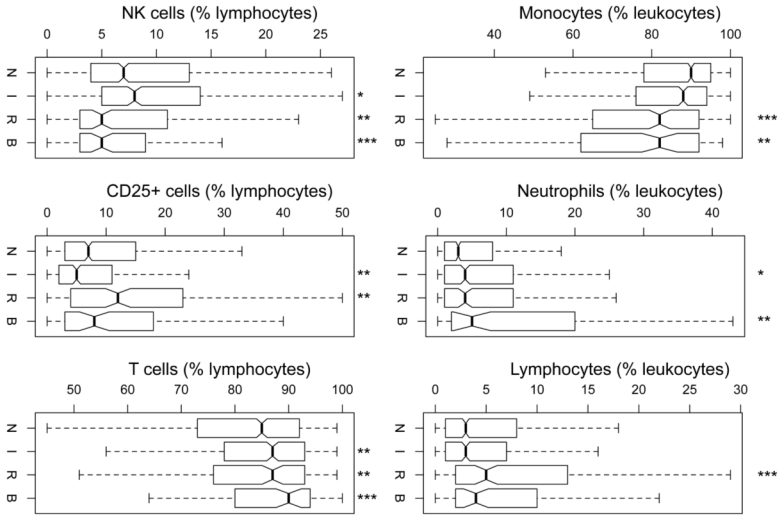
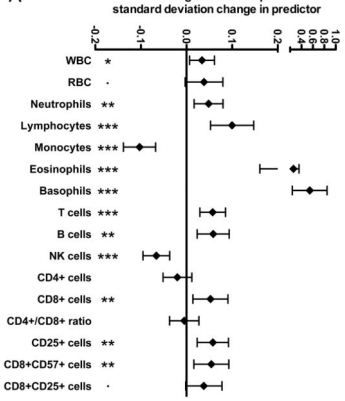


Figure 2. Distributions of BAL fluid cell characteristics

The percentage of cells in the given category at the time of grade ≥A1 acute rejection (R); viral, bacterial, or fungal infection (I); neither (N); or both (B) is shown as box-and-whiskers plots, with notches indicating confidence intervals for the median. Statistical significance for differences was determined using general estimating equations (GEE) and is shown as $P < 0.1$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

A



B

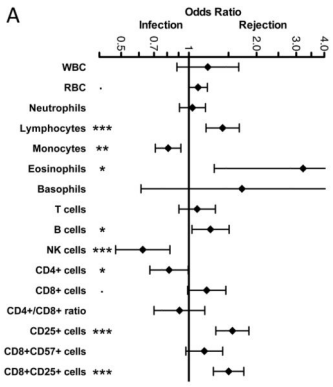
	Univariate			Multivariate		
Predictor	**Cutoff**	**χ2**	***P*-value**	**OR**	**95% CI**	***P*-value**
Monocytes	<75%	60.1	<0.001	2.41	1.84-3.15	<0.001
CD25+ cells	>8%	33.8	<0.001	2.01	1.52-2.66	<0.001
NK cells	<5%	31.2	<0.001	1.93	1.47-2.53	<0.001
Eosinophils	>0%	34.5	<0.001	1.82	1.19-2.77	0.01

C

	Derivation Cohort			Validation Cohort		
Score	**N**	**A ≥1**	**A ≥2**	**N**	**A ≥1**	**A ≥2**
0	506	8%	2%	297	11%	4%
1	710	17%	7%	325	15%	6%
2	314	25%	10%	124	22%	6%
3	77	48%	25%	26	27%	15%
4	10	90%	70%	3	100%	33%
AUC		0.67	0.70		0.60	0.59
Adjusted Odds Ratio		2.10	2.34		1.62	1.57
95% CI		(1.80-2.43)	(1.85-2.97)		(1.22-2.15)	(1.05-2.36)
***P*-value**		<0.001	<0.001		<0.001	0.03

Figure 3. Derivation of a BAL score for acute rejection (R-score)

For the derivation cohort, (A) shows the increase in mean A-score for standard deviation increase in the given BAL fluid characteristic as determined by univariate GEE analysis. The top four dichotomous predictors of a rejection score ≥A1 are shown in (B) as determined by a multivariate GEE model in the derivation cohort. (C) A scoring system summing the predictors in (B) is shown for the prediction of rejection score ≥A1 and ≥A2 in both cohorts. The area under the receiver operating curve (AUC) and odds ratio (OR) per unit increase in this score are shown. Odds ratios are adjusted for subject characteristics in Table 1, number of biopsies a subject received, and time since transplantation. CI denotes confidence intervals; $P<0.1$, $*P<0.05$, $**P<0.01$, $***P<0.001$.



B

		Univariate			Multivariate	
Predictor	Cutoff	χ2	*P*-value	OR	95% CI	*P*-value
CD25+ cells	>9%	60.5	<0.001	2.89	1.93-4.35	<0.001
NK cells	<5%	22.1	<0.001	2.07	1.43-2.98	<0.001
Lymphocytes	>12%	39.7	<0.001	2.04	1.27-3.27	0.003
Monocytes	<71%	25.8	<0.001	1.68	1.06-2.66	0.03

C

	Derivation		Validation	
Score	N	Rejection	N	Rejection
0	232	10%	141	16%
1	263	34%	109	27%
2	105	49%	50	30%
3	38	55%	21	43%
4	12	75%	1	100%
AUC		0.71		0.61
Adjusted Odds Ratio		2.13		1.66
95% CI		(1.76-2.56)		(1.20-2.30)
P-value		<0.001		0.002

Figure 4. Derivation of a BAL score for infection versus acute rejection (IR-score)

(A) For samples with either infection or biopsy-based rejection score ≥A1, the odds ratio (OR) of having rejection per standard deviation increase in the given BAL fluid characteristic is shown as determined by univariate GEE analysis using the derivation cohort. (B) The top four dichotomous classifiers of rejection versus infection are compared by multivariate GEE model in the derivation cohort. (C) A scoring system summing the predictors in (B) is shown for the prediction of ≥A1 rejection in the subset of derivation and validation cohorts having either rejection or infection. The area under the receiver operating curve (AUC) and OR per unit increase in this score are shown. Odds ratios are adjusted for subject characteristics in Table 1, the number of biopsies a subject received, and time since transplantation. CI denotes confidence intervals; $P<0.1$, *$P<0.05$, **$P<0.01$, ***$P<0.001$.

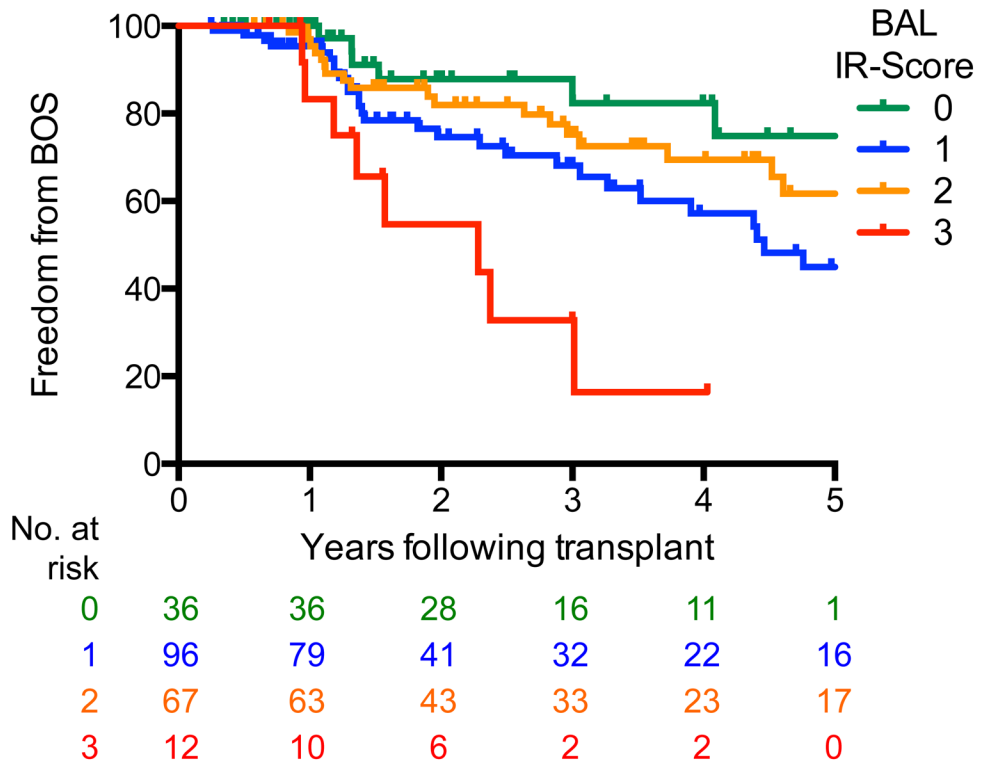


Figure 5. Kaplan-Meier analysis of maximum 90-day BAL IR-score for the prediction of freedom from BOS

Subjects were stratified according to the maximum value of BAL IR-score in the first 90 days excluding samples where infection or ≥A1 rejection was observed. Outcome data were left-truncated at 90 days and right-censored at 5 years. The number of subjects at risk per year is shown. Freedom from BOS curves are non-overlapping by Mantel-Cox test (P <0.001) with increasing maximum score associated with increased risk for BOS (P <0.05 by logrank test for trend).

Table 1

Subject characteristics

	Derivation	Validation	*P*-value
Subjects (n)	212	105	
Demographics			
Age at transplant	55	55	0.93
Male	51.9%	60.0%	0.21
Transplant type			
Double Lung	78.3%	81.9%	
Single Lung	17.9%	14.3%	0.66
Heart/Lung	3.8%	2.9%	
CMV status			
CMV D+/R+	31.1%	24.8%	
CMV D+/R−	13.2%	16.2%	
CMV D−/R+	10.8%	14.3%	0.60
CMV D−/R−	7.1%	9.5%	
CMV Unknown	37.7%	35.2%	
Transplant Indication			
Cystic Fibrosis	10.4%	8.6%	
COPD	21.7%	10.5%	
Pulmonary Fibrosis	35.4%	48.6%	0.14
Pulmonary Hypertension	6.6%	5.7%	
Other	26.0%	26.7%	

Subjects were randomly assigned 2:1 to the derivation and validation cohorts. Subject characteristics for the groups are shown with *P*-values for a χ^2-test of differences between groups.

Table 2

Rejection scores based on BAL immunophenotypes improve prediction of future rejection over current A-score

	Derivation Cohort			Validation Cohort		
	OR[†]	**95% CI**	***P*-value**	**OR**[†]	**95% CI**	***P*-value**
R-score[1]	1.48	1.19 – 1.83	<0.001	1.65	1.22 – 2.23	0.001
Adjusted[‡]	1.49	1.22 – 1.81	<0.001	1.75	1.26 – 2.46	<0.001
Limited to A0	1.54	1.21 – 1.96	<0.001	1.95	1.34 – 2.83	<0.001
IR-score[2]	1.61	1.31 – 1.98	<0.001	1.78	1.27 – 2.50	<0.001
Adjusted[‡]	1.64	1.36 – 1.98	<0.001	1.75	1.24 – 2.47	0.001
Limited to A0	1.56	1.24 – 1.96	<0.001	1.80	1.23 – 2.62	0.002
Current A-score	1.34	1.11 – 1.61	0.002	1.26	0.91 – 1.72	0.16

[†]Odds ratios are shown for ≥A1 rejection on repeat biopsy within 45 days per unit increase in prediction score, CI denotes confidence interval

[‡]Multivariate analysis is adjusted for current A-score, time since transplant, number of biopsies received, and patient characteristics in Table 1.

[1]BAL Rejection score: 1 point per each of monocytes <75%, CD25+ cells >8%, NK cells <5%, and eosinophils >0%

[2]BAL Infection vs. Rejection score: 1 point per each of CD25+ cells >9%, NK cells <5%, lymphocytes >12% and monocytes <71%.