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Inflammation on Bronchoalveolar Lavage Cytology is Associated with Decreased Chronic Lung Allograft Dysfunction-Free Survival

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Abstract

Background: Lung transplant recipients undergo bronchoalveolar lavage (BAL) to detect antecedents of chronic lung allograft dysfunction (CLAD), but routine assessment of BAL cytology is controversial. We hypothesized that inflammation on BAL cytology would predict CLAD-free survival.

Methods: In a single-center retrospective cohort, associations between cytology results and clinical characteristics were compared using generalized-estimating equation-adjusted regression. The association between BAL inflammation and CLAD or death risk was assessed using time-dependent Cox models.

Results: In 3,365 cytology reports from 451 subjects, inflammation was the most common finding (6.2%, 210 cases), followed by fungal forms (5.3%, 178 cases, including 24 cases of suspected *Aspergillus*). Inflammation on BAL cytology was more common in procedures for symptoms (8.5%) versus surveillance (3.2%, P<0.001). Inflammation on cytology was associated with automated neutrophil and lymphocyte counts, acute cellular rejection, infection, and portended a 2.2-fold hazard ratio (CI 1.2–4.0, P=0.007) for CLAD or death. However, inflammation by cytology did not inform CLAD-free survival risk beyond automated BAL cell counts (P=0.57).

Conclusions: Inflammation on BAL cytology is clinically significant, suggesting acute rejection or infection and increased risk of CLAD or death. However, other indicators of allograft inflammation can substitute for much of the information provided by BAL cytology.

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Introduction:

While lung transplant is an important option for the treatment of multiple end stage lung diseases, median post-transplant survival is less than seven years [1]. Chronic lung allograft dysfunction (CLAD) is the major barrier to long term survival, affecting most lung transplant recipients who survive to four years, with substantial impacts on function and quality of life [2]. Lung transplant recipients undergo bronchoscopy to identify acute cellular rejection and infection episodes that might be risk factors for CLAD. These procedures generally include histopathologic examination of transbronchial biopsy samples and microbiologic investigations for infection. Bronchoalveolar lavage (BAL) samples may also be analyzed for cell counts and/or cytology.

The utility of routine BAL cytology assessment is controversial. In an International Society for Heart and Lung Transplant (ISHLT) survey, 61% of participants reported routinely sending cytology for review [3]. Nonetheless, studies have suggested a low diagnostic yield for BAL cytology. A study of 366 bronchoscopies on 65 subjects found only two unique and clinically significant diagnoses [4]. Similarly, a study of 10 lung transplant recipients identified *Candida sp*. as the major diagnostic yield, for which there is uncertain clinical significance [5]. A third study including 155 subjects immunocompromised from malignancy, HIV, solid or bone marrow transplants reported a 35% sensitivity of BAL cytopathology for the diagnosis of infection [6]. Based on currently available literature, ISHLT consensus guidelines on BAL standardization conclude that there is “insufficient evidence to recommend routine morphological microscopic cytology for the detection of infection, malignancy, or rejection,” but that it may be of clinical benefit in cases of high suspicion for atypical infection or malignancy [3]. BAL cytology may demonstrate evidence of neutrophilic or lymphocytic inflammation [7, 8]. Prior studies have identified these findings but without clear clinical relevance. A critical limitation of prior studies on the utility of BAL cytology is the lack of data on progression to CLAD, limiting the assessment of the clinical utility of these cytopathology findings. Here, we hypothesized that inflammation identified on BAL cytology would be predictive of CLAD.

Methods:

This study was approved by the University of California, San Francisco (UCSF) internal review board (IRB approval # 13-10738). All subjects provided written informed consent. We performed a query of the UCSF Pathology Department CoPath database for “Cytology, Non Gynecologic” tests where the report contained the words “Bronchial lavage” between January 1, 2012 and June 30, 2019. From this list, we excluded subjects who had not received a lung transplant and those who did not consent to participate in research. Cytology reports were reviewed and coded based on findings of inflammation, *Aspergillus*, *Candida*, malignancy, or CMV. “*No findings to report*” was coded as benign. Because templated reports result in similar text, identical reports were graded in batch. The cytopathologist of record was noted. Bronchoscopy procedures were classified as for-cause versus surveillance. Additional clinical data were obtained from chart review. Additional subject data were obtained from the United Network for Organ Sharing records. One hundred BAL cytology

cases were selected evenly from inflammation cases and controls for rereview by a single cytopathologist (NYG) to assess the reproducibility of inflammation diagnosis.

Time to CLAD was determined from electronic medical record spirometry data as the days between transplant and sustained forced expiratory volume in 1 second (FEV_1) <20% of post-transplant baseline, as previously described [9]. Samples collected after CLAD onset were not included in the survival models. BAL cell counts were abstracted from electronic medical records, where available. Absolute neutrophil and lymphocyte concentrations were derived by multiplying absolute leukocyte concentrations (as 10^9 cells per L of BAL fluid) by the proportion of neutrophils or lymphocytes identified by automated cell counts.

Bronchoscopy was performed for cause, such as decreased lung function on spirometry or new respiratory symptoms. Surveillance bronchoscopy was typically performed at 2, 4, 8, 12, 25, 52, 78, and 104-weeks post-transplant. Bronchoscopy consisted of airway inspection followed by BAL with five to six 20 ml aliquots of normal saline. BAL return was sequentially sent for microbiologic analysis, cytology, automated cell counts and leukocyte phenotyping [7]. BAL fluid was not pooled, and cytology was sent from the second trap. Transbronchial biopsies were performed following BAL.

Lung transplant recipients were managed per institutional protocols [10]. Induction immunosuppression included prednisone, basiliximab, and mycophenolate, while maintenance included tacrolimus, mycophenolate, and prednisone. Subjects were started on azithromycin 250 mg three times per week for CLAD prophylaxis starting on post-transplant day 30. The institutional protocol of prophylactic azithromycin had started on January 1, 2011, one year prior to the collection of any BAL cytology samples in this study. CMV prophylaxis was started on all participants with intravenous ganciclovir, which was transitioned to oral valganciclovir once able to tolerate oral medications. CMV prophylaxis was continued as long as tolerated, but could be stopped or reduced for side effects, such as leukopenia. Higher risk subjects also received CMV immunoglobulin through post-transplant week 16 [11].

Analysis:

All analyses were performed in R version 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria). Distributions of continuous variables were reported using standard deviation or interquartile range depending on normality, as assessed by Shapiro-Wilk test. Frequency distributions were assessed using Fisher exact tests. Associations between clinical parameters and the finding of inflammation on BAL cytology were determined by univariable generalized-estimating equation-adjusted logistic regression models using clinical parameters as predictor of inflammation on BAL cytology. These parameters included BAL lymphocyte and neutrophil counts greater than median using contemporaneous automated cell count, respiratory or systemic symptoms (acute symptoms, flu-like symptoms, dyspnea, fatigue, cough) as indications for bronchoscopy, bronchoscopy findings of infection for which antimicrobial treatment was initiated, acute cellular rejection (ISHLT A- or B-grade >0) identified on transbronchial biopsy, or bronchoscopy performed

for surveillance. Associations between cytology inflammation and BAL cell counts were determined using Wilcoxon rank sum tests.

Survival analysis was performed using Cox proportional hazards modeling, with inflammation on BAL cytology and absolute lymphocyte and neutrophil counts coded as time-dependent predictors. Multivariable models included recipient age, sex, diagnosis group, and transplant procedure. Cox models were visualized from predicted curves using "survival" and the "survminer" packages.

Results:

We identified cytology reports from 460 lung transplant recipients, of which nine declined to participate in research. Study participant characteristics are shown in Table 1. Relative to national lung transplant data, these subjects were more frequently diagnosis group D, which may reflect UCSF being an interstitial lung disease referral center.

Cytology findings:

BAL cytology specimens were signed out by 20 different cytopathologists, with a range of 3 to 530 cases per cytopathologist. We found a strong effect of cytopathologist on the rates of inflammation called on BAL cytology, with call rates ranging from 0% to 15.8% (chi-squared = 71.5, $p < 0.001$). Rereview of 100 BAL cytology inflammation cases and controls was performed in a blinded manner. There was agreement on 51 cases of inflammation and 42 cases of no inflammation. Six "no inflammation" cases were found to have inflammation on rereview, while only one case with inflammation was not found to have inflammation on rereview. Overall, there was strong agreement, with a Cohen's Kappa of 0.86 (CI 0.76–0.96, $p < 0.001$).

To determine if BAL cytology had a greater yield in procedures done for cause (versus surveillance), we stratified bronchoscopy events based on the pre-procedure clinical indication, which was available for 79% of procedures (Table 2). Overall, 89% of BAL cytology samples were reported as benign, meaning no evidence of inflammation, malignancy, or infection. Benign cytology samples were more common in surveillance procedures (odds ratio 1.9, 95% CI 1.5 – 2.5). The most reported finding was inflammation, which was present on 210 (6.2%) of cytology samples. A representative example of BAL inflammation is shown in Figure 1. Inflammation was more commonly observed during "for cause" bronchoscopies (odds ratio 2.8, 95% CI 2.0 – 4.0). Fungal forms were the second most common finding, appearing on 178 (5.3%) of samples, although much of this reflected 152 (4.5%) cases consistent with *Candida* species, for which there is uncertain clinical significance.

The four cases in which malignancy was identified were distributed among two individuals, for whom there was a known history of cancer and strong suspicion of pulmonary involvement based on radiology findings. Of the 24 patients where Aspergillus was identified on cytology, none was uniquely associated with a change in clinical management. In 18 cases, Aspergillus was also identified on BAL culture or bronchial wash culture. In three cases, a different fungus was identified on culture. In three cases, there was no

culture positivity. Of the three cases in which there was no culture positivity, one patient was already on antifungals. For the other two patients, antifungals were deferred at the provider's discretion. Of the 24 patients who had Aspergillus identified on cytology, nine were already on antifungal therapy.

Association with automated cell counts:

Given that inflammation was the most common finding on BAL cytology, we asked how this finding compared with automated cell counts and differential measurements, which are performed as standard of care. Figure 2 shows the distributions of neutrophil counts and lymphocyte counts stratified by the finding of inflammation on BAL cytology. As would be expected, we observed a significant increase in absolute lymphocyte and neutrophil counts in BAL samples that were found to have inflammation by cytopathology ($P<0.001$). While there was significant overlap between the groups, there were many samples with high lymphocyte and neutrophil counts that were not called as having inflammation on cytology.

Associations between clinical parameters and inflammation on BAL cytology:

As shown in Figure 3, multiple clinical parameters were associated with inflammation on BAL cytology, including elevated BAL cell counts, acute symptoms, infection, and acute cellular rejection. Bronchoscopy performed for surveillance, as opposed to "for cause," was associated with decreased odds of inflammation on BAL cytology. Table 3 shows bronchoscopy indications and outcomes stratified by inflammation on BAL cytology. "For cause" bronchoscopies were more commonly associated with inflammation. Inflammation was associated with the presence of acute symptoms, such as fever, dyspnea, cough, flu-like symptoms, or fatigue. Interestingly, there was no statistically significant association with decreased FEV_1. Similarly, BAL inflammation was associated with treatment for infection, but not with treatment for rejection. Five samples were insufficient for evaluation.

Associations with CLAD-free survival:

We examined whether inflammation on BAL cytology was associated with CLAD-free survival. During the observation period, 253 of 450 included subjects experienced the composite endpoint of CLAD or death, of which 36 died without CLAD. Considered as a time-dependent covariate in a univariable Cox proportional hazards model, the presence of inflammation on BAL cytology was associated with a 2.2-fold (95% CI 1.2–4.0 fold, $P=0.007$) increase in risk of CLAD or death (Figure 4). In a multivariable model including diagnosis group, recipient age and sex, and transplant procedure type, inflammation on BAL cytology was associated with a 2.3-fold (95% CI 1.22–4.3 fold, $P=0.01$) increase in risk of CLAD or death. However, when BAL cell neutrophil and lymphocyte cell counts were added to this multivariable model, there was no association with CLAD-free survival (HR 0.73, 95% CI 0.25–2.14, $P=0.57$). Of note, we only had BAL cell count data for 75% of this cohort. Examining the outcome of CLAD censored on death, inflammation on BAL cytology was associated with a 2.1 fold (95% CI 1.03–4.3, 450 subjects, $P=0.04$) increase in subdistribution hazard for CLAD. When BAL cell neutrophil and lymphocyte cell counts

were added to this multivariable model, there was no association with CLAD censored on death (sub-HR 1.02, 95% CI 0.34–3.1, P=0.97).

In a Cox proportional hazards model examining the percentage of neutrophils and lymphocytes in BAL as a time dependent predictor of CLAD free survival, adjusted for diagnosis group, recipient age and sex, and transplant procedure type, a 1% increase in BAL neutrophils was associated with a 1.01 fold (95% CI 1.00-1.03, P=0.049) increase in risk of CLAD or death, while a 1% increase in BAL lymphocytes was associated with a 1.01 fold (95% CI 0.99-1.02, P=0.28) increase in risk of CLAD or death.

Discussion:

We found that inflammation was the most common feature on BAL cytology and was predictive of time to CLAD or death. However, most BAL studies were benign and the finding of inflammation on BAL adds little beyond BAL automated cell count and culture studies. The yield of BAL cytology is strongly influenced by clinical suspicion, as cytology assessment in surveillance bronchoscopy fluid is highly unlikely to change clinical management.

While we did not assess interobserver reliability for the finding of inflammation on BAL fluid in this study, it is likely to be imperfect, given the substantial overlap in absolute lymphocyte and neutrophil cell counts observed between the inflammation and no inflammation samples. There is no standardized grading system for inflammation on BAL cytology. The wide range in call rates for inflammation and frequency of inflammation upgrading on re-review suggest that cytopathologists may be under calling inflammation on BAL cytology in the absence of established criteria. BAL cell counts and grading of inflammation may also vary depending on BAL fluid dilution. This lack of standardization may bias the association between BAL inflammation and CLAD-free survival towards the null and represents an area in which BAL cytopathology could be improved. We did not observe a clear link between the cell types observed in inflamed BAL samples and CLAD-free survival. Defining the specific cytology features associated with CLAD-free survival would be another area for potential improvement. However, Papanicolaou staining may not be ideal for differentiating inflammatory cell types. While BAL eosinophilia ≥2% has also been shown to be associated with worse CLAD-free and overall survival, these cells can be difficult to identify on Papanicolaou stain [12]. Flow cytometric and gene profiling studies in BAL fluid have shown promise in identifying subjects at increased risk for CLAD, suggesting that BAL immunohistochemical studies could potentially outperform standard Papanicolaou staining [7, 11, 13].

ISHLT consensus guidelines recognize variability in cell counts over the course of BAL return and recommend pooling BAL prior to analysis. Our practice of multiple 20 ml saline installations also differs from ISHLT guidelines. Although practice was consistent over the course of this study, it is possible that these results may not generalize to other centers following the ISHLT protocol [3].

Both neutrophilic and lymphocytic inflammation on cytology were associated with increased risk for CLAD in this cohort. Neutrophilic inflammation has been previously linked to CLAD risk. Elevated neutrophil counts have been reported to precede CLAD onset [8, 14-16]. The syndrome of neutrophilic reversible allograft dysfunction (NRAD) is defined by >15% neutrophils in BAL fluid and a ≥10% decline in FEV_1. NRAD resolves with azithromycin treatment [17], and most of these subjects were on azithromycin for CLAD prophylaxis per institutional protocol. However, we do not have complete medication records in this cohort and so cannot determine what effect azithromycin treatment would have had on these findings. Overall, these data are consistent with the previously described association between neutrophilic airway inflammation and CLAD [18]. Mechanistically, IL-8 and IL-17 are neutrophil chemoattractants released by epithelial cells and alveolar macrophages in the context of CLAD [7, 8]. Similarly, there have been associations reported between BAL lymphocytes and CLAD development [19].

As a single center study, it is not certain how these findings would generalize to other centers where subject characteristics and post-transplant management protocols may differ. For example, our center has a low frequency of CMV pneumonitis, which may be attributable to an aggressive prophylaxis regimen. The utility of cytology for detecting CMV might be greater in centers with higher prevalence, but still may be less than for transbronchial biopsy. At the same time, this study has notable strengths: This is the largest cohort study to evaluate BAL cytology findings in lung transplant recipients, reflects modern post-transplant management practices, and is the first to include associations with long term outcomes.

In summary, this study identified an association between inflammation on BAL cytology fluid and CLAD-free survival. It is unclear whether the presence of inflammation on BAL cytology is an independent risk factor for CLAD or a surrogate marker for other processes such as rejection or infection. BAL cytology is potentially useful for identifying patients at risk for CLAD because of the association between inflammation and CLAD-free survival. However, because automated cell counts on BAL fluid can provide similar risk stratification, centers may not find routine BAL cytology to be a worthwhile use of resources, consistent with ISHLT consensus recommendations. It is possible that improvements in BAL cytology, including standardized criteria for inflammation could improve the yield from this test.

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Data availability:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations:

BAL	bronchoalveolar lavage.
CLAD	chronic lung allograft dysfunction.

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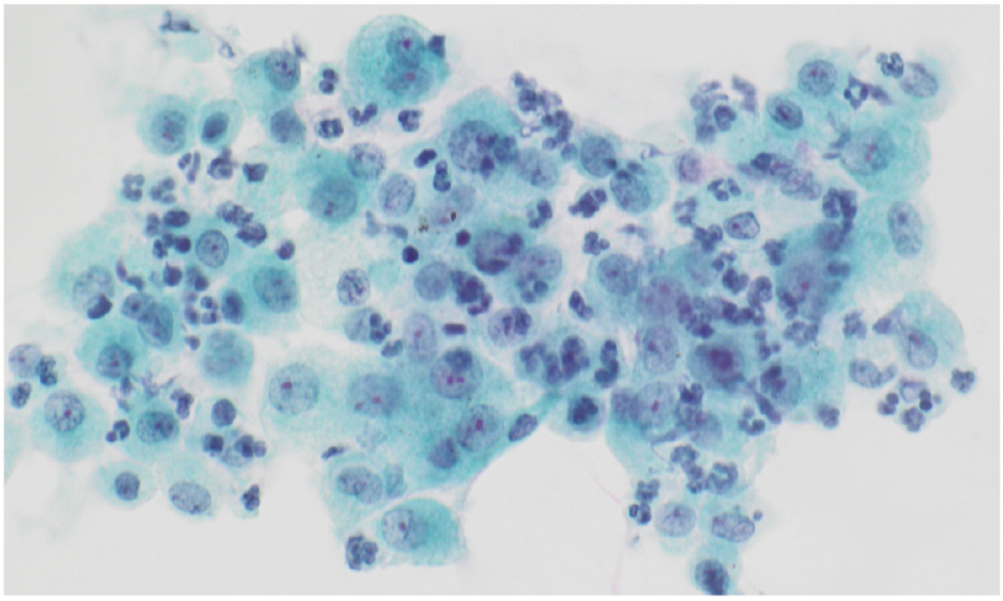

Figure 1.
BAL cytology specimen (Papanicolaou stain) with numerous admixed neutrophils and lymphocytes.

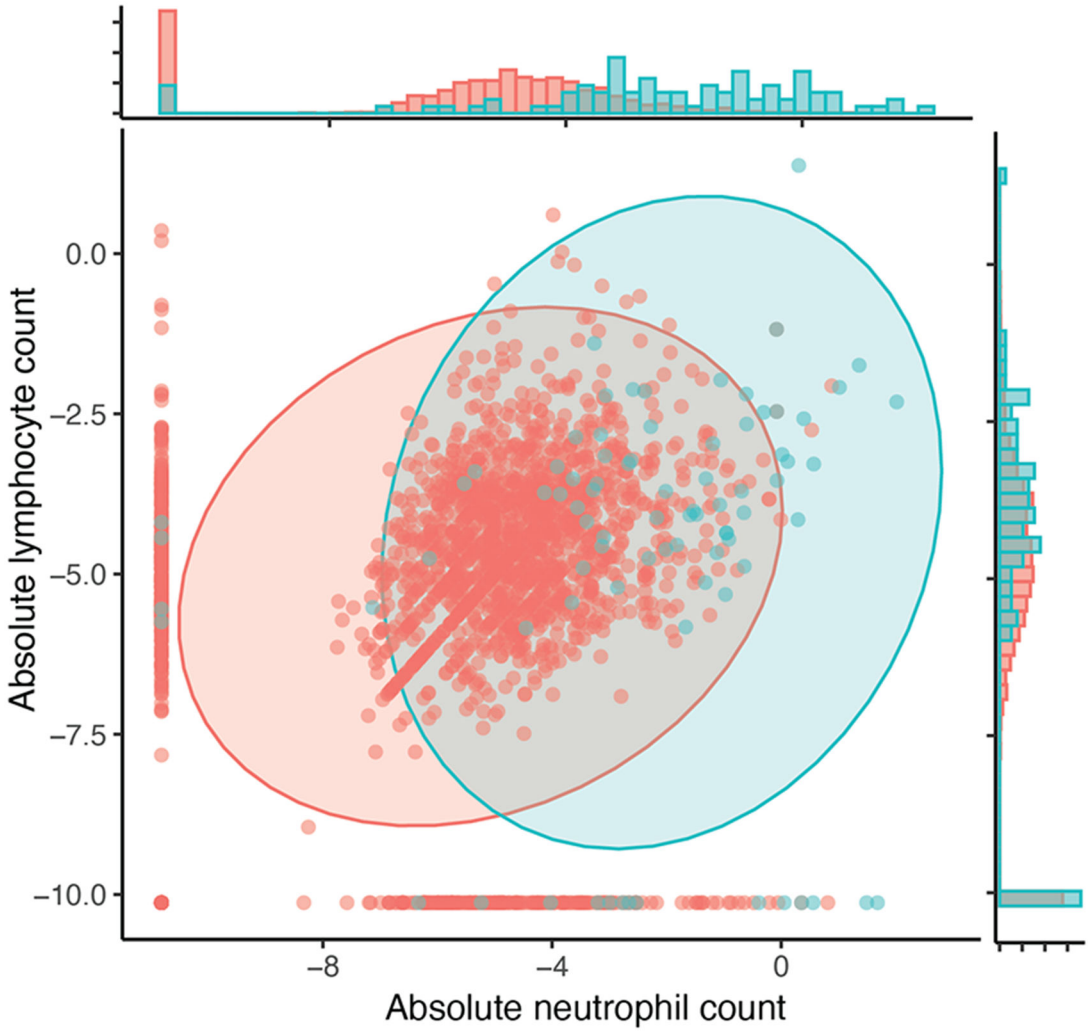

Figure 2.

BAL inflammation by cytology is associated with automated cell counts of BAL neutrophils. Log-transformed absolute neutrophil and lymphocyte count concentrations are on the x- and y-axes, respectively, stratified by the presence of inflammation on the cytology report (indicated in blue) versus no inflammation (red). Cytology reports of inflammation were associated with increased absolute lymphocyte counts ($P<0.001$) and absolute neutrophil counts ($P<0.001$) as assessed by Wilcoxon test.

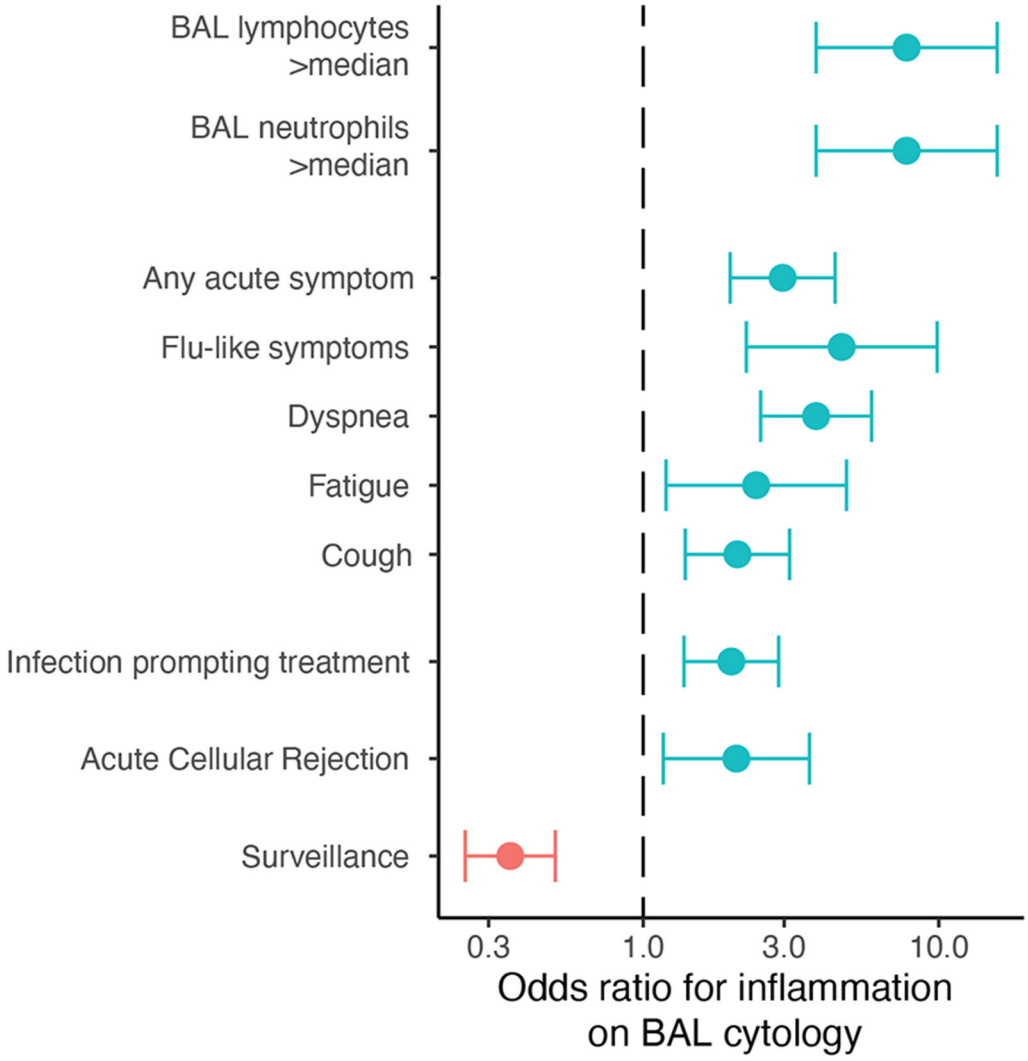

Figure 3:

Associations between clinical parameters and the finding of inflammation on BAL cytology. Odds ratios with 95% confidence interval are shown for univariable generalized-estimating equation-adjusted logistic regression models for the prediction of inflammation on BAL cytology based on paired BAL lymphocyte and neutrophil counts, respiratory or systemic symptoms as indications for bronchoscopy, infection, acute cellular rejection, or bronchoscopy performed for surveillance.

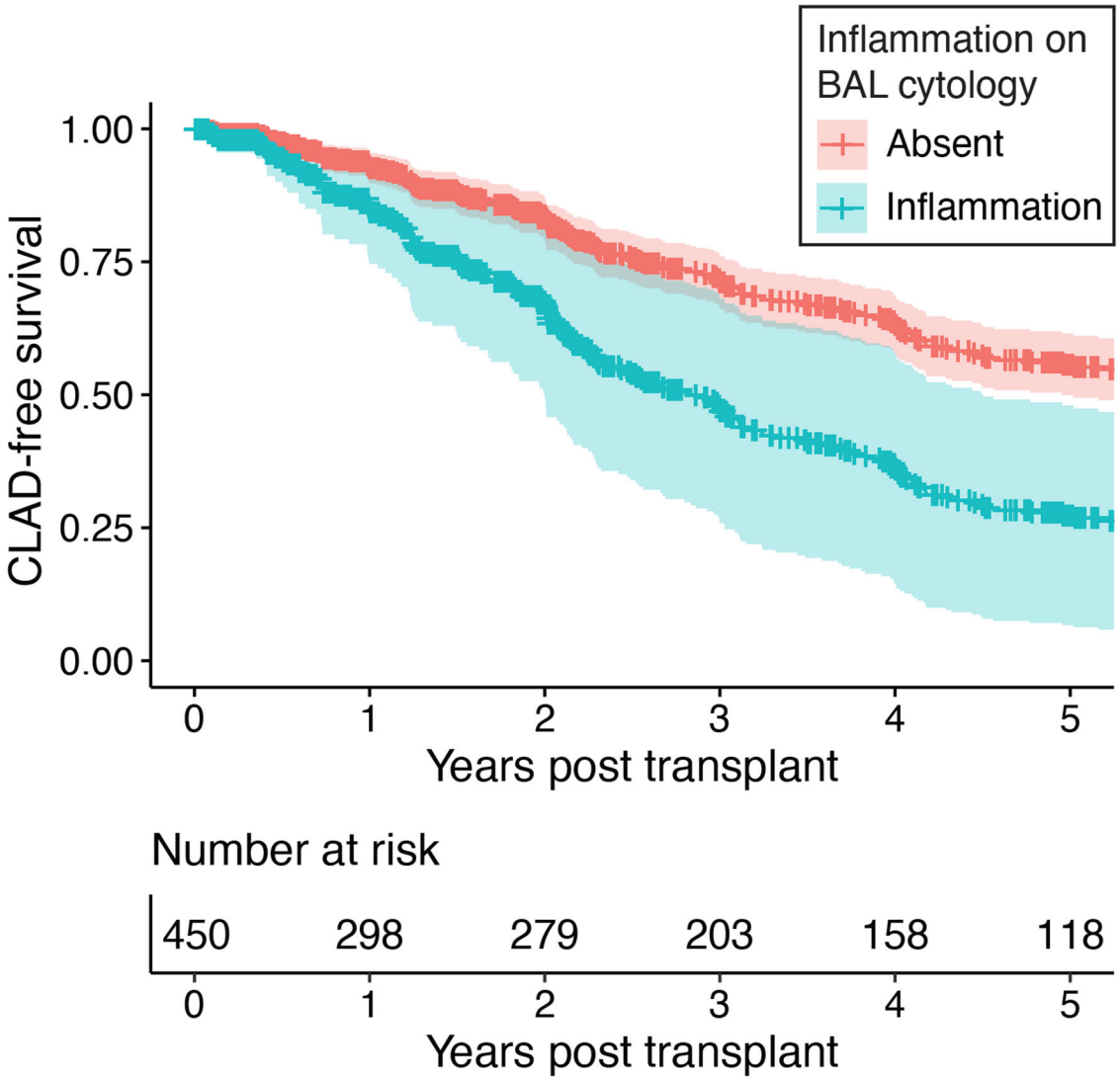

Figure 4:

CLAD-free survival as a function of inflammation on BAL cytology. Estimated distributions for the composite outcome of CLAD or death are shown as a function of inflammation on BAL cytology. Inflammation was modeled as a time-dependent predictor using Cox proportional hazards models. In this model, exposure to inflammation on BAL cytology is associated with a 2.2 fold increase in risk of CLAD or death (95% CI 1.2–4.0, P=0.007).

Table 1.

Cohort characteristics.

IQR: interquartile range, N: number; Variables shown as median and IQR were found to be non-normal by Shapiro-Wilk test.

Subjects, N	451
Cytology samples, N	3365
Cytology samples per subject, median [IQR]	7 [4 – 10]
Recipient age, median [IQR]	58 [48 – 65]
Donor age, median [IQR]	31 [21 – 46]
Recipient sex, N (%)	
Male	257 (57)
Female	194 (43)
Donor sex, N (%)	
Unknown	11 (2.4)
Female	163 (36.1)
Male	277 (61.4)
CMV status, donor/recipient, N (%)	
N/N	60 (13.3)
N/P	89 (19.7)
P/N	110 (24.4)
P/P	159 (35.3)
Unknown	33 (7.3)
Procedure type, N (%)	
Double	409 (90.7)
Heart/Lung	7 (1.6)
Single	35 (7.8)
Diagnosis group, N (%)	
A - Obstructive	70 (15.5)
B - Pulmonary vascular	18 (4.0)
C - Cystic fibrosis	41 (9.1)
D - Restrictive	322 (71.4)
Recipient Ethnicity, N (%)	
American Indian/Alaska Native	2 (0.4)
Asian	27 (6.0)
Black	34 (7.5)
Hispanic	74 (16.4)
Multiracial/Unknown	2 (0.4)
Native Hawaiian/Other Pacific Islander	2 (0.4)
White	310 (68.7)
Donor Ethnicity, N (%)	
Asian	41 (9.1)
Black	42 (9.3)

Hispanic	138 (30.6)
Multiracial/Unknown	21 (4.7)
Native Hawaiian/other Pacific Islander	6 (1.3)
White	203 (45.0)
Lung allocation score, median [IQR]	50.5 [39.5–79.8]

Table 2:

BAL Cytology findings stratified by bronchoscopy indication.

	Unknown	For-cause	Surveillance	P-value*
Finding, N (%)	715	1110	1540	
Inflammation	67 (9.4)	94 (8.5)	49 (3.2)	<0.001
Aspergillus	0 (0.0)	17 (1.5)	7 (0.5)	<0.001
Malignancy	4 (0.6)	0 (0.0)	0 (0.0)	0.001
Candida	33 (4.6)	60 (5.4)	59 (3.8)	0.145
CMV	0 (0.0)	0 (0.0)	2 (0.1)	0.306
Benign	613 (86.1)	953 (85.9)	1418 (92.1)	<0.001
Fungi	33 (4.6)	79 (7.1)	66 (4.3)	0.004
Uninterpretable	3 (0.4)	1 (0.1)	1 (0.1)	0.104

*P-value reflects a Fisher exact test comparing for-cause versus surveillance groups.

Table 3:

Bronchoscopy indication and clinical management stratified by inflammation on BAL cytology.

Five samples were insufficient for evaluation.

	Inflammation Absent	Inflammation Present	p-value
n	3150	210	
Indication (%)			<0.001
For cause	1015 (32.2%)	94 (44.8%)	
Surveillance	1490 (47.3%)	49 (23.3%)	
Not abstracted	645 (20.5%)	67 (31.9%)	
Acute Symptoms	319 (12.7%)	42 (29.4%)	<0.001
Fever	35 (1.4%)	9 (6.4%)	<0.001
Dyspnea	226 (9.0%)	39 (27.7%)	<0.001
Cough	371 (14.9%)	37 (26.2%)	<0.001
Flu like symptoms	40 (1.6%)	10 (7.1%)	<0.001
Fatigue	101 (4.0%)	13 (9.2%)	0.006
Decreased peak flow FEV1	332 (13.3%)	22 (15.6%)	0.511
Treated for Infection	477 (19.1%)	45 (31.9%)	<0.001
Treated for Rejection	163 (6.5%)	13 (9.2%)	0.283