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A Combination of Cytological Biomarkers as a Guide in the Diagnosis of Acute Rejection in Lung Transplant Recipients

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Abstract: The usefulness of bronchoalveolar lavage fluid (BALF) to support the diagnosis of acute cellular (ACR) rejection in lung transplant (LTX) recipients remains controversial. ACR has been associated with blood eosinophil counts (EOS) in other solid organ recipients, but there are few studies in relation to lung transplants. Our aim was to assess the usefulness of the combined analysis of BALF cellularity and EOS for the diagnosis of ACR in lung transplant recipients. This is a retrospective study of findings observed simultaneously in 887 transbronchial biopsies (TBB), BALF, and blood samples obtained from 363 LTx patients transplanted between 2014 and 2020. The variables collected were: demographics, ACR degree, BALF cellularity, and simultaneous blood EOS counts. The lymphocyte count in BALF was significantly higher in patients with ACR than in those without (11.35% vs. 6.11%; $p < 0.001$). In parallel, EOS counts were also significantly higher in patients with ACR than in the non-ACR group (EOS 213 ± 206/mm^3 vs. 83 ± 129/mm^3; $p < 0.001$). Increases in both parameters were associated with an increased risk of ACR (lymphocytes OR 1.100; 95% CI 1.080–1.131; EOS OR 1.460; 95% CI 1.350–1.580). The diagnostic specificity of ACR for a lymphocyte count > 12% was 71.1%, which increased to 95.8% when taking into account a simultaneous blood EOS count > 200/mm^3. Simultaneous assessment of BALF lymphocyte counts and blood eosinophil counts may be useful for diagnosing ACR in patients with risk factors for TBB or in the presence of inconclusive histological samples.

Keywords: lung transplant; acute cellular rejection; bronchoalveolar lavage fluid; blood eosinophil counts

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1. Introduction

Acute cellular rejection (ACR) is one of the most frequent complications in lung transplant (LTx) recipients. According to data from the international society for heart and lung transplantation (ISHLT) registry, up to 30% of patients experience an ACR episode within the first year after transplantation. This complication accounts for 3.6% of mortality within the first 30 days and 1.8% within the first year [1]. In addition, ACR is directly related to the subsequent development of chronic lung allograft dysfunction (CLAD), which is the main cause of mortality after the first year post-transplant [1]. Therefore, it is essential to be able to make an early diagnosis and thus guarantee fast and effective treatment.

ACR has non-specific symptoms and a variable clinical course. Moreover, radiological findings and lung function alterations that it cases may be confused with other very common complications in these patients, such as infections.

The gold standard for diagnosing ACR is a transbronchial biopsy (TBB), although its diagnostic sensitivity varies greatly depending on the clinical indication and the pathologist's experience [2]. TBB is considered a routine technique for monitoring these patients, but it carries a risk of potentially severe complications, such as bleeding (4%) or pneumothorax (2.5%), and may therefore be considered contraindicated in some patients [2]. A less invasive and lower-risk alternative than TBB is bronchoalveolar lavage fluid (BALF). BALF may reflect the inflammatory microenvironment of the lung parenchyma, and its analysis would be useful in the diagnosis of ACR [3]. However, there are variations in the cellular changes in BALF associated with ACR that have limited the consensus regarding its diagnostic usefulness [3,4].

Multiple studies have tried to identify biomarkers of rejection in peripheral blood for the early diagnosis of ACR. In this regard, we can highlight, amongst others, the determination of several cytokines and chemokines, KL6, exosomes, or donor-derived cell-free DNA. However, the cost and complexity of many of these techniques have limited their implementation in routine clinical practice [4–7].

Haemograms are determined routinely and periodically in the follow-up of lung transplant recipients. Increased eosinophil counts in blood (EOS) have been associated with the development of rejection of solid organ transplantation. It has been suggested that in immunosuppressed patients, EOS could be involved in ACR through a T-helper 2–type immune response [8].

In a previous study at our lung transplant unit, we observed that determining EOS counts in blood could be of use to indicate acute rejection and increase the diagnostic effectiveness of TBB [9].

Our aim has been to analyze the usefulness of the combined analysis of lymphocyte counts in BALF and eosinophil counts in blood for the diagnosis of ACR in lung transplant recipients.

2. Materials and Methods

This is a retrospective study of the findings observed in the 887 samples of TBB, BALF, and simultaneous blood obtained from 363 consecutive LTx recipients performed between January 2014 and December 2020.

The variables collected from the electronic medical record registry were: age, sex, underlying pathology, date of transplantation, indication for sample collection, presence and grade of ACR in TBB, and lymphocyte count in BALF and EOS. Other variables collected were the corticosteroid dose at the time of sampling and the median EOS in the three months before sampling. Informed consent was obtained from all subjects involved in the study.

Our program's immunosuppression protocol during the study period was: basiliximab 20 mg on days 0 and 4, and triple therapy with methylprednisolone in decreasing doses to 0.10 mg/kg/day, tacrolimus (levels between 5–15 ng/mL depending on time post-transplant) and mycophenolate (500–1000 mg/12 h depending on effectiveness and adverse effects). Acute rejection episodes were treated with three successive bolus administrations of 500 mg of methylprednisolone.

The indications for performing Fiber-optic-bronchoscopy (FBC) and for sampling were classified as follows:

1. Per protocol in the first month after transplantation
2. Due to a decline in lung function, defined as a $\geq$10% reduction in forced expiratory volume in 1 s (FEV1) with respect to the patient's prior baseline level
3. To verify ACR resolution after treatment
4. Other indications such as the presence of respiratory symptoms or radiological alterations.

FBC with TBB and BALF sampling were performed according to our society's recommendations [10]. After inspecting the airways, three 50 mL aliquots were administered into the segmental bronchi. After each administration, the aliquots were carefully aspirated at a suitable pressure so that the bronchial walls would not collapse. The first 20 mL of

the BALF was discarded, and the rest was processed following the center's criteria for microbiological cultures and cytology.

The cellularity of the BALF was determined in 10 mL of a mixture of the three aliquots after being filtered through a 70 µm cell strainer and centrifuged for 10 min at 300× *g*. The cellular fraction was stained with fluorescence-conjugated monoclonal antibodies (BD Biosciences, San Jose, CA, USA) to identify the different leukocyte subpopulations. Labeled cells were analyzed in a FACSort flow cytometer with the CellQuest and the Paint-a-Gate Pro software (BD Biosciences, San Jose, CA, USA).

The histological diagnosis of acute cellular rejection was classified according to ISHLT recommendations into grade A0 (none), grade A1 (minimal), grade A2 (mild), grade A3 (moderate), and grade A4 (severe) [11].

In the blood samples, we analyzed the absolute and relative EOS count at the time the BALF was performed and the median EOS in the three months before performing the BALF.

A descriptive analysis of categorical variables was performed using absolute and relative frequencies and of numerical variables using the median, 25 and 75 percentiles, minimum and maximum values, mean and standard deviation. To evaluate the association between ACR, absolute EOS counts, and the percentage of lymphocytes in BALF, we started with a variable number of biopsies per patient over time since transplantation. We used generalized estimating equation (GEE) models, which take into account that biopsies from the same patient are more closely associated with one another than biopsies from different patients. The logit function was used as a link function. The percentage of lymphocytes in BALF and absolute and relative EOS counts in blood were introduced in the models as independent variables, with the presence of rejection as a dependent variable.

Stata v17 software was used to perform all the analyses, and the significance level was set at 0.05.

3. Results

The study included a total of 887 TBB, BALF, and blood samples collected from 363 patients, 232 (63.9%) of whom were men with an average age at transplantation of 55 ± 11 years. The main indications for transplantation were chronic obstructive pulmonary disease (COPD), with 146 cases (40.2%), and idiopathic pulmonary fibrosis (IPF), with 76 cases (20.9%) (Table 1).

Table 1. Demographic data at patient level.

	Total ***n* = 363**	**No ACR** ***n* = 211; 58.1%**	**ACR** ***n* = 152; 41.9%**	***p***
Age at transplant	55.0 ± 10.8	54.3 ± 11.3	55.9 ± 9.9	*p = 0.156*
Male	232 (63.9%)	133 (63.0%)	99 (65.1%)	*p = 0.681*
COPD	146 (40.2%)	82 (38.9%)	64 (42.1%)	*p = 0.808*
IPF	76 (20.9%)	47 (22.2%)	29 (19.1%)	
DILD	83 (22.9%)	47 (22.2%)	36 (23.7%)	
CF	37 (10.2%)	23 (10.9%)	14 (9.2%)	
BE	11 (3.0%)	5 (2.4%)	6 (3.9%)	
Other causes	10 (2.8%)	7 (3.4%)	3 (2.0%)	
Post-transplant time (median months)	12 (1–20)	11 (1–25)	12 (1–28)	*p = 0.230*

ACR: acute cellular rejection; COPD: chronic obstructive pulmonary disease; IPF: idiopathic pulmonary fibrosis; DILD: diffuse interstitial lung disease; CF: cystic fibrosis; BE: bronchiectasis.

The mean time between transplant and TBB was 12 (1–20 months), without significant differences between the group with ACR and the group without ACR (Table 1).

The most frequent indication for performing FBC was a decline in lung function (37.4%), followed by those performed per protocol (33.4%) (Table 2).

Table 2. Acute cellular rejection according to basic pathology and indication for transbronchial biopsy. Concurrent steroid dose (*mg*) according to TBB indication.

TBB	Total (n = 887)	No ACR (n = 628; 70.8%)	ACR (n = 259; 29.2%)	p
ACR according to indication				
Per protocol	296 (33.4%)	206 (32.8%)	90 (34.7%)	$p = 0.200$
Decline in lung function	332 (37.4%)	237 (37.7%)	95 (36.7%)	
To confirm ACR resolution	206 (23.2%)	141 (22.5%)	65 (25.1%)	
For other reasons	53 (6.0%)	44 (7.0%)	9 (3.5%)	
ACR according to baseline pathology				
COPD	398 (44.9%)	285 (45.4%)	113 (43.6%)	$p = 0.465$
IPF	189 (21.3%)	129 (20.6%)	60 (23.2%)	
DILD	154 (17.4%)	115 (18.3%)	39 (15.1%)	
CF	90 (10.1%)	62 (9.9%)	28 (10.8%)	
BE	30 (3.4%)	17 (2.7%)	13 (5.0%)	
Other causes	25 (2.8%)	19 (3.0%)	6 (2.3%)	
Concurrent steroid dose (mg) according to indication				
Per protocol	29.4 ± 3.7	29.4 ± 3.5	29.3 ± 3.7	$p = 0.754$
Decline in lung function	10.8 ± 6.9	10.2 ± 6.1	12.5 ± 8.4	$p = 0.007$
To confirm ACR resolution	21.6 ± 8.6	21.1 ± 8.2	22.8 ± 9.2	$p = 0.185$
For other reasons	10.8 ± 7.8	10.0 ± 6.6	15.0 ± 11.5	$p = 0.079$
All	19.5 ± 10.4	18.9 ± 10.4	20.9 ± 10.4	$p = 0.008$
Differential cell count in BALF				
% Neutrophils	9.04 ± 13.08	7.48 ± 11.04	12.90 ± 16.49	$p = 0.106$
% Lymphocytes	7.64 ± 7.38	6.11 ± 5.83	11.35 ± 9.25	$p < 0.001$
% Macrophages	82.92 ± 16.71	85.94 ± 14.26	75.49 ± 19.73	$p = 0.107$
% Eosinophils	0.36 ± 2.12	0.31 ± 1.97	0.48 ± 2.44	$p = 0.311$

ACR: acute cellular rejection; CF: cystic fibrosis; COPD: chronic obstructive pulmonary disease; DILD: diffuse interstitial lung disease; IPF: idiopathic pulmonary fibrosis; TBB: transbronchial biopsy; BE: bronchiectasis; BALF: bronchoalveolar lavage fluid.

ACR was detected in 260 out of 887 TBBs performed (29.31%). Rejection was considered minimal (A1) in 103 cases (39.61%), mild (A2) in 122 cases (46.92%), and moderate-severe (A3–A4) in 35 cases (13.46%). There was no suspicion of AMR in any of the samples included in the study.

No statistically significant differences were observed between the presence or absence of ACR and the indication for FBC, the underlying pathology, or the corticosteroid dose received 14 days before the procedure (Table 2).

The cellularity of BALF in the presence or absence of rejection is shown in Table 2.

The average lymphocyte count in BALF was significantly higher in patients with ACR than in those without (11.35% vs. 6.11%; $p < 0.001$). The higher the percentage of lymphocytes, the higher the risk of developing ACR and the greater its severity (Figure 1A,B), regardless of the underlying pathology, the time post-transplant, the indication for FBC, and the corticosteroid dose (OR 1.10, 95% CI 1.080–1.131).

Absolute and relative EOS counts measured at the time of the FBC were significantly higher in patients with ACR than in patients without ACR (absolute EOS: 213 ± 206/mm^3 vs. 83 ± 129/mm^3; $p < 0.001$; relative EOS: 3% ± 3.8% vs. 1.35% ± 1.8%; $p < 0.001$) regardless of the underlying pathology, the indication for FBC, the corticosteroid dose, the median EOS before BALF (median EOS for the 3 months prior to TBB, or for the last 3 blood tests when TBB was performed less than 1 month after transplant) and the time after transplantation (OR 1.460, 95% CI 1.350–1.580; OR 1.40, 95% CI 1.281–1.522). The severity of rejection was significantly associated with absolute and relative EOS counts (OR 1.38, 95% CI 1.295–1.464; OR 1.32, 95% CI 1.226–1.426, respectively) (Figure 1C,D).

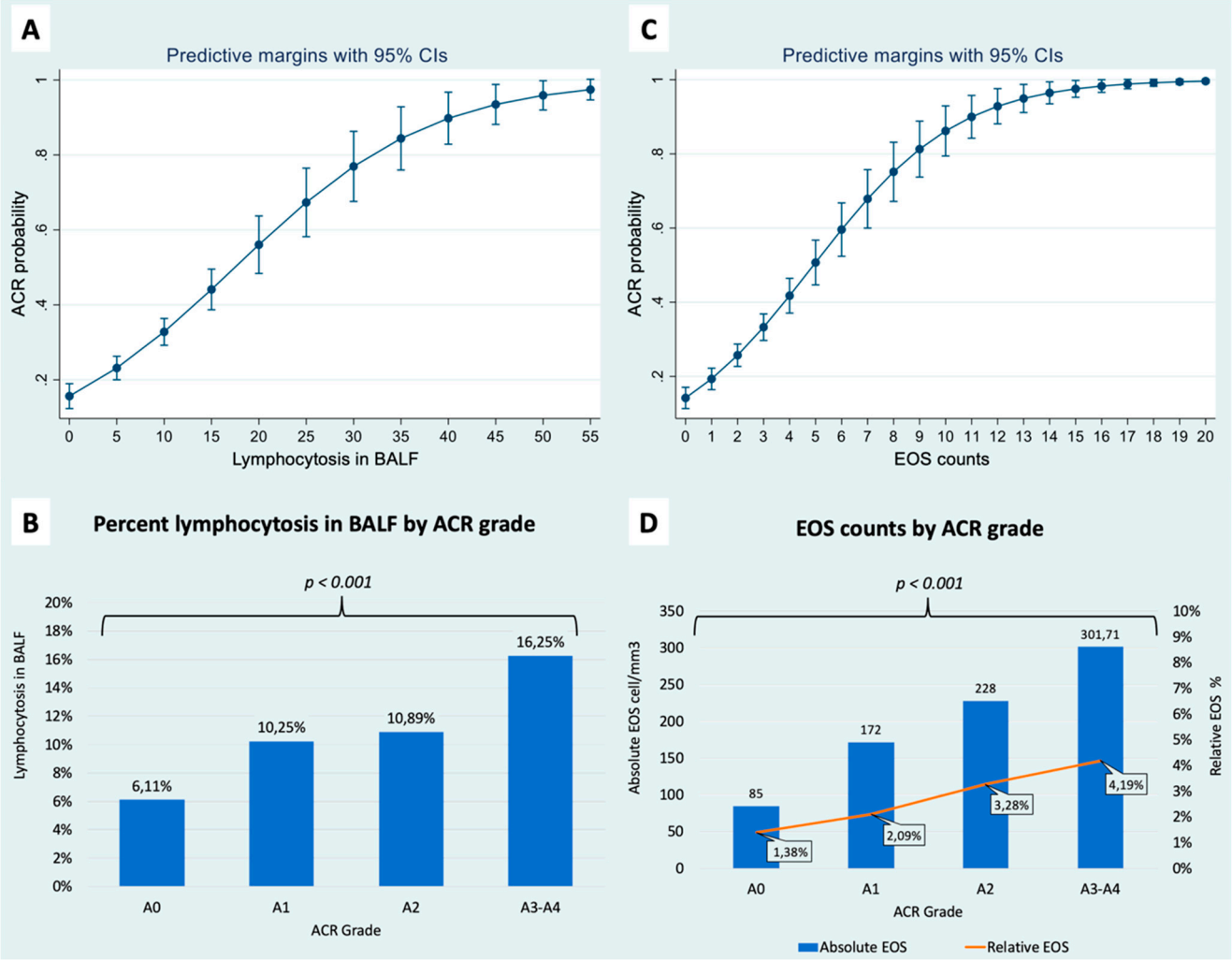

Figure 1. Lymphocytes in BALF samples and eosinophils in peripheral blood and their relationship with the presence of ACR in TBBs from lung transplant recipients. (**A**): the greater the percentage of lymphocytes, the greater the risk of presenting ACR. (**B**): higher percentages of lymphocytes in BALF specimens are associated with greater ACR severity. (**C**): higher eosinophil counts in peripheral blood related to a greater risk of presenting ACR. (**D**): severity of rejection significantly correlated to absolute and relative EOS counts (OR 1.38, 95% CI 1.295–1.464; OR 1.32, 95% CI 1.226–1.426, respectively).

The diagnostic specificity of lymphocyte counts in BALF > 12% increases from 71% (sensitivity 35%, positive predictive value 33%, negative predictive value 73%) to 96% (sensitivity 17%, positive predictive value 62%, negative predictive value 74%) if considered together with an EOS blood count > 200/mm^3.

4. Discussion

In this study, we have observed that lymphocyte counts in BALF and eosinophil counts in blood may be useful for diagnosing ACR in lung transplant recipients. Furthermore, considering both parameters together increases the diagnostic specificity for ACR and can avoid TBBs in patients who may be at risk from the procedure.

ACR is the most common form of rejection in the first months after transplantation [12]. ACR is a serious complication since it is the main risk factor for developing CLAD, in particular, bronchiolitis obliterans syndrome (BOS) [13]. Therefore, early detection and treatment of ACR are essential.

The clinical detection of ACR can be difficult because it can be asymptomatic, present non-specific symptoms or imitate other frequent complications in these patients, such as infections [14].

A decline in lung function, in particular the FEV1, indicates the development of complications in the graft [15,16]. A ≥10% decline of FEV1 with respect to the baseline value lasting more than 48 h indicates allograft dysfunction and the need for new investigations. However, the diagnostic specificity of lung function tests for ACR is low, and, on the other hand, stable lung function does not exclude the presence of underlying ACR, lung infection, or altered bronchial anastomosis.

Imaging techniques such as chest X-rays or high-resolution computerized tomography also have low sensitivity and specificity for ACR diagnosis.

Currently, TBB is the gold standard for differentiating ACR from other common complications in these patients. However, its diagnostic sensitivity for ACR is only 70% and depends largely on the indication for performing the procedure and the experience of the pathologist [2]. It is also an invasive technique that is not without risk, such as reduced SaO_2 (10.5%), bleeding of more than 100 mL (4%), or pneumothorax (0–2.5%) [14].

BALF is a less invasive exploration and presents a lower risk of complications than TBB. The biochemical and cellular analysis of BALF can provide insight into the inflammatory microenvironment of the lung parenchyma that can help us differentiate between ACR, infections, and other complications [3].

In 1975, Achterrath et al. [17] described the cellular profile of BALF during ACR episodes in animal recipients. Since then, other researchers have examined cellular changes in BALF samples of human patients with ACR. A meta-analysis of related published studies showed that a neutrophil count ≥ 12% in BALF should be considered an indication of ACR, with a specificity of 82% and a sensitivity of 74%. This meta-analysis also showed that high BALF lymphocyte counts are associated with ACR with acceptable specificity but with low sensitivity [18].

In our study, in line with what had been previously described by De Hoyos et al. [19], we observed that a lymphocyte count in BALF samples > 12% shows high specificity for ACR diagnosis and, therefore, can support starting therapy in the absence of TBB or with inconclusive histological findings. However, its low sensitivity, also described in De Hoyos' study, does not rule out the presence of ACR, and we should therefore repeat the TBB or look for other diagnostic alternatives in patients who are unresponsive to empirical treatment. As other authors have described [20], we observed that the BALF eosinophil counts did not significantly discriminate among patients with or without ACR. This fact is probably related to the fact that most of the rejections that we have diagnosed are mild (A1) or moderate (A2). Tissue infiltration by EOS is mainly observed in severe rejections (A3 and A4), and BAL is a reflection of the alveolar compartment.

There is growing interest in the identification of biomarkers in blood that can help with the early identification of patients with rejection [7,21]. Several studies have observed the potential usefulness of assessing some cytokines, chemokines, glycoproteins (KL6), exosomes, and mRNA and donor-derived DNA fragments. However, the cost and complexity of many of these techniques have greatly limited their implementation in routine clinical practice.

Haemograms are determined periodically and routinely in the monitoring of lung transplant recipients. In the absence of CD8+ T cell activity as a result of immunosuppression, alternative alloimmune responses could be Th2 type mediated through eosinophil and IL-5 pathways [22]. Several studies have observed that EOS could be involved in graft rejection via a Th2-type immune response and that EOS counts could be an early and specific marker for rejection [8,23,24]. The relationship between the increased blood EOS count and graft rejection was initially recognized in kidney and liver transplant recipients [25–28].

The role of EOS counts in predicting acute rejection in lung transplant recipients is controversial, and there are few studies on the issue. Trull et al. [29] described an association between EOS counts > 140 and ACR. We have previously described those patients with

ACR showed significantly higher EOS counts than patients without ACR (203.6 $\pm$ 248/mm^3 vs. 103.1 $\pm$ 153/mm^3; $p < 0.001$), and that the grade of ACR was higher in patients with higher EOS counts [9]. In this previous study, we calculated the AUROC to know the EOS cut-off point. Only if the AUROC was >0.7, we applied the Liu method to find an optimal cut-off point. Liu's method maximizes the product of sensitivity and specificity. The optimal cut-off point applying Liu's method in these samples was 195/mm^3 (sensitivity 46% and specificity 90%); 0.85% (sensitivity 73% and specificity 59%).

In contrast to what has been observed in kidney and liver transplant recipients, EOS counts in our patients with or without rejection were within normal limits. This fact is probably related to the higher levels of immunosuppression in lung transplant recipients.

Monitoring EOS count evolution in a specific patient may be useful in making the decision whether to perform FBC with TBB and BALF or not. Likewise, when recipients have limitations with regard to performing TBB or when histological specimens are insufficient or inconclusive, lymphocyte counts in BALF can support the start of empirical treatment for ACR. In our study, the diagnostic specificity for ACR of a lymphocyte count > 12% increased up to 95.8% when taking into account simultaneous EOS counts higher than 200/mm^3.

There are no previous studies relating the usefulness of the combination of these two markers for ACR diagnosis, but its high specificity may allow starting empirical therapy and assessing the response before repeating TBB or considering other more aggressive diagnostic tools. Our results could be taken into consideration to produce a score that helps perform FBC when suspecting ACR, as well as to start empirical treatment if the TBB results are inconclusive. However, new prospective studies are necessary to validate its usefulness.

The main limitation of our study is its retrospective nature and that it was carried out at a single center. Additionally, by using lymphocyte counts only at the time TBB was performed, we have not taken into account their variability over time for each patient.

5. Conclusions

Simultaneous assessment of BALF lymphocyte counts and peripheral blood eosinophil counts may be useful for diagnosing ACR in lung transplant recipients. The joint consideration of both parameters could be particularly useful in the presence of inconclusive TBB histology samples or in patients with contraindications for TBB.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are available upon request from the authors.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviation

ACR	Acute cellular rejection
LTx	Lung transplant patients
ISHLT	International Society for Heart and Lung Transplantation
CLAD	Chronic lung allograft dysfunction
TBB	Transbronchial biopsy
BALF	Bronchoalveolar lavage fluid
EOS	Eosinophil counts in blood
FEV1	Forced expiratory volume in 1 s
FBC	Fiber-optic-bronchoscopy
COPD	Chronic obstructive pulmonary disease
IPF	Idiopathic pulmonary fibrosis
DILD	Diffuse interstitial lung disease
CF	Cystic fibrosis
BOS	Bronchiolitis obliterans syndrome
AMR	Antibody-mediated rejection

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