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ORIGINAL ARTICLE

Epithelial cell death markers in bronchoalveolar lavage correlate with chronic lung allograft dysfunction subtypes and survival in lung transplant recipients—a single-center retrospective cohort study

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SUMMARY

Chronic lung allograft dysfunction (CLAD) remains the leading cause of late death after lung transplantation. Epithelial injury is thought to be a key event in the pathogenesis of CLAD. M30 and M65 are fragments of cytokeratin-18 released specifically during epithelial cell apoptosis and total cell death, respectively. We investigated whether M30 and M65 levels in bronchoalveolar lavage (BAL) correlate with CLAD subtypes: restrictive allograft syndrome (RAS) versus bronchiolitis obliterans syndrome (BOS). BALs were obtained from 26 patients with established CLAD (10 RAS, 16 BOS) and 19 long-term CLAD-free controls. Samples with concurrent infection or acute rejection were excluded. Protein levels were measured by ELISA. Variables were compared using Kruskal–Wallis, Mann–Whitney *U* test and Chi-squared tests. Association of M30 and M65 levels with post-CLAD survival was assessed using a Cox PH models. M65 levels were significantly higher in RAS compared to BOS and long-term CLAD-free controls and correlated with worse post-CLAD survival. Lung epithelial cell death is enhanced in patients with RAS. Detection of BAL M65 may be used to differentiate CLAD subtypes and as a prognostic marker in patients with established CLAD. Understanding the role of epithelial cell death in CLAD pathogenesis may help identify new therapeutic targets to improve outcome.

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Key words
bronchoalveolar lavage, epithelial cell death, lung transplant, M30, M65, survival

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Introduction

Lung transplantation has become an important approach in the treatment of end-stage respiratory diseases with more than 4000 transplant procedures reported to the Registry of the International Society for Heart and Lung Transplantation in 2017 [1]. However, survival after lung transplant remains limited primarily due to chronic

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lung allograft dysfunction (CLAD) [1]. Several subtypes of CLAD have been identified, including the long-recognized bronchiolitis obliterans syndrome (BOS) and the more recently described restrictive allograft syndrome (RAS) [2,3]. The pathogenesis of CLAD and its subtypes has been an active area of investigation. Various mechanisms involving both innate and adaptive immune responses and aberrant remodeling and repair processes have been proposed to play a role; however, the exact pathophysiology has yet to be elucidated [4].

The adult human bronchial tree is covered with a continuous layer of epithelial cells that play a critical role in maintaining the conduit for air, and which are central to the defenses of the lung [5]. Beyond providing a key physical barrier, there is a growing body of evidence that epithelial cells at mucosal surfaces play an important role in innate and adaptive immune responses [6] as well as in tissue repair and remodeling. After injury, the lung epithelium may either activate the necessary repair and regeneration pathways for proper repopulation of lost epithelial cells or undergo an aberrant remodeling and differentiation process, which represents the final common pathway for many types of pulmonary diseases [7]. Several lung diseases have been described as involving defective epithelial–mesenchymal interactions, leading to dysfunctional wound healing and abnormal lung remodeling and fibrosis [8–10].

Chronic epithelial injury has been proposed as one of the key mechanisms of CLAD pathogenesis [11–15]. Evidence from animal models suggests that failure of re-epithelialization of the injured airway epithelium may induce an exaggerated fibroblastic response ultimately leading to bronchiolitis obliterans [16]. Markers of epithelial-to-mesenchymal cell transition have been measured in clinically stable lung transplant recipients [14], suggesting that pro-fibrotic processes are activated at baseline, although this remains controversial [15]. The exact role of epithelial cell injury and death in CLAD-related tissue remodeling and fibrosis remains poorly understood.

Epithelial cell death *in vivo* can be detected when epithelial cells release specific intracellular proteins. The cytokeratins are a group of cytoskeleton proteins that maintain the internal organization and dynamic processes of the cell [17]. During necrosis, pulmonary epithelial cells release full-length cytokeratin-18 (CK18), whereas cleaved CK18 is the product of epithelial cell apoptosis (Fig. 1). Detection of M30, a neoepitope exposed only on cleaved CK18 fragments, reflects epithelial cell apoptosis, whereas M65 reflects both caspase-cleaved CK18 and intact CK18, the latter of which is released from cells undergoing necrosis. These markers have previously been detected in the bronchoalveolar lavage (BAL) in Acute Respiratory Distress Syndrome [18], and their presence in the peripheral circulation correlates with lung transplant outcomes [19]. However, M30 and M65 have not been measured in the BAL of lung transplant recipients, and their relationship to CLAD has not been assessed.

This study aimed to evaluate the potential association of BAL markers of epithelial cell death with CLAD status and subtypes (RAS versus BOS), as well as survival after CLAD onset. We specifically measured concentrations of M30 and M65 to detect the modes of epithelial cell death.

Materials and methods

Study design

We conducted a single-center retrospective cohort study based on prospectively collected BAL samples with all relevant clinical data obtained through medical record review. The study was approved by the University Health Network Research Ethics Board. Written consent for the use of excess BAL was obtained from each patient.

CLAD definition and subtype classification

For this study, a minimum of four pulmonary function tests (PFT), including forced expiratory volume in first second (FEV1), forced vital capacity and total lung capacity (TLC) measurements, as well as a chest computed tomography (CT) obtained within 6 months of CLAD onset, were required for defining CLAD status and CLAD subtypes (RAS versus BOS). CLAD, as well as BOS versus RAS status, was retrospectively determined by two clinicians. CLAD was defined as a sustained, greater than or equal to 20% decline in FEV_1, as compared to the average of the two best post-transplant FEV_1 measured at least 3 weeks apart, in the absence of other clinical confounders [2]. RAS was considered in CLAD patients with a restrictive ventilatory defect with an irreversible decline in TLC below 90% of baseline TLC (baseline TLC was defined as the average of the two measurements obtained at the same time as the best two FEV_1 measurements). The diagnosis of RAS was confirmed if this restrictive ventilatory defect was deemed to be a result of a pulmonary process based on radiological findings such as pleural thickening or parenchymal fibrosis. All patients

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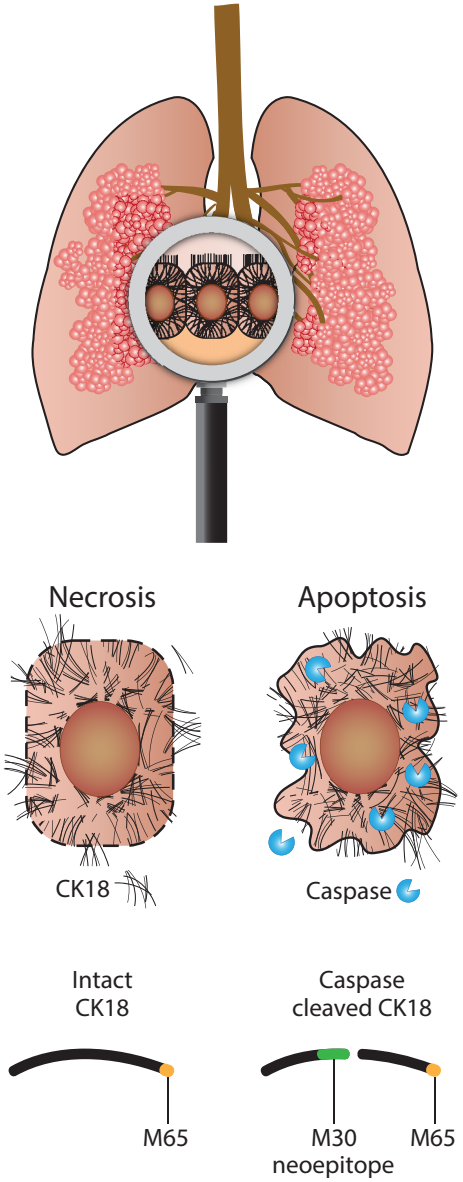

Figure 1 Intact and caspase-cleaved cytokeratin-18 (CK18) serve as epithelial cell death markers. Apoptosis is a process of cellular suicide and represents a physiological controlled form of cell death whereas necrosis, an uncontrolled mode of death, occurs in response to various insults and results in premature cell death. CK18 (black lines) is a part of the epithelial cell cytoskeleton. During apoptosis, CK18 is cleaved by caspase (blue shape) and the released caspase-cleaved fragments can be measured based on the exposed M30 neoepitope (green end) by ELISA. The M65 ELISA detects both caspase-cleaved and intact CK18 that are released from the necrotic cells (yellow end); therefore, M65 level represents both apoptosis and necrosis (total cell death) whereas M30 level denotes apoptosis only.

with CLAD who did not meet criteria for RAS were classified as BOS. Long-term CLAD-free controls were defined as patients who were alive and CLAD-free for at least 4 years after transplant.

Patient and sample selection

The study population was drawn from all first, adult, bilateral lung transplant operations. Patients with definable RAS and BOS, as outlined in the previous paragraph, were eligible for inclusion if they had a post-CLAD onset BAL sample obtained between August 2010 and May 2016, with available excess BAL fluid stored in our biobank (Biobanking at our center began in 2010). Routinely, scheduled surveillance bronchoscopies with BAL and transbronchial biopsies (TBB) collection, are carried out in our program at 0.5, 1.5, 3, 6, 9, 12, 18-, and 24-months post-transplant. Additional diagnostic bronchoscopies are performed if deemed clinically indicated. The current CLAD cohort was limited by sample availability because post-CLAD onset BALs are not routinely collected and were not systematically biobanked over the years. Additionally, BAL samples with evidence of concurrent infection or acute rejection were excluded from this study to avoid potential confounding by these processes. Infection was defined by the presence of pathogenic bacterial, fungal, or viral organisms in the BAL or TBB from the same bronchoscopy (irrespective of symptoms or treatment). Acute rejection was determined based on the presence of a mononuclear infiltrate on a concurrent TBB, graded per ISHLT consensus guidelines [20]. CLAD-free stable controls were eligible if they had an infection- and rejection-free BAL available in the biobank obtained close to 2 years post-transplant (i.e., the last surveillance bronchoscopy). Patients were followed to death, retransplant or the time of data censoring on March 30, 2017.

BAL collection and sample processing

Post-transplant monitoring was performed in accordance with the Toronto Lung Transplant Program protocol as described previously [21,22]. Bronchoscopies were performed via the oropharyngeal route under conscious sedation. The bronchoscope was placed in a wedged position within the targeted segment; when no particular location was targeted, BAL sampling was conducted in the right middle lobe or lingula of the lung allograft, as suggested by the ATS/ERS guidelines [23]. In the case of localized disease processes, the targeted segment was chosen based on radiological examination or visual inspection. After achieving a wedged position with the bronchoscope, 50 ml of normal saline was instilled and then suctioned twice while maintaining the wedged position. After sending BAL fluid for clinical

microbiologic and cytologic analysis, excess fluid was centrifuged for 20 min at 3184G at 4 °C and supernatant was carefully transferred into separate tubes and stored at −80 °C until analysis.

Measurement of BAL protein expressions

For the quantitative determination of total CK18 and caspase-generated neoepitope of CK18, we used the M65® enzyme-linked immunosorbent assay (ELISA) and M30-Apoptosense® ELISA assays respectively (Peviva AB, Bromma, Sweden) according to the manufacturer's instructions. The M65® ELISA measures total CK18 (both cleaved and uncleaved) and represent overall cell death due to both apoptosis and necrosis. The M30-Apoptosense® ELISA measures the caspase-generated neoepitope of CK18, representing cell death due specifically to apoptosis (Fig. 1). BAL concentrations of the antigens in each sample were interpolated from a calibration curve using GRAPHPAD PRISM version 7.00 for Windows (GraphPad Software Inc., La Jolla CA, USA).

Qualitative BAL cytology analyses

To evaluate the correlation between M30 and M65 and concurrent lung inflammation, we assessed BAL neutrophils based on BAL cytology reports obtained at the time of bronchoscopies (our center does not perform quantitative BAL cell differentials). In the clinical cytopathology laboratory, BAL smears were obtained and Papanicolaou stained before being assessed by a trained cytopathologist to identify the cellular profiles. Samples were classified as *acute inflammation* when neutrophils represented >3% of all cells, in accordance with published guidelines [23]. M30 and M65 levels were compared between patients with acute inflammation and without.

Statistical analysis

Baseline characteristics were compared using Kruskal–Wallis tests for continuous variables. The Mann–Whitney *U* test was applied for statistical evaluation of differences between two groups. Comparison of proportions was made using the Chi-squared. Univariable and multivariable linear models were used to examine the association of levels of BAL proteins with CLAD status and subtype. M65 was natural-log transformed to improve normality. As a sensitivity analysis, we restricted the analysis to subjects who had their BAL obtained within 6 months after CLAD onset. To test the discriminatory power of BAL proteins that emerged as associated with CLAD status and subtypes, we performed the receiver operating characteristic (ROC) curve analysis.

The association of M30 and M65 levels with survival post-CLAD onset was assessed only in subjects where BAL was obtained within 6 months after CLAD onset using univariable and multivariable Cox PH models. Hazard Ratios (HR) per one standard deviation increase in M30 and M65 were reported. For illustration purposes, Kaplan–Meier survival curves with M65 categorized by quartiles, with corresponding log-rank tests were provided. To confirm our findings, we performed the same survival analysis using time of BAL as the reference time. A secondary analysis examined the association of BAL protein levels with postbronchoscopy survival in long-term CLAD-free patients using Cox PH models.

Statistical significance was defined as a two-tailed *P* value less than or equal to 0.05. All analyses were conducted using R statistical software version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient characteristics

Twenty-six CLAD (10 RAS and 16 BOS) and 19 long-term CLAD-free controls were included in the study. Baseline characteristics are detailed in Table 1. Median age (years) was 50.5 (43, 55.5), 48.5 (35.5, 53.8), and 59 (46.5, 63.5), for RAS, BOS, and long-term CLAD-free, respectively. The majority of patients were male: 60%, 62.5%, and 57.9% in the RAS, BOS, and long-term CLAD-free groups, respectively. Pulmonary fibrosis was the leading diagnosis in BOS (50%), RAS (20%), and long-term CLAD-free (42.1%) groups. Except for 2 (10.5%) long-term CLAD-free patients who underwent a heart-lung transplant, all others had a bilateral lung transplant. CMV mismatch (D+/R−) incidence was highest in RAS patients (60%), compared to BOS (18.8%) and long-term CLAD-free patients (10.5%). The median (IQR) time in days from transplant to CLAD onset was longer for BOS 592.5 (460, 1130.3) compared to RAS 497 (383.8, 969.5) patients. The median (IQR) time in days from transplant date to bronchoscopy was 882 (389, 1476), 734 (555, 1408.5), and 734 (726.5, 742.5), for the RAS, BOS, and long-term CLAD-free patients, respectively. Only donor-recipient CMV status emerged as significantly different between groups ($P = 0.01$).

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Table 1. Baseline patient characteristics.

	CLAD-free (*n* = 19)	BOS (*n* = 16)	RAS (*n* = 10)	*P* value
Recipient age at transplant, years, median (IQR)	59 (46.5, 63.5)	48.5 (35.5, 53.8)	50.5 (43, 55.5)	0.09
Male sex, *n* (%)	11 (57.9)	10 (62.5)	6 (60)	0.96
Donor-recipient sex mismatch (%)	6 (31.6)	2 (12.5)	2 (20)	0.38
Native lung disease, *n* (%)				
Pulmonary fibrosis	8 (42.1)	8 (50)	2 (20)	0.46
COPD	4 (21.1)	3 (18.8)	1 (10)	
Cystic fibrosis	2 (10.5)	3 (18.8)	2 (20)	
Other	5 (26.3)	2 (12.5)	5 (50)	
CMV serology donor (D)/recipient (R), *n* (%)				
D+/R−	2 (10.5)	3 (18.8)	6 (60)	0.01
D+/R+, D−/R+	13 (68.4)	7 (43.8)	3 (30)	
D−/R−	4 (21.1)	6 (37.5)	1 (10)	
Time (days) from transplant to bronchoscopy, median (IQR)	734 (726.5, 742.5)	734 (555, 1408.5)	882 (389, 1476)	0.97
Time (days) from transplant to CLAD onset, median (IQR)	N/A	592.5 (460, 1130.3)	497 (383.8, 969.5)	0.29
Time (days) from CLAD onset to bronchoscopy, median (IQR)	N/A	98.5 (33.5, 187.5)	134.5 (26.5, 710.5)	0.65
Baseline FEV1, median (IQR)	2.8 (2.6, 3.4)	2.6 (2.3, 3.3)	3.0 (2.5, 3.3)	0.62

BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory value in one-second; N/A, not applicable.

P values determined using chi-squared and Kruskal–Wallis tests. Baseline FEV1 defined as the average of the two highest post-transplant FEV1 values at least 3 weeks apart.

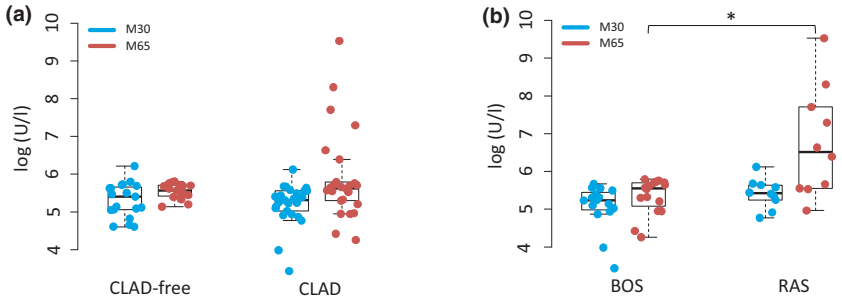

Figure 2 M30 and M65 levels do not differ between patients with chronic lung allograft dysfunction (CLAD) versus CLAD-free but are significantly elevated in restrictive allograft syndrome (RAS) compared with bronchiolitis obliterans syndrome (BOS). Total epithelial cell death and epithelial cell apoptosis were measured in the bronchoalveolar lavage fluid of 16 BOS, 10 RAS, and 19 CLAD-free patients using the M65 (measures necrosis plus apoptosis) and M30 (measures apoptosis) enzyme-linked immunosorbent assay. There were no significant differences in the levels of either M30 ($P = 0.48$) or M65 ($P = 0.24$) between patients with CLAD versus CLAD-free stable controls (a). Among the patients with CLAD, M65 levels were elevated in RAS compared to BOS ($P = 0.001$) (b). Linear models were used to examine the association of levels of bronchoalveolar lavage proteins with CLAD status and subtypes. M65 was natural-log transformed to improve normality.

M65 levels were significantly higher in patients with RAS

There were no statistically significant differences in the levels of either M30 ($P = 0.48$) or M65 ($P = 0.24$) between all patients who developed CLAD versus long-term CLAD-free stable controls (Fig. 2a). Among CLAD patients, BOS patients had an average M30 level of 181.1 ± 70.9 U/l, while in RAS patients, levels were on average 58.9 U/l higher ($P = 0.083$). In addition, BOS patients had an average M65 level of 227.8 ± 83.9 U/l, while RAS patients' levels were higher on average by 312% ($P = 0.001$; Fig. 2b). The results

did not change after adjusting the models separately for age, sex, native lung disease, or CMV mismatch status (data not shown). ROC analysis showed an area under the curve (AUC) of 0.79 (95% CI, 0.59–0.99) and produced an optimal (Youden index-based) M65 cutoff level of 596.4 U/l for discriminating RAS from BOS (Fig. S1).

We performed an additional subanalysis, using only samples that were obtained within 6 months after CLAD onset, with the rationale that these samples may better capture processes that are ongoing during early CLAD evolution. Six RAS and 13 BOS patients were included in the subanalysis. Both M30 and M65 were significantly elevated in RAS compared to BOS. BOS patients had an average M30 level of 172.3 ± 72.5 U/l and M65 level of 219.8 ± 88.2 U/l. M30 was on average 92.1 U/l higher ($P = 0.045$) while M65 was higher on average by 334% ($P = 0.002$) in RAS patients.

M65 & M30 levels were not associated with BAL neutrophilia

Bronchoalveolar lavage M30 and M65 levels did not differ between samples with acute inflammation compared to BAL samples without acute inflammation ($P = 0.67$ and 0.63, respectively; Fig. S2).

M65 level was associated with worse survival among patients with CLAD

We set off to assess the correlation between M30 and M65 levels and survival using only the CLAD patients whose BAL was obtained within 6 months after CLAD onset. Within this subset of patients, at the median patient follow-up time of 1.8 years after CLAD onset, 80% (4/5) of the patients with the highest 25% of M65 levels (>312.2 IU) had died as opposed to 35.7% (5/14) of the patients in the lower 75 percentiles of M65 levels (Fig. 3). In a univariable Cox PH model analysis, a higher level of M65 was associated with shorter post-CLAD survival [HR 2.37, 95% CI (1.27, 4.43)]. The association remained significant after adjusting the models separately for age, sex, native lung disease, or CMV mismatch status. In the same setting, M30 levels were not significantly associated with post-CLAD survival [HR 1.74, CI (0.92, 3.29)]. Neither M30 [HR 1.29; CI (0.70, 2.38)] nor M65 [HR 1.49; CI (0.74, 3.0)] levels were associated with survival post-CLAD independent of CLAD subtype (i.e., after accounting for CLAD subtype in the multivariable Cox PH model). In a secondary analysis where we looked at postbronchoscopy (as opposed to post-CLAD onset) survival in CLAD patients, M65 was associated with higher risk of death while M30 was not [M65: HR 1.69, CI (1.11, 2.58), M30: HR 1.41, CI (0.78–2.54)]. Neither of the two BAL proteins was associated with postbronchoscopy survival in long-term CLAD-free patients (data not shown).

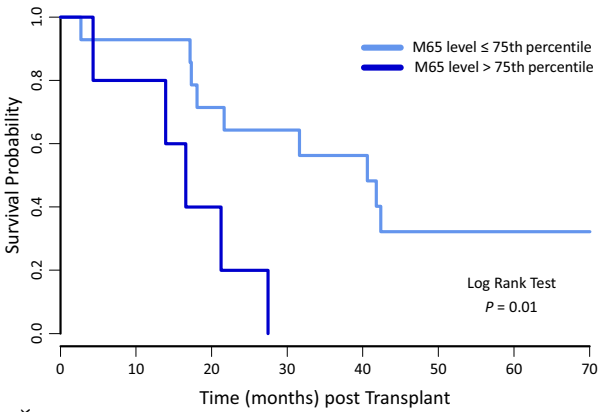

Figure 3 Kaplan–Meier survival curves demonstrating reduced survival for patients with higher M65 (≥312.2 IU). A log-rank test of the null hypothesis of equal survival distributions in both groups was rejected ($P = 0.01$). CLAD, chronic lung allograft dysfunction.

Discussion

This study suggests that higher BAL M30 and M65, representing apoptosis and apoptosis plus necrosis, respectively, measured after CLAD onset, are associated with the RAS phenotype. Furthermore, BAL M65 levels correlate with post-CLAD onset survival and may be useful as a prognostic factor for patients with established CLAD. Based on our findings, in patients with CLAD, one standard deviation rise in BAL M65 level results in a 137% increased risk of death. Lung allograft epithelial cell death may be one of the key differential mechanistic elements driving CLAD subtypes.

Previous data have shown epithelial cell necrosis and apoptosis to be essential factors in the pathogenesis of various disease states, such as in acute liver injury [24] or cancer [25–27]. Bidirectional interactions between epithelial cells, fibroblasts, and interactions with matrix were also shown to play an important role in Idiopathic Pulmonary Fibrosis (IPF) pathogenesis [28,29]. Lung epithelial cell death is thought to be a crucial driver in the development of the disease [29,30] with increased numbers of apoptotic and necrotic cells observed in both the alveolar and bronchial epithelia of IPF patients [31]. Our finding of elevated BAL cell death in RAS

patients is especially interesting since RAS may involve mechanism similar to IPF as the two exhibit progressive parenchymal fibrosis, although the specific histologic patterns differ.

Several pathological conditions in lung transplantation have also been linked to epithelial cell injury. Primary graft dysfunction (PGD) was shown to be associated with lung epithelial injury [32], and death of epithelial cells was shown to be associated with mortality in patients with PGD [19]. Similarly, to our observation, Hashimoto *et al.* were able to show that recipient plasma concentrations of the epithelial cell death markers M30 and M65 at 24 and 48 h after lung transplantation are negatively correlated with early graft performance and long-term survival [19]. With respect to the long-term complications after lung transplantation, the epithelial response to injury through repair, necrosis, and apoptosis is known to be a pivotal event in airway remodeling which characterizes CLAD [11,13,15]. In a study by Kelly *et al.* [12], CLAD was shown to involve a selective alteration in the distribution and function of bronchiolar Club cell secretory protein (CCSP), a marker for epithelial secretory cells which demonstrate anti-inflammatory properties and play a role in the repair of injured epithelial cells. These findings confirmed previously published data revealing that CCSP detection was not only reduced at the time of CLAD but that lung transplant recipients who later developed CLAD had lower BAL CCSP levels as early as 1 month post-transplantation [33]. Altogether, this suggests that several mechanisms may be involved in epithelial cell loss in CLAD development. Although BAL neutrophils may play a role in epithelial cell dysfunction and injury, we were not able to show an association between BAL neutrophilia and levels of M30 or M65 in this study. It is likely that lung inflammation at an earlier stage of CLAD pathogenesis is related to epithelial impairment, but this will require further assessment. This mechanistic complexity may explain our somewhat counter intuitive finding of higher epithelial cell death in RAS while airway involvement is a key feature in the pathogenesis of BOS. Whether differential allograft epithelial cell injury is associated with specific CLAD subtypes and whether epithelial cell death is an initiating event in the pathogenesis of RAS remains to be explored.

Our findings have several potential clinical implications. The current diagnosis of CLAD and its subtypes is a challenging task given that it is based solely on clinical data [2]. Identifying CLAD subtypes is of paramount importance as RAS portends a significantly worse outcome and accurate subtyping could facilitate targeted treatments. Incorporating additional biological variables into estimating CLAD phenotypes and prognosis would add additional objectivity and options for patients who do not have sufficient clinical information, such as PFTs, for assessment. Our results suggest that BAL epithelial cell death biomarkers, in particular, M65, could potentially improve the diagnostic accuracy of CLAD and help distinguish BOS from RAS [34] in the absence of adequate lung volume measurements. Similar to our observations, other BAL proteins have been described recently as having a distinct expression pattern in RAS compared to BOS, with upregulation of innate immune and injury markers [35–37]. Assessing multivariable biomarker signatures will be an important focus in future research.

In this study, with a very distinct RAS population, clearly characterized by full PFT and radiology data, we found that M65 was predictive of survival post-CLAD onset. However, this prediction ability disappeared when adjusted for the RAS phenotype. We postulate that M65 may be as a useful predictor of survival in lung transplant recipients where RAS (and possibly CLAD) cannot be clearly defined. The next step would be to validate these findings in a larger cohort that includes a more diverse CLAD population.

Furthermore, understanding the role of epithelial cell death in CLAD pathogenesis will be important to further advance research of a disease with scant therapeutic options. Since both apoptosis and necrosis are regulated modes of cell death, unraveling the signaling cascades contributing to cell death may permit the development of tools that specifically interfere with the process at predicted levels of signaling [38]. Targeting negative regulators of cell death signaling pathways is currently an active area of interest in cancer research [39].

The current analyses should be interpreted in light of some limitations. First, this is a small, single-center study, which limits its generalizability. Second, since post-CLAD bronchoscopies are not routinely performed or systematically biobanked at our center, a selection bias could not be excluded. The CLAD arm consisted of samples from patients who developed CLAD within the first 2 years after transplant (during which period routine surveillance bronchoscopies are carried out) as well as patients who developed CLAD later and had a specific clinical indication for BAL collection. Additionally, the highly selected patient population was based on a prerequisite requirement for adequate PFTs including TLC measurements, available chest CT, and exclusion of BALs with concurrent infection or acute rejection. These stringent selection criteria may have introduced further bias. To address this limitation, a validation study using

a more comprehensive and contemporaneous cohort is in order. Third, the cross-sectional study design of our analysis did not allow for assessment of longitudinal changes in epithelial death markers. Longitudinal cohort studies will be required to assess the changes in epithelial cell death throughout the allograft's life. Fourth, sampling times in CLAD and long-term CLAD-free controls were not equal, as all CLAD-free controls were sampled around 2-years post-transplant, whereas sampling of BOS and RAS had higher variability. This is an inherent bias that reflects real-life practice at our center: stable CLAD-free patients do not undergo surveillance bronchoscopies beyond 2 years post-transplant, whereas deteriorating patients with BOS or RAS will often have a bronchoscopy as part of their assessment.

In conclusion, detection of BAL M30 and M65 as a marker of epithelial cell death may be useful in differentiating CLAD subtypes and may predict survival in patients with CLAD. Understanding the role of epithelial cell death in CLAD pathogenesis may promote efforts toward personalized treatments to further improve outcome after lung transplant.

Authorship

TM: conceived the research concept, supervised the project, and made critical revisions to the manuscript. LL: conceived the research concept and strategies, designed and supervised the study, analyzed the data, and wrote the manuscript. MS, SK, SJ, and JT: contributed to the conception and design of the research, made critical revisions to the manuscript and approved it prior to publication. MS, KB, and BJ: performed the experiments described in Fig. 2, analyzed the data and contributed to the final version of the manuscript. TS and AT: performed all data collection, biobanking and made revisions to the final manuscript. EH and NM: performed all statistical analyses and made critical revisions to the manuscript. All the authors discussed the results and implications, commented on the manuscript at all stages, and reviewed the final version prior to submission.

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Conflicts of interest

The authors of this manuscript have no conflicts of interest to disclose as described by the Journal.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Receiver operating characteristic (ROC) curve for M65 (cutoff 596.4 U/l).

Figure S2. M65 & M30 levels were not associated with bronchoalveolar lavage neutrophilia.

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