Bronchoalveolar lavage cytokines are of minor value to diagnose complications following lung transplantation

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ABSTRACT

Early diagnosis and treatment of acute cellular rejection (ACR) may improve long-term outcome for lung transplant recipients (LTRs). Cytokines have become valuable diagnostic tools in many medical fields. The role of bronchoalveolar lavage (BAL) cytokines is of unknown value to diagnose ACR and distinguish rejection from infection. We hypothesized that distinct cytokine patterns obtained by surveillance bronchoscopies during the first year after transplantation are associated with ACR and microbiologic findings.

We retrospectively analyzed data from 319 patients undergoing lung transplantation at University Hospital Zurich from 1998 to 2016. We compared levels of IL-6, IL-8, IFN-γ and TNF-α in 747 BAL samples with transbronchial biopsies (TBB) and microbiologic results from surveillance bronchoscopies. We aimed to define reference values that would allow distinction between four specific groups "ACR", "infection", "combined ACR and infection" and "no pathologic process". No definitive pattern was identified. Given the overlap between groups, these four cytokines are not suitable diagnostic markers for ACR or infection after lung transplantation.

1. Introduction

Lung transplantation is an established treatment option for selected patients with end-stage lung disease. Chronic lung allograft dysfunction (CLAD) and infections are the main factors limiting long-term survival in lung transplant recipients (LTRs) [1]. Acute cellular rejection (ACR) is a potential risk factor for the development of CLAD [2–5]. During the first postoperative year, ACR affects 28% of LTRs at least once, necessitating treatment with steroid augmentation [1,6]. Symptoms of ACR are nonspecific, including dyspnea, cough, sputum production, fever and/or hypoxia [7,8]. Non-invasive tests like pulmonary function testing and chest imaging are useful indicators for potential complications, but have no discriminatory value between ACR and infection [9]. For these reasons, transbronchial biopsies (TBB) remain the gold standard for the diagnosis of ACR [10]. However, TBB are invasive and bear

potential risks such as pneumothorax or bleeding [11,12]. Moreover, TBB are prone to sampling error and inter-observer variability [13–15]. The clinical and prognostic role of grade A1 ACR remains unclear. Depending on clinical management guidelines and practice standards at different transplant centers, a finding of grade A1 ACR might be ignored, prompt repeat biopsy or might result in augmented immunosuppression [11,16,17].

Immense work in multiple laboratories worldwide is currently under way to determine the potential role of cytokines in diagnosis, treatment and monitoring disease progression in various fields such as heart failure, neuro-degeneration and gastrointestinal diseases. Cytokine production by BAL T lymphocytes and mast cells has been shown to be part of pro- and anti-inflammatory processes leading to airflow limitation and exacerbations of obstructive lung disease [18,19]. Cytokines are furthermore implicated in rejection after organ

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transplantation and induction of fibrotic pathways [20].

ACR is driven by T cell recognition of foreign major histocompatibility complexes [8,21]. Cytokines play a key role in this process by stimulating proliferation, chemotaxis and activation of cytotoxic T lymphocytes, neutrophils and alveolar macrophages AM [22–24]. We have recently reviewed the potential role of surrogate markers such as cytology and cytokines in bronchoalveolar lavage (BAL) and plasma samples [25,26]. Analyzing cytokines in BAL may provide information on allograft status, a potentially useful diagnostic tool. Advances in detection of biomarkers are urgently needed to identify ACR and reliably predict increased risk for the development of CLAD [27].

In this retrospective single-center study, we analyzed a panel of pro-inflammatory cytokines, including interleukin (IL-)6, IL-8, interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) in a large cohort of LTRs. The aim of this study was to correlate cytokine levels in BAL fluid of surveillance bronchoscopies with the development of complications including ACR and infection during the first year following lung transplantation. Such correlation might provide a feasible and specific diagnostic potentially allowing early recognition and subsequent targeted treatment of complications in LTRs.

2. Patients and methods

2.1. Study population

Starting in 1998, the four cytokines IL-6, IL-8, IFN-γ and TNF-α were analyzed in BAL fluid of LTRs during surveillance bronchoscopies for future research purposes. We enrolled all patients whose medical records included BAL cytokine analyses and concomitantly obtained TBB as well as microbiologic studies. This study was approved by the Cantonal Ethics Committee of Zurich (KEK-ZH number 2016-02148).

2.2. Immunosuppression protocol and prophylaxis regimen

In general, all primary LTRs at University Hospital Zurich receive induction therapy (antithymocyte globulin or basiliximab) and life-long triple immunosuppressive therapy [28]. At our center, cyclosporine A, tapered dose prednisone, and azathioprine or mycophenolate mofetil (since 1999) are used [29]. Anti-infective prophylaxis is used as previously described [30,31]. All patients classified as cytomegalovirus (CMV) intermediate-risk or high-risk received prophylaxis with valganciclovir [32,33]. In case of CLAD, macrolides are given for immunomodulation [29].

2.3. Predictors

At regular intervals BAL fluid and TBB were obtained during routine surveillance bronchoscopies during the first year after transplantation (at one, two, three, four, six and 12 months following lung transplantation) [30]. Levels of C-reactive protein (CRP) were routinely measured for all patients before surveillance bronchoscopies. Patients did not undergo surveillance bronchoscopy if clinically unstable, showing clinical signs of infection or infection-associated lung allograft dysfunction or if elevated inflammatory markers in the laboratory. Bronchoscopies performed for specific clinical indications or events were excluded from the study. Lavage was performed in a sub-segmental bronchus of either the lingula or the middle lobe using three to four 50 ml aliquots of 0.9% saline solution [34]. Five to eight TBB were taken from the lower lobe. After fixation in 4% formaldehyde solution and serial section biopsy specimens were stained with hematoxylin and eosin, elastic Van Gieson and Grocott.

BAL fluid cytokine concentrations of IL-6, IL-8, IFN-γ and TNF-α were determined by University Hospital Zurich Immunology Laboratory using commercially available, validated quantitative sandwich enzyme immunoassays. The immunoassays use microplates precoated with polyclonal antibodies specific for the cytokine and enzyme-linked polyclonal antibodies for their detection in a blinded fashion (R&D Systems; Minneapolis, MN, USA). ELISA kits included Quantikine® ELISA Human IL-6 Immunoassay (catalog # D6050), Quantikine® ELISA Human CXCL8/IL-8 Immunoassay (catalog # D8000C), Quantikine® ELISA Human IFN-γ Immunoassay (catalog # DIF50) and Quantikine® HS ELISA Human TNF-α Immunoassay (catalog # HSTA00D). All cytokines were measured according to the manufacturer's instruction. The absorbance was measured in an enzyme-linked immunosorbent assay (ELISA) reader (Dynex Opsys MR™ Microplate Reader) at 450/630 nm. The respective cytokine concentration was determined by interpolation from standard curves and expressed as pg/ml. Sensitivity of the assays was 0.7 pg/ml for IL-6, 3.5 pg/ml for IL-8, 8 pg/ml for IFN-γ and 0.1 pg/ml for TNF-α. BAL fluid cytokine concentrations were measured once a week. BAL fluid cytokine samples were stored at 4 °C until processed, for a maximum of one week. Starting in 2013, samples were centrifuged for 10 min at a speed of 2370 g at 4 °C immediately prior to the measurements. The supernatant was removed from the pellet after centrifugation. In summary, 50 μl/well (TNF-α) or 100 μl/well (IFN-γ, IL-6, IL-8) of Assay Diluent were added to the well of the cytokine microplates, then 50 μl/well (IL-8), 100 μl/well (IFN-γ, IL-6) or 200 μl/well (TNF-α) of the respective BAL sample, standards and controls were added as suggested by the manufacturer and incubated for 2 h (IFN-γ, IL-6, IL-8) or 3 h (TNF-α). Plates were washed four times (IL-6), five times (IFN-γ, IL-8) or six times (TNF-α). Removing excess liquid and washing thoroughly is essential; hence, plates with IFN-γ and IL-8 were washed once more than suggested by the provider. Then, 100 μl/well (IL-8) or 200 μl/well (IFN-γ, IL-6, TNF-α) of the respective Conjugate was added and incubated at room temperature for one hour (IL-8) or two hours (IFN-γ, IL-6, TNF-α). Plates were washed four times (IL-6), five times (IFN-γ, IL-8) or six times (TNF-α). Only in the case of TNF-α 50 μl/well of Amplifying Solution had to be added before the addition of the Substrate Solution and incubated at room temperature for 30 min. Then, 50 μl/well (TNF-α) or 200 μl/well (IFN-γ, IL-6, IL-8) of the respective Substrate Solutions were added and incubated at room temperature for 30 min (IFN-γ, IL-6, IL-8) or 60 min (TNF-α). 50 μl/well of Stop Solution was added (IFN-γ, IL-6, IL-8, TNF-α). Plates were read at OD 450/630 nm within 30 min.

2.4. ACR and microbial detection

ACR was assessed and graded in the TBB specimens by experienced pathologists using standard International Society for Heart and Lung Transplantation (ISHLT) nomenclature [35,36]. Both Grade A ACR and grade B ACR were considered. Episodes of clinically suspected ACR, antibody-mediated rejection (AMR) and CLAD were not included in the analysis.

Specimens were classified as infected if BAL fluid microbiologic studies identified bacteria, viral pathogens, fungi or mycobacteria. Bacterial cultures were considered positive if cultures showed growth greater than 100000 viable organisms per ml, excluding oral flora. PCR was used for detection of respiratory viruses (adenovirus, bocavirus, coronavirus, enterovirus, influenza A, influenza B, metapneumovirus, parainfluenzavirus, parechovirus, rhinovirus and RSV). CMV culture results were not included in the analysis. For this study we did not include the clinical presentation, radiologic findings, macroscopic appearance of BAL fluid and concomitant antibiotic, antiviral or antifungal treatment. Bronchoscopy samples were classified as "no pathologic process" if both TBB and BAL fluid microbiologic studies did not show a pathologic process, irrespective of clinical or radiologic presentations.

2.5. Statistical analysis

Statistical analysis was performed in R (version 3.4.1; R Foundation for Statistical Computing, Vienna, Austria) using "pROC", "broom",

"lmerTest" and "survival" libraries. Baseline recipient characteristics were expressed as mean (standard deviation) or as median (interquartile range) for continuous variables and as frequency (percentage) for categorical variables. Cytokine concentrations were log transformed prior to analysis to ensure normal distribution. Concentrations too low to detect by ELISA assay were assigned a value of 0.01 pg/ml. Boxplots were generated using default settings in the R graphics library, with whiskers at the default of 1.5 times the interquartile range. Continuous variables were compared using the Mann-Whitney-*U* test or Kruskal-Wallis test. Categorical variables were compared using Fisher's exact test. In a multivariable analysis, we controlled for patient characteristics frequently associated with rejection. These included age, underlying lung disease and type of infection (bacterial, viral or fungal). ROC curves for all four cytokines were calculated using the "pROC" package [37]. Overall survival of LTRs was assessed using the Kaplan-Meier method and compared using the log-rank test, with censoring at 31 December 2016. Because the ACR status was assessed after transplantation, we considered it to be a time-varying co-variate in this part of the analysis and had to reshape the data accordingly.

3. Results

3.1. Study population

During the study period from February 1998 to November 2016, 425 subjects underwent lung transplantation; 106 subjects were excluded because no cytokine data was recorded in the patient health record system and/or no bronchoscopies were performed. In the remaining 319 subjects, 747 BAL fluid samples were analyzed and compared with TBB specimens obtained during the same bronchoscopy. Median number of TBB and BAL samples per patient was 3 (IQR 1–4). Median time to first TBB sample was 43 (IQR 29–83) days. Patient characteristics are provided in Table 1. Compared with ISHLT Thoracic Transplant Registry data, our study population included a greater proportion of patients receiving transplantation for CF (33.5% vs. 22.9%) and pulmonary fibrosis (29.1% vs. 24.7%) [1]. Conversely, the patients included in the study received slightly fewer transplants for chronic obstructive pulmonary disease (COPD) (31.3% vs. 31.8%) and pulmonary hypertension (5.3% vs. 6%) [1]. Overall 31.4% of the total 319 patients experienced at least one episode of ACR during the first year post transplantation (vs. 28% ISHLT) [1].

3.2. Discrimination between specific groups

Bronchoscopy results were grouped based on microbiological and pathological analyses. Of the 747 bronchoscopy specimens, 214 (28.65%) showed "no pathologic process". Of the remaining samples, 69 (9.24%) showed ACR, 358 (47.93%) infection and 106 (14.19%) "combined ACR and infection". Table 2 provides an overview of the

Table 1
Patient characteristics.

Demographics	
Patients, n	319
Female, n (%)	142 (44.5%)
Male, n (%)	177 (55.5%)
Age at transplantation, mean ± standard deviation (range), year	51.8 ± 15.5 (18–77)
CMV high-risk, n (%)	134 (42.1%)
Transplant indication	
COPD, n (%)	100 (31.3%)
Cystic fibrosis, n (%)	107 (33.5%)
Pulmonary fibrosis, n (%)	93 (29.1%)
Pulmonary hypertension, n (%)	17 (5.3%)
Re-Transplantation, n (%)	2 (0.6%)

Table 2
BAL cytokine concentrations for different specific groups. The concentrations [pg/ml] in the given group at the time of grade ≥A1 ACR (R); viral, bacterial or fungal infection (I); "no pathologic process" (None) or "combined ACR and infection" (R + I) is shown as median and interquartile range (IQR). Significance for differences between categories was determined using Kruskal-Wallis test.

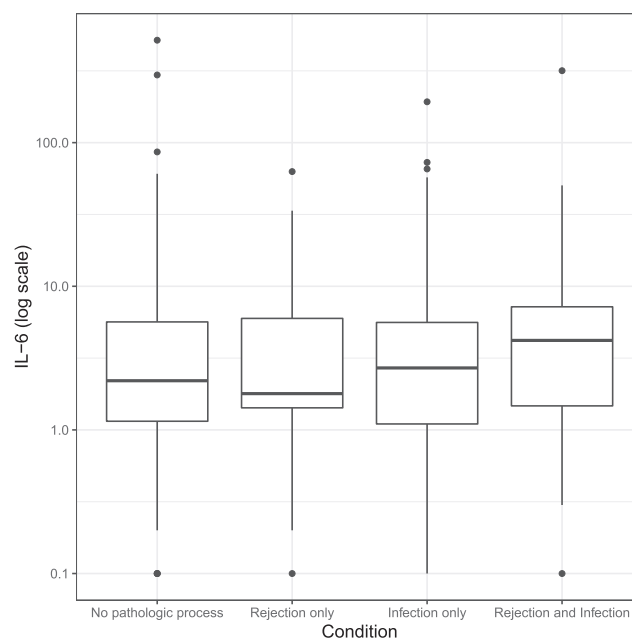|	Group	Median [pg/ml]	IQR [pg/ml]	p-value
IL-6	None	2.20	1.00–5.50	p = 0.045
	R	1.60	1.17–5.93	
	I	2.50	0.90–5.45	
	R + I	4.10	1.40–7.10	
IL-8	None	475.00	212.00–891.00	p = 0.7
	R	453.00	225.00–829.00	
	I	476.00	220.00–1151.50	
	R + I	585.00	255.00–1115.00	
TNF-α	None	0.11	0.01–0.30	p = 0.2
	R	0.10	0.00–0.45	
	I	0.20	0.03–0.50	
	R + I	0.10	0.00–0.68	
IFN-γ	None	0.10	0.00–1.20	p = 0.1
	R	0.10	0.00–0.20	
	I	0.10	0.00–1.02	
	R + I	0.10	0.010–1.00	

Fig. 1. Concentration of IL-6 in BAL fluid for different specific groups. The log transformed concentrations (log([pg/ml]) of IL-6 at the time of ≥A1 rejection; viral, bacterial or fungal infection; "no pathologic process" or "combined ACR and infection" is shown as box-and-whiskers plots. Significance for differences between specific groups was determined using Kruskal-Wallis test. Significance for differences between two categories was determined using Wilcoxon Rank Sum test. Log levels of IL-6 were lowest during ACR and highest during "combined ACR and infection" compared with "no pathologic process" (p = 0.87 and p = 0.11, respectively).

cytokine concentrations in the different groups.

Fig. 1 shows levels of log(IL-6) among the four subgroups; no significant difference was observed. IFN-γ, IL-8 and TNF-α also did not show significant differences by specific group. As shown in Fig. 2, none of these cytokines had an optimal cut-off to diagnose "combined ACR and infection" or isolated episodes of ACR.

Depending on the guidelines of different transplant centers, ACR may only be treated in > A1 ACR episodes. Levels of log(IL-6) during ACR (median 0.37 vs. 0.21) did not differ from "combined ACR and
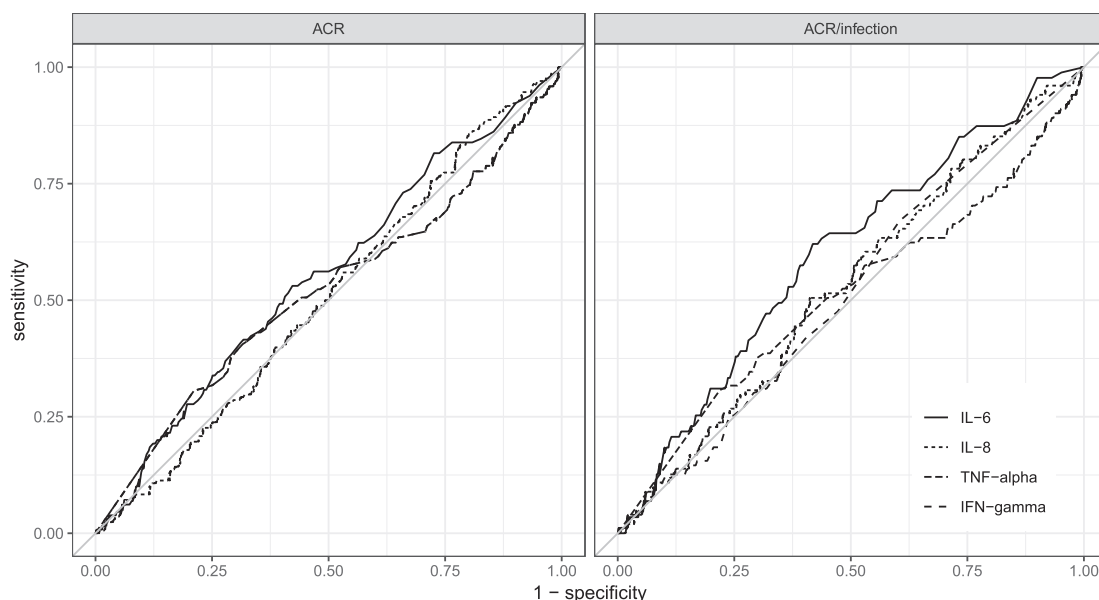
Fig. 2. Left: ROC curves for IFN-γ, IL-6, IL-8 and TNF-α to diagnose isolated ACR in surveillance bronchoscopies with TBB during the first year after transplantation. Area under the curve was 0.48 (IFN-γ), 0.55 (IL-6), 0.51 (IL-8) and 0.52 (TNF-α). No optimal cut-off can be derived from these data. Right: ROC curves for IL-6, IL-8, IFN-γ and TNF-α to diagnose "combined ACR and infection" in surveillance bronchoscopies with TBB during the first year after transplantation. Area under the curve for IFN-γ was 0.517 and for IL-8 was 0.531. Area under the curve for TNF-α was 0.509. No optimal cutoff can be derived from these data, particularly where higher scores of TNF-α do not appear to correspond with higher probability of "combined ACR and infection".
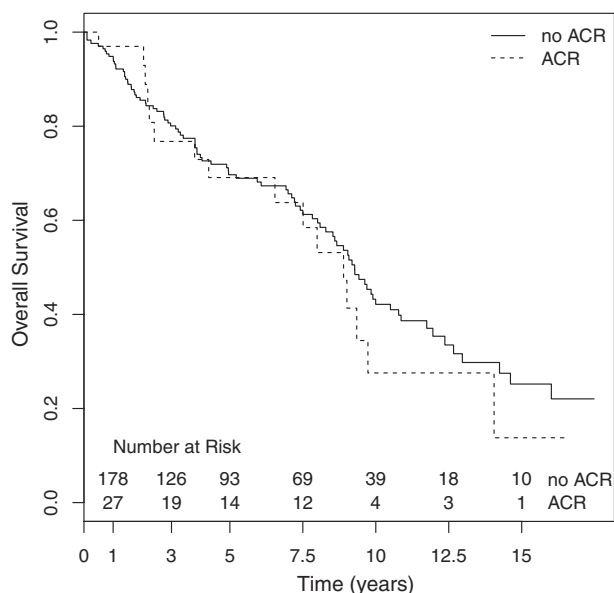

Fig. 3. Kaplan-Meier analysis of survival after lung transplantation. No significant differences in overall survival were observed between patients with and without at least one episode ACR diagnosed in surveillance bronchoscopies during the first year after transplantation (p = 0.54).

infection" (median 0.67 vs. 0.51), when ACR was defined ≥A2 as opposed to ≥A1 ACR (p = 0.22). No differences were observed in the levels of log(IL-8), log(IFN-γ) and log(TNF-α) (p = 0.21, p = 0.52, p = 0.21, respectively).

3.3. Factors influencing cytokine pattern

Using a linear mixed effects model, we found no significant differences in the cytokine levels by specific group for any of the cytokines. After adjusting for age, underlying disease leading to lung transplantation and type of infection at the time of surveillance bronchoscopy,

IL-8 showed significantly lower log(IL-8) levels in patients with infection (2.14) than in patients with "no pathologic process" (2.41, p = 0.02). Also, log(IFN-γ) was lower in patients with ACR only (-1.07), than with "no pathologic process" (−0.77, p = 0.05). log(IL-6) (ACR only 0.39, p = 0.76; infection only 0.19, p = 0.27; "combined ACR and infection" 0.37, p = 0.91, "no pathologic process" 0.35) and log(TNF-α) (ACR only −0.98, p = 0.86; infection only −0.88, p = 0.40; "combined ACR and infection" −0.93, p = 0.65, "no pathologic process" −1.00) did not vary in a significant manner by specific group.

3.4. Number of events

ACR was detected in 175 (23.43%) of all TBB samples (91 showing A1 ACR). 59 patients (18.5%) experienced one event of ACR during the first year after transplantation, 22 patients (7%) experienced two events, and 19 patients (6%) experienced more than two events according to surveillance bronchoscopies. Using a generalized linear mixed model ACR decreased slightly in the first year, with odds ratio of 0.915 per month (p = 0.02). No differences in the rates of ACR event were observed by type of pathogen detected during surveillance bronchoscopies (bacterial infection, p = 0.95; viral infection, p = 0.20; fungal infection, p = 0.79).

3.5. Survival

Among patients undergoing surveillance bronchoscopies with TBB, eight died within the first year after transplantation. Median 1-year conditional survival was 8.6 years for patients without ACR and 7.9 years for patients with a minimum one biopsy-proven ACR in the first year after transplantation, respectively. As shown in Fig. 3, the 5-year survival rate was similar between patients without (70%) or with episodes of ACR (69%) and higher compared to ISHLT Registry data (57% in era 2009 – June 2015) [1]. There were no significant differences in overall survival between patients with and without ACR (hazard ratio 1.18, 95%-confidence interval 0.69–2.02, p = 0.54), and among patients with ACR, between grade A1 ACR and grade > A1 ACR (hazard ratio 1.00, 95%-confidence interval 0.58–1.71, p = 1.00). There was no correlation with ACR events per patient and survival (p = 0.96).

4. Discussion

In this observational study we could not detect a relevant role of IL-6, IL-8, INF-γ and TNF-α in BAL fluid samples of LTRs to identify complications including ACR, infection or both.

After adjustment for age, underlying disease leading to lung transplantation and type of infection at time of surveillance bronchoscopy minor differences in the cytokine levels were observed. These data show that the pattern of BAL cytokines is of minor value as a diagnostic marker in LTRs.

As reported before [26] data on the role of IL-6 in ACR is conflicting. Whereas some experimental and clinical data showed a significant increase in IL-6 in ACR [22,38], other studies found no significant association at all [39,40]. Despite the high number of samples no correlation was found in our analyses. IL-8 was significantly lower during infection than in LTRs with "no pathologic process" after adjustment for age, underlying disease and type of infection. No correlation was found between IL-8 and ACR, which is along the line with most previous studies [22,41,42]. While IL-8 has been linked with the development of CLAD, its role in detecting ACR seems negligible according to our data [43,44]. Levels of IFN-γ were significantly lower during ACR only after correction for age, underlying disease and type of infection. However, in the light of the results from previous studies, these findings should be interpreted with caution [39,45–47]. Levels of TNF-α did not correlate with the four prespecified groups. Accordingly, previous studies have suggested no correlation between TNF-α and ACR [22,39,47]. Of technical note, ELISA is not the best method to detect TNF-α since it is limited in the detection of cytokines that are active in the membrane bound form [48,49].

Survival did not differ between patients with ACR and "no ACR" and no difference was seen in patients with multiple ACR events. However, we only included data obtained from surveillance bronchoscopies without clinical signs of allograft dysfunction. Patients are followed up every 1–2 weeks within the first six months following transplantation and 2–4 weeks thereafter just at our center due to the limited size of Switzerland. Any clinical signs of infection or allograft rejection prompt immediate treatment. In addition, patients with multiple ACR events and early signs of CLAD are treated with extracorporeal photophoresis, potentially explaining the higher survival rate compared to the data from the ISHLT registry. In summary, no correlation was found between the cytokines studied here and survival of patients suggesting that these cytokines are not suitable markers for monitoring.

This study has several limitations: First, this is a retrospective study. Thus, even though we planned surveillance bronchoscopies at regular intervals, a selection bias can't be excluded and patients with clinically relevant allograft dysfunction or infection did not undergo surveillance bronchoscopies. This might explain why we derived data from 319 patients, missing the potential of 425 subjects. Also, while we obtained total cell count in BAL samples we did not include this information in our analysis. An increase in total cell count is non-specific after lung transplantation and has also been found in periods with no infection or rejection [50]. Second, we restricted cytokine analysis to a small number of cytokines. When this study was initiated in 1998, these were the only commercially available and validated kits used at our center. All four cytokines had been associated with rejection in previous studies [26]. Also, we did not perform immunologic analyses reflecting the innate immune system such as alpha defensins or matrix metalloproteinases. Further, combining BAL cytokine and cytology levels might contribute to a composite score, increasing diagnostic accuracy for BAL to diagnose ACR. However, evaluating such a score was not part of the study. Third, due to the fact, that AMR was an ill-defined condition before the publication of the 2016 ISHLT Consensus Report [51] we did not include patients with AMR in our study. Fourth, we did not perform analyses to associate cytokines with CLAD development [52,53]. The diagnosis of CLAD is an indication to treat patients with an immunomodulatory macrolide. As such, even if the absolute number of patients receiving macrolides at our institution during the first year after lung transplantation is low, a bias of such treatment for cytokine distribution in BAL cannot be excluded [54]. Fifth, statistical methods were not used to adjust for storage time of the BAL samples, number of analyses per patient and date of collection after transplantation. Sixth, infection was defined based on microbiological findings in BAL fluid only and did not take into account clinical symptoms, imaging studies, macroscopic appearance of BAL fluid and concomitant antibiotic, antiviral or antifungal medication. Detecting infection and differentiating infection from ACR based on clinical symptoms may be difficult in LTRs [33,55]. Thus, in patients on multiple immunosuppressive drugs the mere presence of pathogens in BAL fluid in the absence of clinical deterioration may fulfill criteria of infection and prompt anti-infective treatment. Indeed, at our center, all pathogenic findings in BAL fluid except for oral flora and candida species prompted anti-infective treatment [30,31].

Some aspects of our immunological analysis warrant further discussion. We did not centrifuge BAL specimens before 2013. In a recent internal review conducted by our Immunology Lab comparing cytokine concentrations in BAL samples with and without prior centrifugation, no difference was seen in the majority of samples (unpublished data). Further, we measured BAL cytokine levels once a week and therefore not necessarily on the day they were obtained. Finally, BAL fluid samples were stored at 4 °C until processed, at a maximum of one week. An internal analysis performed in our Immunology Lab in 2012 showed a difference in cytokine concentration < 20% between BAL samples stored at 4 °C and samples stored at −20 °C immediately after arrival until processing one week later.

There is a need of standardization in BAL technique in LTRs. The ISHLT has recently established a Working Group to address this issue as far as the BAL procedure is concerned, the quotient of instilled volume and aspirated volume should be calculated and provided. This facilitates comparability of cellular and protein concentrations between studies. This would be particularly important for cytokines, as their concentrations are typically low. To further correct for dilution factors of BAL fluid, the urea method has been described, whereby the measured BAL fluid cytokine concentration is adjusted to a urea plasma/BAL coefficient [56]. We did not apply this method in our study to normalize the data. Levy and colleagues recently showed that sequential BAL samples reflect distinct pulmonary compartments [57]. This might have implications for future research. Along this line, centers should agree on a standardized cytokine detection method as comparability between methods is low [48]. ELISA has been a reliable method at our center. However, it is costly and requires strict adherence to time protocols. FACS or Luminex® assays may be alternatives for selected cytokines, yet these methods have yielded less reliable results in our laboratory. Also, numerous potential confounders related to medication (dosage of immunosuppression, anti-infective prophylaxis and treatment, additional individual medication) and immunological analysis make interpretation of cytokine data in LTRs challenging. In the field of rheumatology attempts have been made to harmonize autoantibody nomenclature, thereby optimizing antinuclear antibody usage [58].

Based on the data shown here, the identification of a single biomarker or a profile of different biomarkers is unlikely to provide conclusions on lung allograft function. Whether computed algorithms and precision medicine might help to translate this complex data into the clinical setting is unclear at the moment. In our opinion, this can only be achieved in a collaborative approach by using standardized and validated methods in large prospective multi-center cohort studies. This effort is crucial, however, to optimize survival and quality of life for LTRs [27].

In summary, this is one of the largest retrospective studies to analyze BAL fluid cytokine profiles in LTRs. The cytokines investigated here have no role to diagnose complications after lung transplantation. It is unlikely that further attempts to study these BAL fluid cytokines in the context of ACR, infection or both in LTRs will add novel valuable insights.

5. Authorship

NES conducted data collection and statistical analysis, provided draft versions and revised the manuscripts. EP supervised the laboratory analyses, reviewed all versions of the manuscript and assisted to write the final version. SH performed statistical analysis and assisted to write the final version. CB and MK reviewed all versions of the manuscript and assisted to write the final version. LCH designed the project, revised all versions of the manuscript and assisted to write the final version. CAR designed and supervised the project, revised all versions of the manuscript and provided the final version. All authors read and approved the final manuscript.

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8. Credit author statement

NES conducted data collection and statistical analysis, provided draft versions and revised the manuscripts. EP supervised the laboratory analyses, reviewed all versions of the manuscript and assisted to write the final version. SH performed statistical analysis and assisted to write the final version. CB and MK reviewed all versions of the manuscript and assisted to write the final version. LCH designed the project, revised all versions of the manuscript and assisted to write the final version. CAR designed and supervised the project, revised all versions of the manuscript and provided the final version. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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