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Immune Responses in the Lung Preceding a Diagnosis of Chronic Lung Allograft Dysfunction

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Abstract

Background: Chronic Lung Allograft Dysfunction (CLAD) is the major limitation to long-term survival following lung transplantation and the pathogenesis is thought to involve dysregulated immune responses to various lung injuries. Type 1, Type 2, and Type 17 immune responses have all been implicated.

Objective: Determine the relationship between immune response markers measured in Bronchoalveolar Lavage (BAL) fluid and the development of CLAD.

Methods: We measured BAL concentrations by bead-based multiplex or ELISA for Type 1 (IL-12p70, IFN-γ, CXCL9, CXCL10, CXCL11, CCL5), Type 2 (IL-4, IL-13, CCL2, CCL11), and Type 17 (IL-17, IL-6, TGF-β and CCL20) markers. For each marker, the estimated concentration at 6 months and composite variables for Type 1, Type 2, and Type 17 markers were generated using Principal Component (PC) analysis. We used cumulative incidence competing risk regression to determine associations with CLAD and death unrelated to CLAD.

Results: 57 lung transplant recipients had BAL samples before and after 6 months post-transplant; 19 never developed CLAD and 38 developed CLAD. Type 1 (1st PC) and Type 2 (1st PC) immune response markers were associated with CLAD development. In addition, Type 1 (2nd PC) and Type 17 (2nd PC) immune response markers were associated with death unrelated to CLAD.

Conclusion: We demonstrate that alterations in Type 1, Type 2, and Type 17 immune responses precede CLAD and mortality in lung transplant recipients. Larger longitudinal studies to assess prognostic utility are warranted.

Keywords: Lung transplantation; Chronic lung allograft dysfunction; Immune response

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Introduction

Lung transplant is a therapeutic option for end-stage pulmonary disorders, but long-term survival is dependent upon remaining free from Chronic Lung Allograft Dysfunction (CLAD), which affects greater than 50% of recipients within 5 years. CLAD is characterized by the inexorable loss of lung function, and the typical survival following a CLAD diagnosis is less than 3 years [1]. Unfortunately, by the time a CLAD clinical diagnosis is made, treatment is usually ineffective [2].

Both animal models and human studies demonstrate that a Type 1 immune response plays a key role in acute and chronic rejection [3-9]. Prototypical Type 1 cytokines include Interleukin (IL)-12 and Interferon-γ (IFN- γ) [10]. The leukocyte influx associated with a Type 1 immune response is mediated in part by interferon inducible ELR- CXC chemokines CXCL9, CXCL10, and CXCL11 [11-15], as well as by the CC chemokine CCL5 [16]. Analogous to Type 1, a Type 17 response plays a role in inflammatory diseases, especially autoimmune diseases, and has been associated with allograft rejection [17-21]. Key markers of a Th17 response include IL-17, IL-6, Transforming Growth Factor- β (TGF-β), and CCL20.

Excessive inflammation can lead to uncontrolled tissue damage, so there needs to be mechanisms to counteract this. The classic paradigm balances Type 1/17 immune responses with Type 2 responses. While a Type 2 immune profile has favored the acquisition of tolerance in some animal models, there is also evidence implicating a role for Type 2 responses in fibroproliferative disease

and chronic rejection [22-24]. The Type 2 cytokines include IL-4 and IL-13, which promote mucosal, allergic and humoral immunity [10]. Related chemokines include CCL2, which influences Th2 polarization [25], and CCL11, which is induced by Type 2 cytokines and directly contributes to airway remodeling [26].

We have demonstrated that the risk of CLAD increases with most acute lung allograft injuries after 6 months post lung transplantation [27]. Based on this, we hypothesized that immune responses in the lung allograft surrounding the 6 months post-transplant period, as measured by cytokine and chemokine concentrations in BAL fluid, will be associated with the development of CLAD, as well as mortality. We serially measured Type 1, Type 17, and Type 2 immune response proteins in BALF in the first post-transplant year from lung transplant recipients at the University of California Los Angeles (UCLA). In order to assess the immune response around 6 months post-transplant, we used 2 serial samples to estimate the immune response at 6 months and we determined the relationship of these immune pathways to the development of CLAD.

Ethics Statement

The UCLA Biologic changes in Lung Transplant Patients Study were approved by the UCLA Institutional Review Board (IRB# 10-001492) and all subjects were provided written informed consent to participate in the study.

Materials and Methods

Patient selection

A subset of lung transplant recipients at the University of California, Los Angeles (UCLA) was enrolled in an observational registry study that included the collection of left-over BAL fluid for research purposes at the time of standard of care bronchoscopies. This study included standardized medical record abstraction including demographic, transplantation, and outcome related variables. CLAD was defined as a sustained drop in Forced Expiratory Volume in 1 second (FEV1) by at least 20% from the average of the 2 best post-transplant measurements [28].

For this study, eligible patients were transplanted between 3/2/2001 and 9/30/2007 and had sufficient spirometry testing (at least 6 tests) to determine CLAD. From this cohort, eligible BAL samples were those collected prior to 3/23/2008 and prior to a diagnosis of CLAD. Follow-up data were collected through January 2017. A total of 768 BAL samples from 187 patients were included in our biorepository. However, 10 patients were excluded because of insufficient data to make a CLAD determination, leaving 177 possible patients and 752 total BAL samples. After further exclusion of samples before 80 days or after 400 days, we were left with 307 samples from 156 patients. Of these 307 samples, 210 samples from 140 patients were collected before 6 months (80 to 199 days), and 97 samples from 75 patients were collected after 6 months (200 to 400 days). The final study cohort included 57 patients with a sample from both before and after 6 months (Figure 1). In this cohort 19 never developed CLAD and 38 developed CLAD.

Immunosuppression, anti-microbial prophylaxis and treatment of acute rejection were administered in accordance with UCLA protocol as previously described [14,29]. Three 60 ml aliquots of isotonic saline were instilled into the sub-segmental bronchus in the lingula, right middle lobe or area of interest and pooled. After centrifugation, the supernatant was collected and stored unconcentrated at -80ºC.

BAL biomarker assays

For most cytokine and chemokine markers, the BAL concentrations were determined by bead based multiplex assays. Specifically, the concentrations of the Type 1 markers (IL-12p70, IFN-γ, CXCL9, CXCL10, CXCL11, CCL5), the Type 2 markers (IL-4, IL-13, CCL2, CCL11), and the Type 17 markers (IL-17 and IL-6) were all assayed *via* Luminex according to manufacturer instructions (Millipore, Billerica MA). In addition, the BAL concentrations of the Type 17 markers (TGF-β1 and CCL20) were measured by ELISA Duo Set kits according to manufacturer instructions (R & D Systems, Minneapolis, MN). Limits of detection for each marker were determined by the average of 4 blank measurements plus 3 standard deviations [30].

Statistical methods

Subject characteristics are described as proportions or medians plus range. Comparisons between the final analysis cohort and patients not included used either Fisher's exact or Mann-Whitney U tests. Biomarker concentrations are described using medians and interquartile ranges, as well as the percentage of samples above the limit of detection. When less than 50% of samples were detectable for a given marker, that marker was modeled as a binary (detectable or undetectable) variable. The remaining marker concentrations were log-transformed and modeled as continuous variables. Data trends for each continuous marker were further explored with loess curves generated from scatter plots over time using the SAS LOESS procedure (smooth =0.25). Loess curves were generated from predicted values using a nonparametric method for estimating regression surfaces. We selected the final list of protein markers for inclusion in inferential models after inspection of the descriptive data.

To further simplify longitudinal data, we estimated the log-concentration of each marker surrounding 6 months post-transplant using simple linear regression between the sample immediately before day 200 and the sample immediately after day 200 for each subject. In order to reduce the number of variables for survival models, we generated composite variables for each immune pathway by principal component analyses of the estimated 6-month log-concentrations. Separate cumulative incidence competing risk regression models for CLAD and death unrelated to CLAD were generated for each pathway (e.g., model for Type 1 pathway included 1st and 2nd principal components as covariates) [31]. We also constructed Cox Proportional Hazard (PH) models for mortality. As sensitivity analyses, Cox models also examined time to CLAD (mortality censored) and time to CLAD or mortality (composite endpoint of CLAD or mortality). For each Cox model we calculated the C-index as a measure of model discrimination, which can be interpreted as the probability that a subject from the event group (e.g., CLAD) has a higher predicted probability of having an event than a subject from the non-event group. Statistical analyses were conducted using SAS (version 9.4 for Windows, SAS Institute Inc. Cary NC, USA). P values less than 0.05 were considered statistically significant.

Results

Patient and sample characteristics

Our biorepository included 307 samples collected between 80- and 400-days post lung transplant from 156 patients. The characteristics of the 156 patients are shown in Table 1, grouped by whether or not the subject was included in the final analyses. The concentrations of each potential biomarker are described in Table 2. IL-4 was not detectable

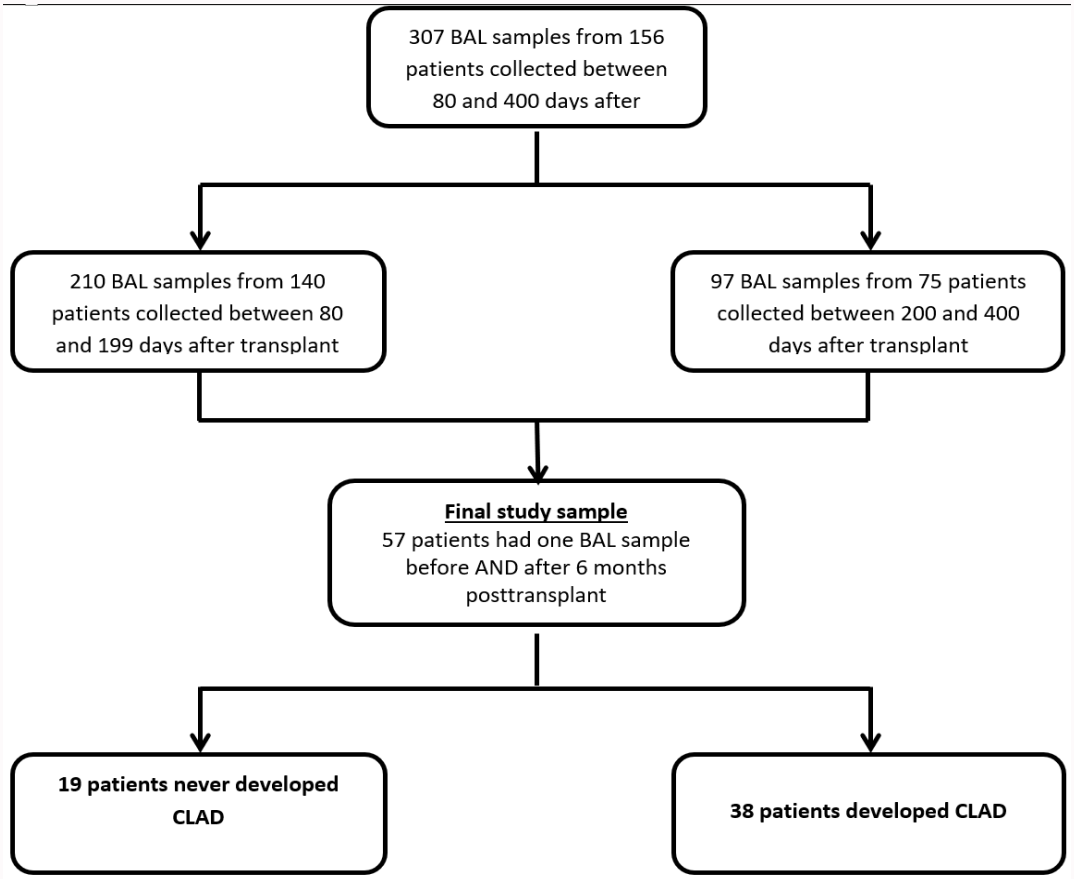

Figure 1: Study Cohort derivation. After selecting patients with BAL samples available both before and after 6 months (200 days), 57 subjects were included: 38 that developed CLAD and 19 that never developed CLAD.

in 98% of the samples and was not examined any further. Of the 13 remaining analytes, 7 (CXCL9, CXCL10, CCL5, IL-13, CCL2, IL-6, and CCL20) were considered routinely detectable and analyzed as continuous variables. The remaining 6 analytes assayed (IL12p70, IFN-γ, CXCL11, CCL11, TGF-β, and IL-17) were dichotomized as detectable and undetectable. For each potential marker, we plotted log concentrations over time as scatter plots and LOESS curves for continuous markers or bar charts for dichotomized markers (Figures S1-S14).

The final survival analysis cohort included 57 patients with samples and marker data from both before and after 6 months. The analytic cohort was more likely to receive Anti-Thymocyte Globulin (ATG) and less likely to receive basiliximab than patients not included in the final analyses, but otherwise the 2 groups were not significantly different. The clinical characteristics of the included and excluded patients are shown in Table 1. In the final study cohort, 38 developed CLAD and 19 remained CLAD free at the end of follow up (Figure 1).

In order to reduce the number of variables for survival models, we performed principal component analyses for each predefined pathway; Type 1, Type 2, and Type 17 immune responses. The first PC for Type 1, 2, and 17 were highly correlated (Table S1).

Immune response markers and risk of clad and mortality

In cumulative incidence competing risk models, CLAD and death unrelated to CLAD were treated as competing risks. Type 1 (1st PC) and Type 2 (1st PC) immune response markers in BAL fluid were significantly associated with CLAD, while Type 1 (2nd PC) and Type 17 (2nd PC) markers were associated with death unrelated to CLAD (Table 3).

As sensitivity analyses, we also constructed Cox PH models for time to death, time to CLAD, and time to the combined endpoint of CLAD or death. Type 1 (1st and 2nd PC), Type 2 (1st and 2nd PC), and Type 17 (1st PC) markers were each associated with time to death (Table 4). Cox PH models for CLAD and the combined endpoint of CLAD or mortality generated similar results (Tables S2 and S3). Given the high correlation between Type 1, 2, and 17 immune response, multivariable analyses including all three immune responses were not conducted.

Discussion

In this study, we tested our hypothesis that Type 1, 2, and 17 immune responses in the BAL fluid would be associated with long-term outcomes after lung transplant. We measured 14 total markers in the BAL fluid and ultimately included 11 in the final models after inspection of descriptive statistics for each. In order to quantify each immune response, we performed principal component analyses and used the first and second PC scores in survival models. Our main findings implicate both Type 1 and 2 immune responses in the development of CLAD, while the Type 1 and 17 immune response may be associated with mortality independent of CLAD.

Classically, wound repair following any injury is thought to be the result of a delicate balance between pro- and anti- inflammatory

Table 1: Clinical characteristics of the study cohort.

	Not Included (N=99)	Included (N=57)	P value
Age at Transplant, Median (Range)	60 (23-79)	59 (31-75)	0.63
Sex, N (%)			0.06
Male	63 (64%)	27 (47%)	
Female	36 (36%)	30 (53%)	
Race, N (%)*			1.00
White	74 (75%)	43 (75%)	
Black	7 (7%)	4 (7%)	
Other	18 (18%)	10 (18%)	
Ethnicity			0.79
Hispanic	10 (10%)	7 (12%)	
Not Hispanic	89 (90%)	50 (88%)	
Pre-transplant disease†, N (%)			0.31
Group A (obstructive lung disease)	39 (39%)	19 (33%)	
Group B (pulmonary vascular disease)	6 (6%)	2 (4%)	
Group C (cystic fibrosis)	1 (1%)	0	
Group D (restrictive lung disease)	53 (54%)	36 (63%)	
Transplant Type			1.00
Single	37 (37%)	21 (37%)	
Bilateral	62 (63%)	36 (63%)	
Induction Type			0.004
ATG	49 (49%)	42 (74%)	
Basiliximab	50 (51%)	15 (26%)	

*P-value from Fisher's exact test for comparison of White vs. non-White Race
†P-value from Fisher's exact test for comparison of Group D *vs.* others (A, B, and C combined)

Table 2: Marker concentrations (pg/ml) in bronchoalveolar lavage samples including detection limits, proportion detectable, median concentrations, and interquartile ranges for each potential marker.

Marker	LOD	MIN	Q1	MEDIAN	Q3	MAX	% Detectable
IL12p70	2.7	0.28	0.44	1.27	2.76	32.57	25.8
IFN-γ	1.88	0	0.19	0.19	0.75	52.23	11.6
CXCL9/MIG	3.45	5.82	105.46	330.58	2729.56	9764.1	100
CXCL10/IP-10	5.03	0.78	58.93	117	327	10666.07	98
CXCL11/ITAC	123.62	34.76	65.73	82.84	106.75	2317.94	17.1
CCL5/RANTES	6.66	0.53	2.95	17.9	63.61	1758	64.9
IL-4	16.82	0.64	0.64	0.64	0.64	181.16	1.7
Il-13	1.4	0.24	2.72	4.69	8.04	49.01	90.4
CCL2/MCP-1	7.52	0.49	54.37	109	229	7909.61	98.7
CCL11/Eotaxin	5.91	1.26	1.26	2.19	9.46	88.65	33.1
IL-17	1.86	0.23	0.23	0.23	0.23	13.64	5.6
IL-6	1.56	0.46	2.79	7.47	19.99	15334	88.7
TGF-b	52.44	5.52	31.95	51.59	84.35	607.07	48.6
CCL20/MIP3a	11.18	0.24	28.2	62.74	144.66	3207.59	92.9

cytokines. In this paradigm, Type 1 and 2 cytokine expression profiles have distinct counterbalancing functions [24,32-36]. However, our findings are in line with other data suggesting that the counterbalancing immune responses may not hold true for allograft rejection [24,37-47]. We find relatively high correlation between the first PC of each of the 3 immune responses examined, arguing against a case for immune polarization. We also find that both Type 1 and Type 2 immune responses are associated with the development of CLAD. These findings suggest Type 1 and Type 2 responses may work in parallel. The prototypical Type 1 cytokine, IFN-γ, in BAL fluid is associated with acute and refractory lung allograft rejection, both risk factors for CLAD [4,7]. A significant correlation has been detected between the presence of a "high" expressing human IFN-γ polymorphism and CLAD [5]. The Type 2 response may be

Table 3: Cumulative incidence competing risk models for CLAD, or death unrelated to CLAD.

		CLAD		Death unrelated to CLAD	
	PC	Hazard Ratio	P-value	Hazard Ratio	P-value
Type 1 (IL-12p70†, CXCL10, CCL5, IFN-γ†)	1st	1.518	0.0080	0.578	0.1345
	2nd	0.960	0.8617	1.656	0.0199
Type 2 (IL-13, CCL2, CCL11†)	1st	1.387	0.0396	0.717	0.3077
	2nd	1.310	0.0505	0.556	0.0772
Type 17 (IL-17†, IL-6, TGF-β†, CCL20)	1st	1.175	0.3817	1.245	0.5421
	2nd	0.850	0.3035	1.790	0.0011

†Indicates markers modeled as categorical detectable or undetectable concentration. All other markers modeled as continuous

Table 4: Cox proportional hazard models for time to death.

	PC	HR (95% CI)	P-value	C-index
Type 1 (IL-12p70†, CXCL10, CCL5, IFN-γ†)	1st	1.416 (1.072, 1.870)	0.0142	0.862
	2nd	1.453 (1.049, 2.012)	0.0246	
Type 2 (IL-13, CCL2, CCL11†)	1st	1.349 (1.018, 1.787)	0.0373	0.851
	2nd	1.468 (1.122, 1.921)	0.0052	
Type 17 (IL-17†, IL-6, TGF-β†, CCL20)	1st	1.666 (1.175, 2.363)	0.0042	0.641
	2nd	1.128 (0.787, 1.616)	0.5120	

†Indicates markers modeled as categorical detectable or undetectable concentration. All other markers modeled as continuous

driving fibroproliferation as opposed to counterbalancing a Type 1 response. For example, IL-13 can induce fibroblast proliferation and procollagen expression [48,49]. Other work has shown that driving an allogeneic response toward a Type 2 cytokine cascade can accelerate rejection [24,37-47]. We cannot rule out that our data represents an inadequate attempt to counterbalance the Type 1 response, but this seems unlikely given the prior studies indicating a role for Type 2 response in promoting fibroproliferation and rejection. Taken together, our findings support the importance of both Type 1 and Type 2 immune responses in the pathogenesis of CLAD [11,13,14,23]. Thus, prevention of CLAD may depend on the down regulation of both Type 1 and 2 immune responses [50]. To our knowledge this is a novel observation with important pharmacological implications.

Type 17 immune responses have commonly been associated with autoimmunity and CLAD in prior studies [17,18,20]. In this study, we did not find an association between the Type 17 immune response and the development of CLAD. The lack of significance may be a consequence of too small a sample size. We did find an association with non-CLAD mortality (from competing risk models) overall mortality, and the composite of CLAD and mortality (from Cox model). We also found a Type 1 immune response was associated with death independent of CLAD. The mechanism by which Type 1 and Type 17 immune responses might be associated with non-CLAD death is not clear from this study, but high levels of Type 1 and Type 17 markers may indicate inadequate control mechanisms and set the stage for excessive inflammation in response to any form of insult. Eleven deaths occurred in patients who were never diagnosed with CLAD, but there was no consistent mechanism of death in these patients. One patient experienced a rapid respiratory decline and acute lung injury from unknown etiology. In the remaining cases, the cause of death was infection (n=4), malignancy (n=2), cerebrovascular accident (n=1), or unknown (n=3).

The limitations of our study include the relatively small final cohort due to our requirement for samples collected before and after 6 months post-transplant. By requiring samples before and after 6 months, our intention was to model a single measurement at 6 months. Our prior experience suggested that evaluation of BAL markers at approximately 6 months post-transplant would be informative of outcomes [51]. Future studies could explore true longitudinal study of impact of within patient changes. Finally, we selected markers based on our prior experience. Other markers could be studied for each pathway and may improve performance. However, our study supports that Type 1, 2, and 17 immune responses can inform about the pathogenesis and risk of CLAD, and possibly for other outcomes after lung transplant.

In summary, we showed that Type 1, Type 2, and Type 17 immune responses are associated with important outcomes after transplant including CLAD and mortality. These data suggest potential for earlier recognition and possibly intervention to reduce the risk of CLAD and mortality after lung transplantation.

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