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Plasma CXCL9 and CXCL10 at Allograft Injury Predicts Chronic Lung Allograft Dysfunction

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Abstract

Histopathologic lung allograft injuries are putative harbingers for chronic lung allograft dysfunction (CLAD). However, the mechanisms responsible are not well understood. CXCL9 and CXCL10 are potent chemoattractants of mononuclear cells and potential propagators of allograft injury. We hypothesized that these chemokines would be quantifiable in plasma, and would associate with subsequent CLAD development. In this prospective multi-center study, we evaluated 721 plasma samples for CXCL9/CXCL10 levels from 184 participants at the time of transbronchial biopsies during their first-year post-transplantation. We determined the association between plasma chemokines, histopathologic injury and CLAD risk using Cox proportional hazards models. We also evaluated CXCL9/CXCL10 levels in bronchoalveolar lavage (BAL) fluid and compared plasma to BAL with respect to CLAD risk. Plasma CXCL9/CXCL10 levels were elevated during the injury patterns associated with CLAD, acute rejection and acute lung injury, with a dose-response relationship between chemokine levels and CLAD risk. Importantly, there were strong interactions between injury and plasma CXCL9/CXCL10, where histopathologic injury associated with CLAD only in the presence of elevated plasma chemokines. We observed similar associations and interactions with BAL CXCL9/CXCL10 levels. Elevated plasma CXCL9/CXCL10 during allograft injury may contribute to CLAD pathogenesis and has potential as a minimally invasive immune monitoring biomarker.

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1. INTRODUCTION:

Chronic lung allograft dysfunction (CLAD) remains the major factor limiting long-term survival after lung transplantation.[1] Since treatment options for CLAD remain limited, the identification and treatment of key modifiable risk factors for CLAD may be the most effective way to improve outcomes for lung transplant recipients. Prior single center studies have consistently identified the histopathologic injury patterns of acute cellular rejection (AR)[2–8] and acute lung injury (ALI)[9,10] as two of the strongest risk factors associated with CLAD development. However, the mechanisms responsible for this association remain poorly understood.

The Clinical Trials in Organ Transplantation-20 (CTOT-20) study, was a prospective multicenter observational cohort study of newly transplanted lung recipients with the objective of identifying the risk factors associated with CLAD development. The primary mechanistic aim of CTOT-20 was to better understand CLAD pathogenesis by leveraging multi-center clinical samples to evaluate cytokine / chemokine interactions during histopathologic allograft injury and CLAD development. Histologically, AR and ALI both involve recruitment and infiltration of mononuclear cells into the area of injury. AR is defined by the presence and extent of perivascular and interstitial infiltration of mononuclear cells.[11] ALI, the most severe acute allograft injury pattern, is defined by an initial exudative phase and a subsequent proliferative phase with infiltration of mononuclear cells into the interstitium and alveoli.[12,13]

The chemokines CXCL9 (MIG) and CXCL10 (IP10) are induced by interferon-γ in a Type I (Th1) immune response and act as potent chemoattractants for mononuclear cells through a common receptor, CXCR3. The key role of CXCL9 and CXCL10 in the pathogenesis of AR and CLAD has been demonstrated in animal models.[14,15] Single center clinical studies from our group and others evaluating bronchoalveolar lavage (BAL) fluid from lung transplant recipients show BAL CXCL9 and CXCL10 elevation during both AR and ALI, with higher BAL chemokine levels predicting increased CLAD risk.[8,9,16,17] Confirming these single center studies, we recently reported that BAL CXCL9 and CXCL10 are elevated during both AR and ALI in this CTOT-20 multi-center cohort.[18] Despite evidence supporting the prognostic importance of BAL CXCL9 and CXCL10 levels for CLAD risk, the use of the less invasively obtained plasma CXCL9 and CXCL10 levels has not been well studied to date in lung transplantation.

The current study extends these findings by evaluating the association between histopathologic allograft injury, plasma CXCL9 and CXCL10 levels with CLAD development in a prospective multi-center cohort. We hypothesized that the injury patterns of AR and ALI would be associated with increased CLAD risk and that this association would be mediated by CXCL9 and CXCL10, quantifiable in the plasma compartment. The goals of this study were four-fold: 1) determine the association between histopathologic injury and CLAD development, 2) evaluate plasma CXCL9 and CXCL10 levels during episodes of histopathologic injury, 3) explore the impact of plasma CXCL9 and CXCL10

levels during histopathologic injury on CLAD risk, and 4) compare the significance of CXCL9 and CXCL10 levels in the plasma with the BAL compartment.

2. MATERIALS AND METHODS:

Cohort:

Among the first 200 CTOT-20 patients enrolled and transplanted, 184 had at least one transbronchial biopsy during the first post-transplant year with contemporaneous BAL sampling and plasma sample collected within a ±7-day window of the biopsy (Figure 1). IRB approval was obtained for the study at each center.

Assessments:

Recipients received standard of care management according to practices at each study center as previously described.[18,19] Four to five "Surveillance" bronchoscopies were performed during the first-year after transplant according to each center's protocol (at 2–4 weeks, 3 months, 6 months and 12 months post-transplant). Bronchoscopies were considered "for cause" if performed due to new symptoms, decline in pulmonary function testing (PFT) or radiographic changes. The primary endpoint of CLAD was defined as a ≥20% decline in FEV1 as compared to the average of the two best posttransplant FEV1s measured at least three weeks apart in the absence of an alternate explanatory etiology for the decline.[20]

Histopathologic Assessment:

Transbronchial biopsies were reviewed by a pulmonary pathologist at the enrolling study center.[11] A pathology working group, including pathologists from each center, met several times before study initiation and reviewed representative digital images and harmonized the nomenclature, grading and reporting of each histopathologic injury. These consensus terms and descriptors were included in the histopathology case report forms. The term "allograft injury" refers to the presence of either: AR, lymphocytic bronchiolitis (LB), organizing pneumonia (OP) or ALI. Ungradable biopsies for AR or LB were considered missing and excluded from the analysis.

Infection Assessment:

As standard of care, BAL samples were evaluated for microbiologic findings. "Pathogen" was defined as presence of an organism known to cause lung infections. "Infection" was defined as: 1) pathogen detection with symptoms (fever, dyspnea, viral prodrome, cough or sputum) or signs (radiographic findings, PFT decline); or 2) purulence noted on the bronchoscopy. All bronchoscopic findings with associated symptoms and signs were documented at time of bronchoscopy. Biopsies were classified as "normal" if there was no histopathologic evidence of allograft injury or infection.

Cytokine Assessment:

Plasma and BAL levels of CXCL9 and CXCL10 were measured using luminex bead assays (MilliporeSigma). Chemokine levels are reported as median fluorescence intensities (MFIs) to minimize variability introduced by standard curve interpolation.[21,22]

Analysis:

The baseline analysis dataset included 721 biopsies with matching BAL samples and plasma samples within a ± 7 day-window (Figure 1). BAL chemokines analysis was performed on a more inclusive dataset of 884 biopsies with matching BAL samples without the matching plasma restriction. Chemokine differences during the histopathologic injury patterns and "normal" biopsies were evaluated using mixed effects models with random intercepts to account for repeated measurements from both recipient and study center.[18] Chemokine levels were log2 transformed given the non-normal distribution. Covariates for time from transplant (≥6 months, "late biopsy") and clinical indication ("surveillance") were a priori chosen to be included in multivariable models based on biologic plausibility.

To evaluate the risk of CLAD development, Cox proportional hazards models were constructed with time-dependent dichotomous variables for each histopathologic injury pattern and infection (Table 5). For example, the variable for AR started at 0 for each recipient and increased to 1 at the first acute rejection episode. The AR variable remained at 1 until the next biopsy showing no AR, at which point it decreased from 1 to 0. At the next episode of AR, the AR variable would again increase from 0 to 1. The impact of plasma CXCL9 and CXCL10 levels during histopathologic injury on CLAD risk was determined using time-dependent variables for the injuries with quartile cutoffs of the chemokines measured at the time of the injury (Table 6). To further explore the association between the injury patterns and plasma chemokines on subsequent CLAD risk, we constructed Cox proportional hazards models with interaction terms for the injury patterns and "high" CXCL9 and CXCL10, where "high" was defined as greater than the 50th percentile for each chemokine during allograft injuries (Table 7). Thus, the variables for the injury only (with low chemokines), high chemokine only (without injury) and the interaction term (injury with high chemokine) were compared with the reference group (no injury with low chemokine). All analysis was performed using SAS v9.4 (Cary, NC).

3. RESULTS:

3.1 Cohort characteristics

721 biopsies with matching BAL and plasma samples from 184 recipients were included in the baseline analysis dataset (Figure 1). 49 (27%) recipients developed CLAD, while 135 (73%) did not during a median (Q1, Q3) follow-up period of 3 years (1.6, 3.3) post-transplant. The median time to CLAD onset was 1.6 (1.1, 2.6) years. 22 (12%) recipients died during the follow-up period without a diagnosis of CLAD with median (Q1, Q3) time to death of 1.4 (0.9, 2.7) years. Table 1 describes the recipient characteristics by CLAD development. Overall, those who developed CLAD were similar to those who did not. The median number of transbronchial biopsies and the time to first biopsy were similar between the CLAD and no CLAD recipients.

3.2 Incidence of Histopathologic Injury During the First-Year Post-Transplant

Frequency of each histopathologic diagnosis is described in Table 2 for the entire cohort and by bronchoscopy clinical indication. There were 188 (26%) episodes of "allograft injury" (AR, LB, OP or ALI) from 115 recipients. There were 129 (18%) episodes of AR, 22

(3%) episodes of LB, 39 (5%) episodes of OP, and 29 (4%) episodes of ALI. 641 (89%) were “surveillance” biopsies, while 80 (11%) were performed for clinical symptoms (“for cause”). The frequency of the histopathologic diagnoses did not differ significantly between “surveillance” and “for cause” biopsies. 29 (4%) biopsies had more than one concurrent histopathologic diagnosis observed.

234 (32%) BALs were positive for the detection of pathogenic organisms: 104 (44%) bacterial, 26 (15%) mycobacterial, 90 (38%) fungal and 67 (9%) viral. There were 94 (13%) episodes of “infection” based on either: 1) pathogen detection with clinical symptoms or signs, or 2) purulence noted on bronchoscopy. 42 (53%) of the “for cause” bronchoscopies and 52 (8%) of the “surveillance” bronchoscopies were classified as infection.

3.3 Chemokine Levels During Histopathologic Injury

Table 3 describes CXCL9 and CXCL10 levels from plasma samples collected within a ± 7-day window of the bronchoscopies, as well as the BAL samples collected at the bronchoscopy. Median plasma CXCL9 and CXCL10 levels were higher during episodes of “allograft injury” versus “healthy” biopsies: 428.8 vs 330.5 and 913.0 vs 760.0, respectively. BAL CXCL9 and CXCL10 measurements showed a similar pattern with higher levels during “allograft injury” versus “healthy” biopsies: 222.0 vs 91 and 470.0 vs 196.5, respectively.

To evaluate differences in CXCL9 and CXCL10 levels between the allograft injuries and healthy biopsies, multivariable mixed effects models were constructed with time from transplant and clinical indication as covariates (Table 4). AR and ALI were associated with plasma CXCL9 fold-increases (95% CI) of: 1.3 (1.1–1.5) and 1.5 (1.1–2.1), compared with healthy bronchoscopies. Similarly, AR and ALI were associated with plasma CXCL10 fold-increases (95% CI) of 1.3 (1.1–1.6) and 1.5 (1.0–2.2), respectively. Plasma CXCL9 and CXCL10 levels were not elevated during LB, OP or infection.

Similarly, BAL CXCL9 levels were increased during AR, ALI and infection with the following fold-increases (95% CI): 1.5 (1.2–2.0), 2.1 (1.3–3.5) and 2.6 (1.9–3.7), respectively. BAL CXCL10 levels were increased during these same injury patterns with fold-changes (95% CI): 1.9 (1.4–2.6), 1.8 (1.0–3.2), and 1.8 (1.2–2.6), respectively. BAL CXCL9 and CXCL10 levels were not elevated during LB or OP.

3.4 Risk of CLAD after Histopathologic Injury

Univariable Cox proportional hazards models were constructed to evaluate the risk of CLAD development after each of the histopathologic injury patterns and infection (Table 5). ALI was associated with increased CLAD risk (HR 5.0 95% CI 1.9–13.0), while AR did not meet statistical significance (HR 2.0 95% CI 0.97–4.1). LB, OP and infection were not associated with increased CLAD risk. In the multivariable model including the injury patterns, infection, time from transplant and clinical indication, AR (HR 2.2 95% CI 1.0–4.8) and ALI (HR 5.9 95% CI 2.2–15.9) were associated with CLAD, while LB, OP and infection were not.

3.5 Chemokine Levels During Histopathologic Injury on CLAD risk

To explore the impact of plasma and BAL CXCL9 and CXCL10 levels during histopathologic injury on CLAD risk, Cox proportional hazards models for CLAD were constructed with time-dependent variables for the injury patterns associated with CLAD (allograft injury, AR and ALI) using quartile cutoffs of CXCL9 and CXCL10. Overall, there was a “dose-response” relationship noted between increasing CXCL9 and CXCL10 levels and CLAD risk (Table 6). The HR (95% CI) of CLAD for an episode of allograft injury with plasma CXCL9 greater than the 25th (252.0 MFI), 50th (428.8 MFI) and 75th (828.5 MFI) percentiles was 2.9 (1.6–5.2), 2.9 (1.5–5.3) and 4.3 (2.2–8.5), respectively. The HR (95% CI) for AR with these same plasma CXCL9 cutoff were: 2.3 (1.1–4.7), 2.5 (1.2–5.3) and 4.0 (1.7–9.5), respectively. The HR (95% CI) for ALI with these CXCL9 cutoffs were: 5.1 (2.0–13.3), 9.6 (3.3–27.9) and 9.0 (2.7–29.8), respectively.

Similarly, the HR (95% CI) of CLAD for an episode of allograft injury with plasma CXCL10 greater than the 25th (478.5 MFI), 50th (913.0 MFI) and 75th (2558.0 MFI) percentiles was: 3.2 (1.7–5.8), 3.6 (1.9–6.9) and 6.4 (3.0–13.3), respectively. The HR (95% CI) for AR with these plasma CXCL10 cutoff were: 3.2 (1.5–6.8), 2.9 (1.3–6.5) and 3.7 (1.3–10.5), respectively. The HR (95% CI) for ALI with these CXCL10 cutoffs were: 5.1 (2.0–13.3), 4.9 (1.7–13.8) and 8.6 (2.6–28.7), respectively.

The dose-response relationship between increasing CXCL9 and CXCL10 level during allograft injury and CLAD risk was more variable in the BAL. The HR (95% CI) of CLAD for an episode of allograft injury with BAL CXCL9 greater than the 25th (81.0 MFI), 50th (222.0 MFI) and 75th (732.5 MFI) percentiles was 1.9 (1.0–3.4), 2.3 (1.2–4.4) and 2.4 (1.1–5.1), respectively. Similarly, the HR (95% CI) of CLAD for an episode of allograft injury with BAL CXCL10 greater than the 25th (124.0 MFI), 50th (470.0 MFI) and 75th (1710.5 MFI) percentiles was: 1.6 (0.9–3.0), 2.0 (1.1–3.8) and 2.5 (1.2–5.2), respectively. The dose-response relationship for the BAL chemokines during AR and ALI was more variable and often not statistically significant (Table 6).

3.6 Histopathologic Injury and Chemokine Interactions

To further explore the association between the injury patterns and plasma chemokines, Cox proportional hazards models were constructed with three dummy variables for the combination of injury (yes or no) and high plasma chemokines (≥50th percentile or no), compared to the reference group: “No injury with low chemokine levels”. For the injury patterns associated with CLAD (allograft injury, AR and ALI), there was a strong interaction between the injury pattern and high plasma chemokines (Table 7). A single episode of allograft injury with high CXCL9 was associated with HR of CLAD of 3.0 (95% CI 1.5–5.9), while episodes of allograft injury only (with low CXCL9) and high CXCL9 only (without allograft injury) were not associated with CLAD risk. Similarly, AR with high CXCL9 was associated with CLAD (HR 2.5 95% CI 1.1–5.6), while AR only and high CXCL9 only were not. ALI with high CXCL9 had a HR of 10.8 (95% CI 3.6–32.6), while ALI only and high CXCL9 only were not associated with CLAD. Evaluation of the interaction between LB, OP and plasma chemokines was limited by low sample size in the injury with high chemokine groups (data not shown).

There were also strong interactions observed between the injury patterns (allograft injury, AR and ALI) and plasma CXCL10 levels (Table 7). An episode of allograft injury with high CXCL10 had a HR (95% CI) of 4.4 (2.2–9.1), while allograft injury only and high CXCL10 only were not associated with CLAD risk. Similarly, AR with high CXCL10 had a HR of 3.4 (1.4–8.1), while AR only and high CXCL10 only were not significant. ALI with high CXCL10 had a HR of 6.0 (2.0–17.8), while ALI only and high CXCL10 only were not significant.

BAL CXCL9 and CXCL10 also showed a similar interaction between the injury patterns associated with CLAD (allograft injury, AR and ALI) and chemokine levels (Table 7). Allograft injury with high BAL CXCL9 had a HR of 3.2 (1.5–6.5), while allograft injury only and high CXCL9 only were not associated with CLAD risk. AR with high CXCL9 had a HR of 2.6 (1.1–6.4), while AR only and high CXCL9 only were not significant. ALI was associated with CLAD regardless of BAL CXCL9 levels: ALI only, High BAL CXCL9 only and ALI with high BAL CXCL9 had HRs for CLAD of 10.0 (1.3–78.9), 1.8 (1.0–3.1) and 5.6 (1.9–16.4), respectively.

Similarly, allograft injury with high BAL CXCL10 had a HR of 2.9 (1.4–6.2), while allograft injury only and high CXCL10 only were not associated with CLAD (Table 7). AR with high BAL CXCL10 had a HR of 2.5 (1.0–6.3), high CXCL10 only had a HR of 1.9 (1.0–3.4), while AR only was not significant. ALI with high CXCL10 had a HR of 6.0 (2.0–17.8), while ALI only and high CXCL10 only were not significant. Evaluation of the interaction between LB, OP and BAL chemokines was limited by low sample size in the injury with high chemokine groups.

3.7 Respiratory Infection and Chemokine Interactions

In keeping with our prior single center results showing the association between BAL CXCL9 and CXCL10 elevation during various respiratory infections with CLAD development, we evaluated the interaction between respiratory infection, plasma / BAL chemokines and CLAD risk. For plasma CXCL9 and CXCL10, there was no interaction observed between infection and chemokines with CLAD development (Table 8). There was however, an interaction noted between BAL CXCL9 and infection on CLAD risk. The HR (95% CI) for CLAD for infection with high BAL CXCL9, infection only and high BAL CXCL9 only was 2.6 (1.1–5.9), 0.8 (0.1–5.9), and 1.8 (1.0–3.3) respectively. Similarly, the HR (95% CI) for CLAD for infection with high BAL CXCL10, infection only and high BAL CXCL10 only was 3.0 (1.3–7.0), 0.6 (0.1–4.3), and 1.6 (0.9–2.9) respectively.

4. DISCUSSION:

This prospective multi-center study evaluated 721 biopsies with matching plasma and BAL samples from 184 recipients at 5 large North American lung transplant centers. In multivariable Cox models with time-dependent covariates, there was a strong association between AR and ALI with subsequent CLAD development. Plasma CXCL9 and CXCL10 levels were significantly elevated during these two injury patterns with a dose-response relationship between increasing CXCL9 and CXCL10 levels and CLAD risk. To further evaluate the association between allograft injury, plasma chemokines and CLAD

development, we modeled interaction terms for the injury patterns and chemokine levels. There were strong interactions found between allograft injury, AR and ALI and high plasma chemokine levels. The significance of these interaction term indicates a non-additive effect between the histopathologic injury and plasma chemokines on CLAD risk. More specifically, the increase in CLAD risk associated with AR and ALI are dependent on the presence of elevated plasma CXCL9 and CXCL10.

The explanation for this interaction remains unclear, but naïve T-cells require three distinct signals for activation: allorecognition (signal 1), costimulation (signal 2) and inflammatory cytokines (signal 3). Cytokines including IFN-γ and IL-12 have been shown to provide the critical third signal to determine whether antigen presentation with costimulation to naïve T-cells will lead to tolerance versus clonal expansion, development of effector function and establishment of a memory population.[23–26] Several prior studies have shown that although IFN-γ levels remain low in the blood, there is amplification of the downstream chemokines CXCL9 and CXCL10.[27,28] Thus, plasma CXCL9 and CXCL10 may reflect the IFN-γ dependent signaling pathway required for the development of a sustained immune response against the allograft, ultimately leading to CLAD. The current study confirms the prognostic importance of AR and ALI in terms of CLAD risk, and supports the involvement of a Th1 immune response mediated by CXCL9 and CXCL10.

These findings add to our current understanding regarding the significance of BAL cytokines on CLAD risk. Two prior single center studies from our group found AR and ALI to be the strongest histopathologic predictors of subsequent CLAD development.[8,9] In these single center studies, BAL CXCL9 and CXCL10 levels were elevated during these histopathologic injuries, with elevations reflecting the relative risk of CLAD development: ALI had the highest HR for CLAD with the highest BAL CXCL9 and CXCL10 levels, followed by AR.

The current multicenter study confirms our prior single center results. BAL CXCL9 and CXCL10 were again increased during AR and ALI. However, the dose-response relationship between increasing CXCL9 and CXCL10 levels and CLAD risk was more variable in the BAL compared with the plasma with several chemokine cutoffs not reaching statistical significance. We speculate that this increase variability may be due to differences in BAL techniques by the bronchoscopists across the study centers.[18] Importantly, allograft injury and chemokine interactions were similar between the BAL and plasma compartments. Elevated BAL CXCL9 and CXCL10 during allograft injury, AR and ALI increased CLAD risk with similar effect size and significance as the plasma. Taken together, both the plasma and BAL CXCL9 and CXCL10 levels appear to provide prognostic data regarding CLAD risk.

Similar to the current study, we previously found that lymphocytic bronchiolitis[8,9] and organizing pneumonia[29] did not predict CLAD development in multivariable models adjusted for the other injury patterns. However, episodes of OP with elevated BAL CXCL9 and CXCL10 levels were associated with CLAD with a dose-response relationship. Evaluation of the interaction between LB, OP and chemokine levels was limited in the current study by sample size.

To our surprise, infection was not associated with CLAD in the univariable or multivariable models. This reason for this lack of association was unclear, but we speculate that it may have been due to the heterogeneity of the various respiratory infections captured with our definition. Stratification by infection types was not possible due to the available sample size. The current study evaluated chemokine levels during infection, given the prior studies by our group demonstrating this association.[30–33] We found that CXCL9 and CXCL10 levels were not elevated in the plasma during infections, but significantly elevated in the BAL. This may be due to the fact that most infections occur in the distal airways making BAL sampling easy, while only severe episodes of infection affecting the alveoli would be captured in the plasma. We furthermore found no interaction between infection and plasma chemokines, while infection with elevated BAL chemokines was associated with increased CLAD risk. Thus, the BAL compartment may be preferable to risk stratify infections regarding CLAD risk. We feel that further studies evaluating the relative significance of CXCL9 and CXCL10 in the plasma and BAL compartments are warranted.

To our knowledge, our study is the first to evaluate plasma chemokine levels during the histopathologic allograft injuries of AR, LB, OP and ALI. The ability to evaluate and potentially use a plasma biomarker has several advantages over BAL biomarkers. Blood draws are less invasive than bronchoscopy, can be obtained more frequently, and not as easily affected by procedural factors such has BAL technique and lavage volume.[18] Other strengths of the study include robust serial prospective data collection in a multi-center present-day cohort undergoing regular surveillance biopsies and blood draws. Significant effort was made by the study investigators before the start of the study to harmonize the clinical data collection, histopathologic grading and the primary CLAD endpoint.

A major limitation of this study is the potential for confounding given the observational design, especially with regards to differences in practice by center. This includes differences in transbronchial biopsy procedures, pathologic evaluation, clinical management, and other factors that are unmeasured. Given the limited episodes of allograft pathology, we were unable to stratify the analysis by study center. We attempted to control for unmeasured study center differences by including study center as a random effect variable for all mixed effects models, but not the Cox proportional hazards models (due to sample size). Samples sizes for LB and OP were limited and the analysis was underpowered to detect smaller differences in chemokine levels and effects on CLAD risk. The limited number of CLAD endpoints in this cohort did not allow for stratification by the CLAD phenotypes. Finally, adjusting the analysis for all known CLAD risk factors and individual treatments administered for allograft injuries was beyond the scope of this study.

Despite these limitations, this study improves our understanding of the association between histopathologic allograft injury and CLAD development. It supports our hypothesis that this association is mediated at least in part by a Th1 immune response involving CXCL9 and CXCL10, and that this response can be quantified as plasma and BAL CXCL9 and CXCL10 levels. The identification of a biomarker that can stratify allograft injuries in terms of subsequent CLAD risk could significantly improve outcomes by allowing for augmented therapy for high risk injuries while minimizing treatment related side effects for

low risk injuries. We believe that further work on this topic as a strategy to decrease CLAD development is warranted.

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Abbreviations:

AR	acute rejection
ALI	acute lung injury
BAL	bronchoalveolar lavage
CLAD	chronic lung allograft dysfunction
FEV1	forced expiratory volume in 1 second
IP10	interferon-γ induced protein 10
LB	lymphocytic bronchiolitis
MFI	median fluorescence intensities
MIG	monokine induced by interferon-γ
NK	natural killer
OP	organizing pneumonia
PFT	pulmonary function test

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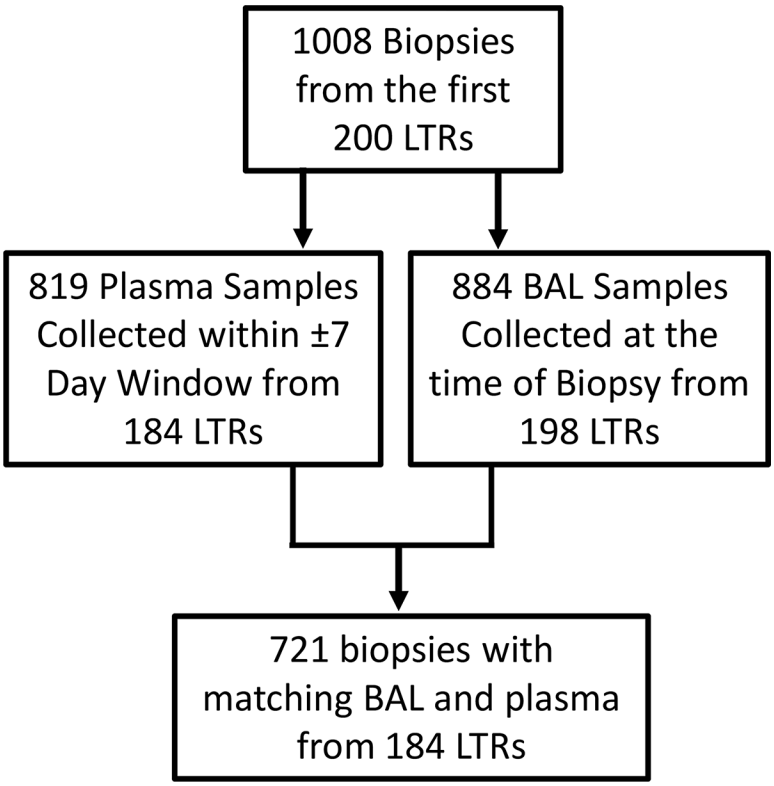

figure 1:
Flow diagram of patient and sample size used in the analysis. BAL, bronchoalveolar lavage; LTR, lung transplant recipients.

Table 1.

Baseline Patient Characteristics

	Patients Without CLAD		Patients With CLAD	
	n	%	n	%
Number of patients:	135	73%	49	27%
Pre-Transplant Characteristics				
Race				
White	121	90%	41	84%
Black	9	7%	6	12%
Other	5	4%	2	4%
Mean age at transplant (sd)	59	(14)	61	(16)
Female Sex	43	32%	20	41%
Native lung disease				
Restrictive	76	56%	25	51%
Obstructive	37	27%	13	27%
Cystic Fibrosis	17	13%	8	16%
Pulmonary Vascular	5	4%	3	6%
Bilateral lung transplant	103	76%	35	71%
LAS score at transplant (sd)	37	(11)	39	(10)
Post-Transplant Characteristics				
Induction immunosuppression				
ATG	9	7%	3	6%
Basiliximab	66	49%	27	55%
None	60	44%	19	39%
Primary Graft Dysfunction ǂ				
Grade 0	15	11%	8	16%
Grade 1	66	49%	21	43%
Grade 2	29	21%	9	18%
Grade 3	25	19%	11	22%
Primary immunosuppression ǂǂ				
Tacrolimus	109	81%	37	76%
Cyclosporine	26	19%	13	27%
Secondary immunosuppression ǂǂ				
Mycophenolate	119	88%	42	86%
Azathioprine	14	10%	5	10%
Other	2	1%	2	4%
Total number of biopsies:				
Median days to first biopsy (range)	44	(16–363)	37	(18–275)
Median # biopsies per subject (range)	4	(1–9)	4	(1–7)

Definition of abbreviations: COPD = chronic obstructive lung disease, ILD = interstitial lung disease, ATG = anti-thymocyte globulin.

ǂHighest at 72 hours.

At post-transplant discharge.

Table 2.

Bronchoscopy Findings by Clinical Indication

	Surveillance		For Cause		Total	
	n	%	n	%	n	%
Total Number of Biopsies	**641**	**89%**	**80**	**11%**	**721**	**100%**
Allograft Injury ǂ	**167**	**26%**	**21**	**26%**	**188**	**26%**
Acute Rejection	**116**	**18%**	**13**	**16%**	**129**	**18%**
None (A0)	478	75%	59	74%	539	75%
Minimal (A1)	84	13%	10	13%	94	13%
Mild (A2)	32	5%	3	4%	35	5%
Moderate (A3)	0	0%	0	0%	0	0%
Severe (A4)	0	0%	0	0%	0	0%
Ungradable (AX)	45	7%	8	10%	53	7%
Lymphocytic Bronchiolitis	**18**	**3%**	**4**	**5%**	**22**	**3%**
None (B0)	450	70%	48	60%	500	69%
Low-grade (B1R)	18	3%	4	5%	22	3%
High-grade (B2R)	0	0%	0	0%	0	0%
Ungradable (BX)	171	27%	28	35%	199	28%
Organizing pneumonia	**32**	**5%**	**7**	**9%**	**39**	**5%**
Acute lung injury	**24**	**4%**	**5**	**6%**	**29**	**4%**
Pathogen detection ǂǂ	**195**	**30%**	**39**	**49%**	**234**	**32%**
Infection ǂǂǂ	**52**	**8%**	**42**	**53%**	**94**	**13%**

ǂAllograft injury: Acute rejection, lymphocytic bronchiolitis, organizing pneumonia or acute lung injury.

ǂǂPathogen detection is defined as the detection of a pathogenic organism.

ǂǂǂInfection is defined as (pathogenic detection with clinical symptoms or radiographic findings) or purulence observed on bronchoscopy.

Table 3.

Plasma and BAL Cytokine Median MFIs By Histopathologic Findings

	Plasma			BAL		
	n	CXCL9	CXCL10	n	CXCL9	CXCL10
Healthy	243	330.5	760.0	301	91.0	196.5
Allograft Injury	188	428.8	913.0	249	222.0	470.0
AR ♯	129	427.5	1,107.0	173	263.0	543.5
LB	22	333.0	936.8	30	201.5	494.0
OP	39	414.0	695.0	52	119.0	307.3
ALI	29	505.5	1,129.0	42	333.3	1,030.0
Pathogen ♯♯	234	377.0	820.5	30	361.0	591.0
Infection ♯♯♯	94	473.8	1,042.3	126	475.0	827.3

♯AR includes ≥A1 rejection.

♯♯Pathogen is defined as the detection of a pathogenic organism

♯♯♯Infection = pathogen detection (with symptoms or radiolographic findings) or purulence on bronchoscopy.

Table 4.

Multivariable Models for Plasma and BAL MFIs By Histopathologic Findings

	Plasma CXCL9			BAL CXCL9		
	Fold chang	95% CI	p-value ǂ	Fold chang	95% CI	p-value ǂ
AR	1.3	1.1 – 1.5	0.0062	1.5	1.2 – 2.0	0.0015
LB	1.2	0.8 – 1.7	0.3878	1.6	0.9 – 2.7	0.0930
OP	1.0	0.8 – 1.4	0.7920	1.0	0.6 – 1.5	0.9062
ALI	1.5	1.1 – 2.1	0.0213	2.1	1.3 – 3.5	0.0028
Infection ǂǂ	1.1	0.9 – 1.4	0.4532	2.6	1.9 – 3.7	0.0001
Late Biopsy ǂǂǂ	1.1	1.0 – 1.2	0.2332	0.9	0.7 – 1.1	0.3289
Surveillance	0.8	0.6 – 1.0	0.0616	0.9	0.6 – 1.3	0.5361
	Plasma CXCL10			**BAL CXCL10**		
	Fold chang	95% CI	p-value ǂ	Fold chang	95% CI	p-value ǂ
AR	1.3	1.1 – 1.6	0.0063	1.9	1.4 – 2.6	0.0001
LB	1.0	0.7 – 1.5	0.9528	1.0	0.6 – 1.9	0.9648
OP	0.8	0.6 – 1.1	0.1040	0.7	0.5 – 1.2	0.2491
ALI	1.5	1.0 – 2.2	0.0337	1.8	1.0 – 3.2	0.0434
Infection ǂǂ	1.2	1.0 – 1.6	0.0887	1.8	1.2 – 2.6	0.0049
Late Biopsy ǂǂǂ	0.9	0.8 – 1.1	0.2116	1.2	0.9 – 1.5	0.2308
Surveillance	0.8	0.6 – 1.0	0.1079	0.7	0.5 – 1.0	0.0751

ǂP-values based on mixed effects model. Log2 MFIs used.
Study center and subject are included as a random intercept.
Covariables are as listed in table: AR, LB, OP, ALI, infection, late biopsy and surveillance.

ǂǂInfection = pathogen detection (with symptoms or radiographic findings) or purulence on bronchoscopy.

ǂǂǂLate biopsy: ≥ 6-months from transplant.

Table 5.

Risk of CLAD By Histopathologic Findings

Univariable Models:	**n**	**HR**	**95% CI**	**p-value** ≠
Allograft Injury	188	2.6	1.5 – 4.6	0.0013
AR (>=A1)	129	2.0	1.0 – 4.1	0.0605
LB	22	1.0	0.1 – 7.2	0.9907
OP	39	1.4	0.4 – 4.5	0.5730
ALI	29	5.0	1.9 – 13.0	0.0009
Infection	94	1.6	0.7 – 3.8	0.2903
Multivariable Model:	**n**	**HR**	**95% CI**	**p-value** ≠
AR (>=A1)	93	2.2	1.0 – 4.8	0.0499
LB	21	0.6	0.1 – 4.8	0.6605
OP	30	1.1	0.3 – 3.7	0.8919
ALI	23	5.9	2.2 – 15.9	0.0004
Infection	59	1.5	0.6 – 3.7	0.3790
Late Biopsy ##	274	0.6	0.2 – 1.7	0.3589
Surveillance	459	2.8	0.4 – 20.5	0.3125

≠P-values based on Cox proportional hazards models.
Multivariable model includes: AR, LB, OP, ALI, infection, late biopsy and surveillance.

##Late biopsy: ≥ 6-months from transplant.

Table 6.

Risk of Chronic Lung Allograft Dysfunction By Allograft Injury and Chemokine Levels

	Plasma CXCL9				BAL CXCL9			
Univariable Models:	**n**	**HR**	**95% CI**	**p-value ǂ**	**n**	**HR**	**95% CI**	**p-value ǂ**
Allograft Injury with CXCL9 > 25th:	141	2.9	1.6 – 5.2	0.0003	188	1.9	1.0 – 3.4	0.0358
Allograft Injury with CXCL9 > 50th:	94	2.9	1.5 – 5.3	0.0009	125	2.3	1.2 – 4.4	0.0084
Allograft Injury with CXCL9 > 75th:	47	4.3	2.2 – 8.5	0.0001	54	2.4	1.1 – 5.1	0.0233
AR with CXCL9 > 25th:	98	2.3	1.1 – 4.7	0.0245	140	1.6	0.8 – 3.5	0.2048
AR with CXCL9 > 50th:	64	2.5	1.2 – 5.3	0.0191	92	2.1	0.9 – 4.9	0.8860
AR with CXCL9 > 75th:	35	4.0	1.7 – 9.5	0.0016	46	2.7	1.1 – 6.8	0.0354
ALI with CXCL9 > 25th:	26	5.1	2.0 – 13.3	0.0007	31	4.2	1.5 – 11.9	0.0067
ALI with CXCL9 > 50th:	17	9.6	3.3 – 27.9	0.0001	25	4.3	1.5 – 12.1	0.0063
ALI with CXCL9 > 75th:	9	9.0	2.7 – 29.8	0.0004	13	3.0	0.7 – 12.8	0.1375
	Plasma CXCL10				**BAL CXCL10**			
Univariable Models:	**n**	**HR**	**95% CI**	**p-value ǂ**	**n**	**HR**	**95% CI**	**p-value ǂ**
Allograft Injury with CXCL10 > 25th:	141	3.2	1.7 – 5.8	0.0002	187	1.6	0.9 – 3.0	0.1093
Allograft Injury with CXCL10 > 50th:	94	3.6	1.9 – 6.9	0.0001	125	2.0	1.1 – 3.8	0.0322
Allograft Injury with CXCL10 > 75th:	47	6.4	3.0 – 13.3	0.0001	63	2.5	1.2 – 5.2	0.0118
AR with CXCL10 > 25th:	98	3.2	1.5 – 6.8	0.0031	135	1.5	0.7 – 3.3	0.3271
AR with CXCL10 > 50th:	71	2.9	1.3 – 6.5	0.0089	94	1.8	0.8 – 4.3	0.1608
AR with CXCL10 > 75th:	35	3.7	1.3 – 10.5	0.0134	47	2.4	0.9 – 6.6	0.0965
ALI with CXCL10 > 25th:	26	5.1	2.0 – 13.3	0.0007	30	4.5	1.6 – 12.7	0.0048
ALI with CXCL10 > 50th:	16	4.9	1.7 – 13.8	0.0031	23	4.6	1.6 – 13.0	0.0041
ALI with CXCL10 > 75th:	10	8.6	2.6 – 28.7	0.0005	20	4.8	1.7 – 13.7	0.0031

ǂ P-values based on Univariable Cox proportional hazards models.

ǂǂ P-values: * < 0.05, ** <0.01, *** <0.001.

ǂǂ Variables are not mutually exclusive: “>25th” = 25th-100th percentile, “>50th” = 50th to 100th percentile, etc.

Table 7.

Risk of Chronic Lung Allograft Dysfunction By Histopathologic Findings and CXCL9 and CXCL10 Interaction

	Plasma CXCL9				BAL CXCL9			
	n	HR ǂ	95% CI	p-value ǂ	n	HR ǂ	95% CI	p-value ǂ
Allograft injury only	94	1.8	0.7 – 4.7	0.2569	124	1.8	0.8 – 4.4	0.1747
High CXCL9 only	133	0.9	0.4 – 2.0	0.8494	161	1.9	1.0 – 3.8	0.0587
Allograft Injury with CXCL9	94	3.0	1.5 – 5.9	0.0016	125	3.2	1.5 – 6.5	0.0016
AR only	65	0.8	0.1 – 6.0	0.8367	81	1.3	0.4 – 4.4	0.6543
High CXCL9 only	213	1.0	0.5 – 1.9	0.9409	261	1.7	1.0 – 3.1	0.0639
AR with high CXCL9	64	2.5	1.1 – 5.7	0.0280	92	2.6	1.1 – 6.4	0.0365
ALI only	12	1.9	0.2 – 14.7	0.5373	17	10.0	1.3 – 78.9	0.0295
High CXCL9 only	280	1.3	0.7 – 2.3	0.4218	361	1.8	1.0 – 3.1	0.0470
ALI with high CXCL9	17	10.8	3.6 – 32.6	0.0001	25	5.6	1.9 – 16.4	0.0018
	Plasma CXCL10				BAL CXCL10			
	n	HR ǂ	95% CI	p-value ǂ	n	HR ǂ	95% CI	p-value ǂ
Allograft injury only	94	1.7	0.7 – 4.4	0.2424	124	2.2	1.0 – 5.2	0.0637
High CXCL10 only	155	1.5	0.7 – 3.2	0.2563	154	1.9	1.0 – 3.7	0.0656
Allograft Injury with CXCL10	94	4.4	2.2 – 9.1	0.0001	125	2.9	1.4 – 6.2	0.0052
AR only	58	1.0	0.2 – 4.2	0.9812	79	1.7	0.0 – 5.6	0.4207
High CXCL10 only	236	1.2	0.7 – 2.3	0.5142	254	1.9	1.0 – 3.4	0.0401
AR with high CXCL10	71	3.4	1.4 – 8.1	0.0060	94	2.5	1.0 – 6.3	0.0479
ALI only	13	6.5	0.8 – 53.0	0.0783	19	6.4	0.8 – 49.9	0.0746
High CXCL10 only	310	1.5	0.8 – 2.7	0.1710	357	1.6	0.9 – 2.9	0.0879
ALI with high CXCL10	16	6.0	2.0 – 17.8	0.0013	23	6.0	2.0 – 17.8	0.0012

ǂP-values based on Multivariable Cox proportional hazards models.

Table 8.

Risk of Chronic Lung Allograft Dysfunction By Respiratory Infection and CXCL9 and CXCL10 Interaction

	Plasma CXCL9				BAL CXCL9			
	n	HR ǂ	95% CI	p-value ǂ	n	HR ǂ	95% CI	p-value ǂ
Infection only	45	1.0	0.1 – 7.3	0.9793	40	0.8	0.1 – 5.9	0.8187
High CXCL9 only	247	1.3	0.7 – 2.5	0.3332	298	1.8	1.0 – 3.3	0.0389
Infection with high CXCL9	49	2.2	0.8 – 5.8	0.1154	86	2.6	1.1 – 5.9	0.0301
	Plasma CXCL10				BAL CXCL10			
	n	HR ǂ	95% CI	p-value ǂ	n	HR ǂ	95% CI	p-value ǂ
Infection only	42	1.5	0.3 – 6.4	0.5895	55	0.6	0.1 – 4.3	0.5958
High CXCL10 only	272	1.6	0.9 – 2.9	0.1199	307	1.6	0.9 – 2.9	0.1098
Infection with high CXCL10	52	2.3	0.8 – 6.8	0.1277	71	3.0	1.3 – 7.0	0.0132

ǂP-values based on Multivariable Cox proportional hazards models.