Published in final edited form as:

Am J Transplant. 2021 October ; 21(10): 3401–3410. doi:10.1111/ajt.16601.

Correlation between BAL CXCR3 Chemokines and Lung Allograft Histopathologies: A Multi-Center Study

Michael Y. Shino[1], **Ning Li**[1], **Jamie L. Todd**[2], **Megan L. Neely**[2], **Heather Kopetskie**[3], **Michelle L. Sever**[3], **Jerry Kirchner**[2], **Courtney W. Frankel**[2], **Laurie D. Snyder**[2], **Elizabeth N. Pavlisko**[2], **Tereza Martinu**[4], **Lianne G. Singer**[4], **Wayne Tsuang**[5], **Marie Budev**[5], **Pali D. Shah**[6], **John M. Reynolds**[2], **Nikki Williams**[7], **Mark A. Robien**[7], **Scott M. Palmer**[2], **S. Sam Weigt**[1], **John A. Belperio**[1]

[1] –University of California Los Angeles; Los Angeles, CA

[2] –Duke University Medical Center; Durham, NC

[3] –Rho; Durham, NC

[4] –University of Toronto; Ontario, Canada

[5] –Cleveland Clinic; Cleveland, OH

[6] –John Hopkins University; Baltimore, MD

[7] –National Institute of Allergy and Infectious Diseases; Washington DC

Abstract

The histopathologic diagnosis of acute allograft injury is prognostically important in lung transplantation with evidence demonstrating a strong and consistent association between acute rejection (AR)(1–7), acute lung injury (ALI)(8, 9) and the subsequent development of chronic lung allograft dysfunction (CLAD). The pathogenesis of these allograft injuries however, remains poorly understood. CXCL9 and CXCL10 are CXC chemokines induced by interferon-γ and act as potent chemoattractants of mononuclear cells. We hypothesized that these chemokines are involved in the mononuclear cell recruitment associated with AR and ALI. We further hypothesized that the increased activity of these chemokines could be quantified as increased levels in the bronchoalveolar lavage fluid. In this prospective multicenter study, we evaluate the incidence of histopathologic allograft injury development during the first-year post-transplant and measure bronchoalveolar CXCL9 and CXCL10 levels at the time of the biopsy. In multivariable models, CXCL9 levels were 1.7-fold and 2.1-fold higher during AR and ALI compared with “normal” biopsies without histopathology. Similarly, CXCL10 levels were 1.6-fold and 2.2-fold higher during these histopathologies, respectively. These findings support the association of

Correspondence: Michael Y. Shino mshino@mednet.ucla.edu.

Disclosure

The authors of this study have no conflicts of interest to disclose as described by the *American Journal of Transplantation.*

Supporting Information

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

CXCL9 and CXCL10 with episodes of AR and ALI, and provide potential insight into the pathogenesis of these deleterious events.

1. INTRODUCTION

Chronic lung allograft dysfunction (CLAD) is the leading cause of death and the major factor limiting long-term survival after lung transplantation (10). Since there are no known effective therapies for CLAD, the identification and avoidance of key modifiable risk factors for CLAD is a crucial step towards improving long-term graft function and survival. Prior single-center studies have found acute cellular rejection (AR)(1–7) and acute lung injury (ALI)(8, 9) to be the histopathologic injury patterns most consistently associated with subsequent CLAD development. Despite the clinical importance of these histopathologic diagnoses, their incidence has not been well defined in multi-center studies. Furthermore, the pathobiology of these injury patterns remains poorly understood.

AR is defined by perivascular mononuclear cell infiltration that may extend to the adjacent alveolar septa with elevated grades of rejection (11). AR is graded from A0 to A4 based on the extent of mononuclear cell infiltration. ALI, the most severe form of acute allograft injury, has an initial exudative phase with alveolar hyaline membrane formation leading to a proliferative phase with marked mononuclear cell infiltration into the interstitum and alveoli (11). Histologically, these allograft injury patterns share a common theme involving the extravasation and infiltration of leukocytes into the area of injury. CXCL9 (MIG) and CXCL10 (IP10) are CXC chemokines which are induced by interferon-γ and signals through a G protein-coupled receptor, CXCR3. These CXCR3 chemokines are potent chemoattractants for mononuclear cells (e.g., activated T-cells and NK cells) in a Type I immune response. In animal models, we and others have demonstrated the key role of CXCR3 and its ligands in the pathogenesis of AR and CLAD, with in vivo neutralization of CXCR3 or its ligands leading to profound attenuation of both AR and CLAD development (12, 13). In clinical studies, we showed augmented immunohistochemical expression of CXCL9 and CXCL10 by alveolar and airway epithelial cells, as well as augmented CXCR3 expression by infiltrating mononuclear cells during ALI (8). These single center studies also showed AR and ALI to be the strongest histopathologic risk factors for CLAD development (7, 8). Importantly, bronchoalveolar lavage (BAL) fluid CXCL9 and CXCL10 levels were significantly elevated during these histopathologic events compared to “normal” biopsies. BAL CXCL9 and CXCL10 elevations reflected the relative risk of CLAD development: ALI had the highest hazards ratio (HR) for CLAD with the highest CXCL9 and CXCL10 levels, followed by AR (8). We also found that biopsies with higher grade AR were associated with higher CXCL9 and CXCL10 levels, as well as higher risk of CLAD development (7).

The current prospective multi-center study extends these findings by evaluating BAL CXCL9 and CXCL10 concentrations during acute allograft histopathology in the first-year post-transplant. Given their role in the recruitment of mononuclear cells, we hypothesized that CXCL9 and CXCL10 concentrations would be quantifiably increased in the BAL fluid during acute allograft pathology. Furthermore, we hypothesized that ALI would have the highest chemokine levels followed by AR, with high grade AR having higher levels than

low grade AR, reflecting the severity of the injury pattern and extent of mononuclear cell infiltration.

2. MATERIALS AND METHODS

Clinical Trials in Organ Transplantation (CTOT)-20 (NCT02631720), is a prospective multi-center observational study collecting serial clinical data and biologic samples from 5 transplant centers: Cleveland Clinic, Johns Hopkins Medical Center (Hopkins), University of Toronto Health Network (Toronto), Duke University Medical Center (Duke) and University of California Los Angeles (UCLA). This study cohort consisted of the first 198 lung transplant recipients enrolled in CTOT-20 who had at least one concurrent transbronchial biopsy and BAL collected (Figure 1). The transplant dates for this cohort ranged from December 17, 2015 to September 12, 2016. The study was approved by the IRB at each center. Recipients were managed according to clinical practices at each study center as summarized in the clinical management supplement. Bronchoscopies were classified as surveillance if performed as part of the surveillance protocol or “for cause” if performed due to clinical symptoms, pulmonary function test (PFT) decline or radiographic findings. Recipients received a minimum of 4 surveillance bronchoscopies during the first-year post-transplant (at 2-4 weeks, 3 months, 6 months and 12 months post-transplant). Biopsies were reviewed by a pulmonary pathologist at the enrolling center (11). The terms “any injury” or “allograft injury” were used to refer to any of the following four histopathologic diagnoses: AR, lymphocytic bronchiolitis (LB), organizing pneumonia (OP) or ALI. Biopsies with no histopathologic evidence of allograft injury were classified as “normal”. Biopsies ungradable for AR or LB were considered missing and excluded from the analysis. Prior to the start of the study, pathologists from each of the five study centers convened a working group which met several times to harmonize nomenclature, grading and prospective reporting of allograft histopathology.

Immunosuppression, anti-microbial prophylaxis and treatment of the allograft pathologies were administered according to each center’s protocol as described in the clinical management supplement. Maintenance immunosuppression medications were assumed to be unchanged from the immunosuppression that the recipient received on discharge after the transplant. BAL samples were sent for microbiologic evaluation as standard of care. Positive detection of an organism known to cause pulmonary infections were classified as a “pathogen”. Infection was defined as pathogen detection with clinical symptoms (fever, cough, dyspnea, sputum, viral prodrome) or signs (PFT decline, radiographic findings), all of which were documented at the time of the bronchoscopy. Recipients also consented to the collection of BAL for research purposes. The study protocol recommended two 50 to 60 ml aliquots of isotonic saline to be instilled into the sub-segmental bronchus in the lingula, right middle lobe or area of interest and the returns pooled. The supernatant was collected and stored unconcentrated at −80°C after straining and centrifugation. BAL CXCR3 chemokine levels (CXCL9 and CXCL10) were measured using luminex bead assays (Millipore, Billerica MA). Chemokine levels were reported as median fluorescence intensities (MFIs) to minimize variability associated with standard curve interpolations (14, 15).

To evaluate differences in CXCR3 chemokine levels between allograft histopathology and "normal" biopsies, mixed effects models were constructed taking into account repeated measurements from both recipients and study center. This model assumes non-independence among these repeated observations and accounts for this correlation with random intercepts assigned at both the recipient and study center level. Chemokine levels were log2 transformed given the non-normal distribution. In addition to the allograft histopathologies, we a-priori postulated that several factors would affect BAL CXCR3 chemokine levels: allograft infection, clinical indication ("surveillance" vs "for-cause"), BAL volume instilled, as well as maintenance and induction immunosuppression medications. Univariable mixed effects models were performed to compare each chemokine by these additional factors of interest. Variables associated with significant chemokine differences in the univariable models were included in the final multivariable mixed effects models. All analyses were performed using SAS v9.4 (Cary, NC).

3. RESULTS

3.1 Cohort characteristics

135 (68%) of 198 recipients developed an episode of acute allograft injury during the first year of follow-up, while 63 (32%) of recipients did not. Table 1 describes the overall cohort characteristics as well the characteristics of those who did vs did not develop allograft injury. Overall, recipients who developed allograft injury were similar to recipients who did not. The time to first biopsy and median number of biopsies were also similar between those who developed allograft injury vs. those who did not.

3.2 Frequency of Allograft Histopathology During the First-Year Post-Transplant

Table 2 describes the frequency of each histopathologic diagnosis observed per recipient for the entire cohort as well as by study center. In total, there were 104 (53%) recipients who developed AR, 24 (12%) recipients with LB, 45 (23%) recipients with OP and 37 (19%) recipients with ALI. There were differences in the frequency of histopathologic diagnosis across study centers. Hopkins had a lower incidence of AR (15% of recipients), Duke had a lower incidence of LB (2%), and UCLA had a lower incidence of OP (5%). Compared with the other histopathologies, the incidence of ALI was more similar across centers. Mean number of pathologic diagnoses per recipient also reflected these differences across study centers.

There were also differences in the frequency of pathogen detection and clinical infection across study centers (Table 2). Overall, 129 (65%) recipients had 287 episodes of BAL pathogen detection: 132 (46%) were bacterial, 35 (12%) were mycobacterial, 107 (37%) were fungal and 75 (26%) were viral. 52 episodes of pathogen detection from 43 (22%) recipients were classified as infection based on the presence of clinical symptoms or signs (radiology, PFT decline).

Table 3 describes the total number of each histopathologic diagnosis, stratified by the clinical indication of the bronchoscopy. In total, there were 844 biopsies with 249 (28%) episodes of allograft injury observed. There were 173 (20%) episodes of AR, 30 (3%)

episodes of LB, 52 (6%) episodes of OP, and 42 (5%) episodes of ALI. The following AR grades were observed: 126 (14%) A1, 46 (5%) A2, 1 (0%) A3, 0 (0%) A4, with 67 (8%) biopsies which were ungradable for AR. The following LB grades were observed: 29 (3%) B1R, 1 (0%) B2R, with 255 (29%) biopsies which were ungradable for LB. ALI was graded as 23 (3%) acute, 13 (1%) organizing and 6 (1%) acute and organizing. 767 (87%) biopsies were performed for “surveillance”, while 116 (13%) were performed due to clinical symptoms (“for cause”). The incidence of AR and LB was not significantly different between “surveillance” and “for cause” biopsies, but there was a higher incidence of OP and ALI observed in “for cause” biopsies. 46 (5%) biopsies had more than one concurrent histopathologic diagnosis observed. The highest frequency of concurrent histopathologic findings was between LB, OP and ALI with AR: Of the 173 biopsies with AR, there were 18 (10%) with concurrent LB, 15 (9%) with concurrent OP and 10 (6%) concurrent ALI. There was also high concurrence between pathogen detection and AR. Of the 287 episodes of pathogen detection, there were 54 (19%) with concurrent AR, 5 (2%) with concurrent LB, 17 (6%) with concurrent OP and 7 (2%) with concurrent ALI. Similarly, of the 52 episodes of clinical infection, there were 10 (3%) with concurrent AR, 1 (0.3%) with concurrent LB, 5 (2%) with concurrent OP and 3 (1%) with concurrent ALI.

3.3 BAL CXCR3 Chemokine MFI Measurement During Allograft Histopathology

We next evaluated BAL CXCR3 chemokine levels by histopathologic diagnosis using univariable mixed effects models with random effect variables for recipient and study center. Median CXCL9 MFIs were higher during “any injury” (AR, LB, OP or ALI) compared to “normal” biopsies: 222.0 vs 91.0, respectively (p=0.0001, Table 4). Similarly, median CXCL10 MFIs for “any injury” vs “normal” biopsies were: 470.0 vs 196.5, respectively (p=0.0003). For the individual injury patterns, CXCL9 MFIs were elevated during ALI (333.3, p=0.0001), AR (263.0, p=0.0001) and LB (201.5, p=0.0009), compared with “normal” biopsies. Similarly, CXCL10 MFIs were elevated during ALI (1030.0, p=0.0003), AR (543.5, p=0.0001) and LB (494.0, p=0.0017), compared with “normal” biopsies. OP was the only histopathologic allograft injury which was not associated with elevated CXCL9 or CXCL10 levels.

Importantly, higher grade AR was associated with higher CXCL9 and CXCL10 levels. Median CXCL9 MFIs for A1 and ≥A2 were 199.5 (p=0.03) and 315.0 (p=0.0001) compared with 145.0 for A0 rejection grade, respectively (Table 5). Similarly, CXCL10 MFIs for A1 and ≥A2 were 498.8 (p=0.0001) and 648.0 (p=0.0129) compared with 290.5 for A0 rejection grade, respectively.

3.4 BAL CXCR3 Chemokine MFI Measurement During Respiratory Infection

BALs with detected pathogens were associated with elevated CXCL9 and CXCL10 MFIs compared with “normal” biopsies: 361.0 vs 91.0 (p=0.0001) and 591.0 vs 196.5 (p=0.0013), respectively (Table 6). BALs associated with clinical infections had even higher CXCL9 and CXCL10 levels with median MFIs of 709.5 (p=0.0001) and 857.0 (p=0.0004), respectively.

3.5 Univariable Models Evaluating BAL CXCR3 Chemokine MFIs

Univariable mixed effects models were also constructed to estimate “fold-change” increases in CXCR3 chemokines during episodes of allograft histopathology, compared with “normal” biopsies. An episode of “any injury” was associated with a 1.6-fold increase in CXCL9 (95% CI 1.3-2.0) and 1.4-fold increase in CXCL10 (95% CI 1.1-1.8), compared with “normal” biopsies (Table 6). The individual injury patterns AR, LB and ALI were all associated with increased CXCL9 MFIs with fold-changes of: 1.7 (95% CI 1.4-2.2), 2.4 (95% CI 1.4-4.0) and 2.0 (95% CI 1.3-3.1), compared with “normal” biopsies, respectively. For CXCL10, AR and ALI were the only injury patterns associated with increased MFIs with fold changes of: 1.7 (95% CI 1.3-2.1) and 2.2 (95% CI 1.3-3.5, respectively. CXCL9 levels were not elevated during OP, and CXCL10 was not elevated during LB or OP.

We next performed univariable comparisons of BAL CXCR3 chemokine MFIs by several factors, which were a-priori postulated to potentially influence the MFIs: respiratory infection, clinical indication (“surveillance” vs “for cause”), bronchoalveolar lavage volume, induction immunosuppression, and maintenance immunosuppression. BALs with pathogens detected were associated with increased CXCL9 and CXCL10 with fold-changes of: 1.7 (95% CI 1.4-2.0) and 1.3 (95% CI 1.1-1.7), respectively. Episodes of clinical infection were associated with higher CXCL9 and CXCL10 levels with fold-changes of: 2.2 (95% CI 1.5-3.2) and 1.8 (95% CI 1.2-2.8), respectively.

There were no significant differences in CXCL9 or CXCL10 levels based on the clinical indication of the biopsy (Supplemental 1). There were differences in the saline lavage volume instilled during the bronchoscopy across study centers. UCLA had the highest median lavage volumes at 120 ml, followed by Hopkins (100 ml), Toronto (100 ml), Duke (80 ml) and Cleveland (70 ml). The volume of lavage fluid recovered was more similar across centers. UCLA (55 ml) and Toronto (49 ml) had the highest volumes recovered, followed by Cleveland (45 ml), Hopkins (45 ml) and Duke (30 ml). There was a trend towards higher CXCL9 and CXCL10 MFIs with higher lavage volumes but the difference was not statistically significant (Supplemental 2).

Induction immunosuppression used at the time of transplantation was another factor which we postulated would affect BAL CXCR3 chemokines MFIs. Almost all patients at Cleveland (98%) and Toronto (91%) received no induction immunosuppression. All patients at Hopkins (100%) and Duke (100%) received basiliximab, while patients at UCLA received either basiliximab (67%) or ATG (33%). For “normal” biopsies, those recipients that received basiliximab had higher CXCL9 and CXCL10 levels, followed by ATG, then no induction (Supplemental 3). However, these differences were not statistically significant.

There were also differences in the use of maintenance immunosuppression across study centers. Most patients at Cleveland (97%), Hopkins (100%), UCLA (94%) and Duke (89%) received tacrolimus, while most patients at Toronto (91%) received cyclosporine. Compared with tacrolimus, cyclosporine use was associated with higher CXCR3 chemokine levels, but the differences were not statistically significant (Supplemental 4).

3.6 Multivariable Models Evaluating BAL CXCR3 Chemokine MFIs

Multivariable mixed effects models were then constructed using the variables which were significant predictors of CXCL9 and CXCL10 in the univariable models.

The multivariable model for CXCL9 included the variables: AR, LB, ALI and infection. An episode of AR, ALI and infection were associated with fold-increases of: 1.7 (95% CI 1.3-2.2), 2.1 (95% CI 1.3-3.5) and 3.1 (95% CI 1.9-4.9), respectively (Table 7). LB was not associated with elevated CXCL9 in the multivariable model. The multivariable model for CXCL10 included the variables: AR, ALI and infection. All three variables remained significant predictors of CXCL10 with fold-changes of: 1.6 (95% CI 1.3-2.1), 2.2 (95% CI 1.4-3.7) and 1.4 (95% CI 1.1-1.8), respectively. Other factors which we postulated to affect MFIs (clinical indication, lavage volume instilled, induction and maintenance immunosuppression) were not significant in univariable models and thus not included in the multivariable models.

3.7 BAL CXCR3 Chemokine MFIs after Treatment of Histopathology

To evaluate changes in BAL CXCL9 and CXCL10 levels after treatment of the allograft histopathology, we determined if the recipient received augmented steroids (IV or PO) within 7 days after the histopathologic diagnosis. For the 249 episodes of allograft injury (from 135 recipients), 113 (45%) episodes (from 79 recipients) received augmented steroids, while 136 (55%) episodes (from 98 recipients) did not. 69% of allograft injury episodes were followed up with a repeat bronchoscopy in the next 90 days. The follow-up BAL chemokine levels overall trended lower for recipients who received treatment compared to those who did not: 106.0 vs 235.0 and 239.0 vs 532.5, for CXCL9 and CXCL10 respectively. However, the median change in BAL levels for the treated vs untreated groups were not significantly different using the Wilcoxon Signed-Rank Test: −34.5 vs −32.0 ($p=0.48$), and −68.5 vs −38.0 ($p=0.12$), for CXCL9 and CXCL10 respectively.

4. DISCUSSION

The histopathologic diagnosis of acute allograft injury is prognostically important with several studies showing a strong association between AR (1–7), ALI (8, 9) and subsequent CLAD development. The pathogenesis of these allograft injuries however, remains poorly understood. In this multi-center study, we sought to evaluate the incidence of allograft histopathology development during the first-year post-transplant and evaluate CXCR3 chemokine expression as measured in the BAL at the time of the biopsy. Consistent with our prior single center studies (7, 8), we found significant BAL CXCL9 and CXCL10 elevations during episodes of AR and ALI. In multivariable models, CXCL9 levels were 1.7-fold and 2.1-fold higher during AR and ALI compared with “normal” biopsies without histopathology. Similarly, CXCL10 levels were 1.6-fold and 2.2-fold higher during these histopatholgies, respectively. Furthermore, these BAL chemokine elevations reflect the severity of the injury and inflammation: CXCL9 and CXCL10 levels were highest for ALI, the most severe form of allograft injury, followed by higher grade AR (≥A2), then lower grade AR (A1). These findings support our hypothesis that CXCL9 and CXCL10

are involved in the recruitment of injurious mononuclear cells, which defines AR and ALI histologically.

Two prior UCLA single center studies that found AR and ALI were the strongest histopathologic predictors of subsequent CLAD development (7, 8). BAL CXCL9 and CXCL10 levels were significantly elevated during these histopathologic diagnoses compared with normal biopsies, with levels that correlated with the intensity of injury (eg, CXCL9 and CXCL10 levels were highest for ALI, followed by AR and then LB) (8). Similarly, CXCL9 and CXCL10 levels were higher for higher grade AR: A3 > A2 > A1 (7). Consistent with these studies, the current study found pronounced elevations of BAL CXCL9 and CXCL10 concurrent with AR and ALI in multivariable models, and also LB in univariable analysis. Also consistent with the prior studies, there was a strong correlation between BAL CXCL9 and CXCL10 levels and the severity of the allograft injury.

Although there was no association between the diagnosis of OP and elevated CXCR3 chemokines in the current study, this may be due to the relatively small number of OP episodes (n=52) in the current study. Our prior single center study of 1856 biopsies with 169 episodes of OP did not find an overall association between OP and CLAD risk. However, we found that elevated BAL CXCR3 chemokines during OP increased CLAD risk in a dose-response manner (16). Similarly, we previously found that although low grade AR (A1) was not associated with CLAD risk, low grade AR with elevated BAL CXCL9 increased CLAD risk in a dose-response manner (7).

The current study has completed enrollment with patients now accruing follow-up time towards CLAD development. We intend to evaluate the effect of CXCR3 chemokine elevations during allograft pathology on CLAD risk after completion of the accrual period. We anticipate, based on our prior single center studies, that allograft pathologies with elevated BAL CXCR3 chemokines will significantly increase CLAD risk. This may allow for the risk stratification of allograft injuries to better guide treatment decisions. For example, low grade AR with elevated chemokine will require treatment, while low grade AR with lower chemokine levels will not. The current study supports the possibility of using BAL chemokines to better risk-stratify allograft injuries for future adverse events and may improve our ability to optimize post-transplant care.

Overall, we found that 53% of recipients developed AR, 12% developed LB, 23% developed OP and 19% developed ALI over the first-year post-transplant. We attempted to standardize the pathologic evaluation by distributing a protocol for the bronchoscopy and pathologic review to all participating investigators. Prior to the initiation of the study, we convened a working group of pathologists from each study center. These pathologists met several times to review representative cases and reach a consensus on the histopathologic classification criteria. Despite these efforts, there was variability in the diagnosis of allograft injuries between study centers, highlighting the difficulty of standardizing histopathologic interpretation. Interestingly, there was more consistency in the frequency of ALI diagnosed compared with the other histopathologies, including AR. This may be explained by the severe and diffuse nature of ALI, compared with AR which is often patchy and heterogeneous. Several other studies have reported poor interobserver

agreement of transbronchial biopsy assessments for the histopathologic diagnosis of acute rejection (17, 18). Thus, the evaluation and use of immunologic biomarkers, in conjunction with or in place of histopathologic evaluation, for the risk stratification of patients for adverse events may be preferable. We feel that BAL CXCR3 chemokine expression after lung transplantation deserves further study for this purpose.

We postulated that concurrent pathogen detection and respiratory infection would increase BAL CXCL9 and CXCL10 levels. In univariable models, we found that pathogen detection was associated with increased CXCL9 and CXCL10 levels compared with “normal” biopsies. The subset of pathogen positive BALs associated with clinical symptoms or signs (respiratory infection) was associated with even higher CXCL9 and CXCL10 levels. In the final multivariable models, respiratory infection remained a significant determinant of CXCL9 and CXCL10 levels with fold changes of: 3.1 (95% CI 1.9-4.9) and 1.4 (95% CI 1.1-1.8), respectively. Several prior studies have reported an association between respiratory infections and CLAD development (19–24). Our group previously demonstrated BAL CXCL9 elevations during respiratory infections, as well as increased with CLAD risk with increasing CXCL9 levels in a dose-response manner (25). The association between respiratory infection and elevated CXCL9 and CXCL10 levels in the current study confirms the findings from our prior single center study.

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 bronchoscopy procedures, pathologic evaluation, clinical management, and other factors that are unmeasured. We postulated that several of these center-specific factors would affect BAL chemokine levels: lavage volume instilled, maintenance immunosuppression and induction immunosuppression. Given the limited episodes of allograft pathology, we were unable to stratify the analysis by study center. However, we attempted to control for these study center differences by including study center as a random effect variable for all mixed effects models. We found that, for biopsies without evidence of histopathology, there were no statistically significant differences in CXCR3 chemokine levels based on these factors, after adjustment for study center as a random effect variable.

Despite these limitations, this multi-center study refines our current understanding of the patterns of elevated BAL CXCR3 chemokines associated with prognostically important histopathologic diagnoses, as previously described in single-center studies. To our knowledge, this is the first prospective multi-center study to evaluate BAL chemokine levels after lung transplantation, and the largest BAL chemokine study to date for lung transplant recipients. We demonstrate BAL CXCL9 and CXCL10 elevations during episodes of AR and ALI, the strongest histopathologic predictors of CLAD development. Consistent with our prior results, we find that these chemokine elevations reflect the severity of allograft injury. These findings support our hypothesis that CXCL9 and CXCL10 are involved in the recruitment of injurious mononuclear cells which defines AR and ALI histologically, and confirm the generalizability of these results across study centers. Improving our understanding of key receptor-chemokine interactions responsible for the pathogenesis of allograft injury may lead to novel options to prevent and treat these deleterious events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The research described was supported by NIH K23HL138256-01 (MYS), UM2AI117870 (MS and HK), 5U01AI113315-02 (SSW, SMP and JAB) and 2P01HL108793-06 (SSW and JAB).

Abbreviations:

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

References

1. Burton CM, Iversen M, Carlsen J, Andersen CB. Interstitial inflammatory lesions of the pulmonary allograft: A retrospective analysis of 2697 transbronchial biopsies. Transplantation 2008;86:811–819. [PubMed: 18813106]
2. Davis WA, Finlen Copeland CA, Todd JL, Snyder LD, Martissa JA, Palmer SM. Spirometrically significant acute rejection increases the risk for BOS and death after lung transplantation. Am J Transplant 2012;12:745–752. [PubMed: 22123337]
3. Estenne M, Maurer JR, Boehler A, Egan JJ, Frost A, Hertz M, Mallory GB, Snell GI, Yousem S. Bronchiolitis obliterans syndrome 2001: An update of the diagnostic criteria. J Hear Lung Transplant 2002;21:297–310.
4. Hachem RR, Khalifah AP, Chakinala MM, Yusen RD, Aloush AA, Mohanakumar T, Patterson GA, Trulock EP, Walter MJ. The significance of a single episode of minimal acute rejection after lung transplantation. Transplantation 2005;80:1406–1413. [PubMed: 16340783]
5. Hopkins PM, Aboyoun CL, Chhajed PN, Malouf MA, Plit ML, Rainer SP, Glanville AR. Association of minimal rejection in lung transplant recipients with obliterative bronchiolitis. Am J Respir Crit Care Med 2004;170:1022–1026. [PubMed: 15297270]
6. Sharples LD, McNeil K, Stewart S, Wallwork J. Risk factors for bronchiolitis obliterans: A systematic review of recent publications. J Hear Lung Transplant 2002;21:271–281.

7. Shino MY, Weigt SS, Li N, Derhovanessian A, Sayah DM, Saggar R, Huynh RH, Gregson AL, Ardehali A, Ross DJ, Lynch JP, Elashoff RM, Belperio JA. The Prognostic Importance of Bronchoalveolar Lavage Fluid CXCL9 During Minimal Acute Rejection on the Risk of Chronic Lung Allograft Dysfunction. Am J Transplant 2018;18:136–144. [PubMed: 28637080]
8. Shino MY, Weigt SS, Li N, Palchevskiy V, Derhovanessian A, Saggar R, Sayah DM, Gregson AL, Fishbein MC, Ardehali A, Ross DJ, Lynch JP, Elashoff RM, Belperio JA. CXCR3 ligands are associated with the continuum of diffuse alveolar damage to chronic lung allograft dysfunction. Am J Respir Crit Care Med 2013;188:1117–1125. [PubMed: 24063316]
9. Sato M, Hwang DM, Ohmori-Matsuda K, Chaparro C, Waddell TK, Singer LG, Hutcheon MA, Keshavjee S. Revisiting the pathologic finding of diffuse alveolar damage after lung transplantation. J Hear Lung Transplant 2012;31:354–363.
10. Chambers DC, Yusen RD, Cherikh WS, Goldfarb SB, Kucheryavaya AY, Khusch K, Levvey BJ, Lund LH, Meiser B, Rossano JW, Stehlik J. The Registry of the International Society for Heart and Lung Transplantation: Thirty-fourth Adult Lung And Heart-Lung Transplantation Report—2017; Focus Theme: Allograft ischemic time. J Hear Lung Transplant 2017;36:1047–1059.
11. Stewart S, Fishbein MC, Snell GI, Berry GJ, Boehler A, Burke MM, Glanville A, Gould FK, Magro C, Marboe CC, McNeil KD, Reed EF, Reinsmoen NL, Scott JP, Studer SM, Tazelaar HD, Wallwork JL, Westall G, Zamora MR, Zeevi A, Yousem SA. Revision of the 1996 Working Formulation for the Standardization of Nomenclature in the Diagnosis of Lung Rejection. J Hear Lung Transplant 2007;26:1229–1242.
12. Belperio JA, Keane MP, Burdick MD, Lynch JP, Xue YY, Li K, Ross DJ, Strieter RM. Critical Role for CXCR3 Chemokine Biology in the Pathogenesis of Bronchiolitis Obliterans Syndrome. J Immunol 2002;169:1037–1049. [PubMed: 12097412]
13. Belperio JA, Keane MP, Burdick MD, Lynch JP, Zisman DA, Xue YY, Li K, Ardehali A, Ross DJ, Strieter RM. Role of CXCL9/CXCR3 Chemokine Biology during Pathogenesis of Acute Lung Allograft Rejection. J Immunol 2003;171:4844–4852. [PubMed: 14568964]
14. Breen EJ, Tan W, Khan A. The Statistical Value of Raw Fluorescence Signal in Luminex xMAP Based Multiplex Immunoassays. Sci Rep 2016;6:1–13. [PubMed: 28442746]
15. Breen EJ, Polaskova V, Khan A. Bead-based multiplex immuno-assays for cytokines, chemokines, growth factors and other analytes: Median fluorescence intensities versus their derived absolute concentration values for statistical analysis. Cytokine 2015;71:188–198. [PubMed: 25461398]
16. Shino MY, Weigt SS, Li N, Palchevskiy V, Derhovanessian A, Saggar R, Sayah DM, Huynh RH, Gregson AL, Fishbein MC, Ardehali A, Ross DJ, Lynch JP, Elashoff RM, Belperio JA. The prognostic importance of CXCR3 chemokine during organizing pneumonia on the risk of chronic lung allograft dysfunction after lung transplantation. PLoS One 2017;12:1–15.
17. Chakinala MM, Ritter J, Gage BF, Aloush AA, Hachem RH, Lynch JP, Patterson GA, Trulock EP. Reliability for grading acute rejection and airway inflammation after lung transplantation. J Hear Lung Transplant 2005;24:652–657.
18. Bhorade SM, Husain AN, Liao C, Li LC, Ahya VN, Baz MA, Valentine VG, Love RB, Seethamraju H, Alex CG, Bag R, DeOliveira NC, Vigneswaran WT, Garrity ER, Arcasoy SM. Interobserver variability in grading transbronchial lung biopsy specimens after lung transplantation. Chest 2013;143:1717–1724. [PubMed: 23370547]
19. Valeri M, Piazza A, Torlone N, Poggi E, Adorno D, Casciani CU. Immunologic monitoring in kidney transplantation. Transplant Proc 1991;23:2275–2276. [PubMed: 1871869]
20. Vaughan CP, Goode PS, Burgio KL, Markland AD, Atlanta B. Urinary Incontinence in Older Adults Address Correspondence to: 2011;558–570.doi:10.1002/MSJ.
21. Allyn PR, Belperio JA. GRAFT LOSS AND CLAD ONSET IS HASTENED BY VIRAL PNEUMONIA AFTER LUNG TRANSPLANTATION. Transplantation 2016;100:2424–2431. [PubMed: 27467538]
22. Gregson AL, Wang X, Injean P, Weigt SS, Shino M, Sayah D, DerHovanessian A, Lynch JP, Ross DJ, Saggar R, Ardehali A, Li G, Elashoff R, Belperio JA. Staphylococcus via an interaction with the ELR+ CXC chemokine ENA-78 is associated with BOS. Am J Transplant 2015;15:792–799. [PubMed: 25683785]

23. Gregson AL, Wang X, Weigt SS, Palchevskiy V, Lynch JP, Ross DJ, Kubak BM, Saggar R, Fishbein MC, Ardehali A, Li G, Elashoff R, Belperio JA. Interaction between pseudomonas and CXC chemokines increases risk of bronchiolitis obliterans syndrome and death in lung transplantation. Am J Respir Crit Care Med 2013;187:518–526. [PubMed: 23328531]

24. Khalifah AP, Hachem RR, Chakinala MM, Schechtman KB, Patterson GA, Schuster DP, Mohanakumar T, Trulock EP, Walter MJ. Respiratory viral infections are a distinct risk for bronchiolitis obliterans syndrome and death. Am J Respir Crit Care Med 2004;170:181–187. [PubMed: 15130908]

25. Shino Michael Y, DerHovanessian Ariss, Sayah David M., Saggar Rajan, Ying Ying Xue, Abbas Ardehali, Stripp Barry R., Ross David J., Lynch Joseph P. III, Elashoff Robert M., Weigt S. Samuel JAB. The Impact of Allograft CXCL9 during Respiratory Infection on the Risk of Chronic Lung Allograft Dysfunction. OBM Transpl 2018;2:.

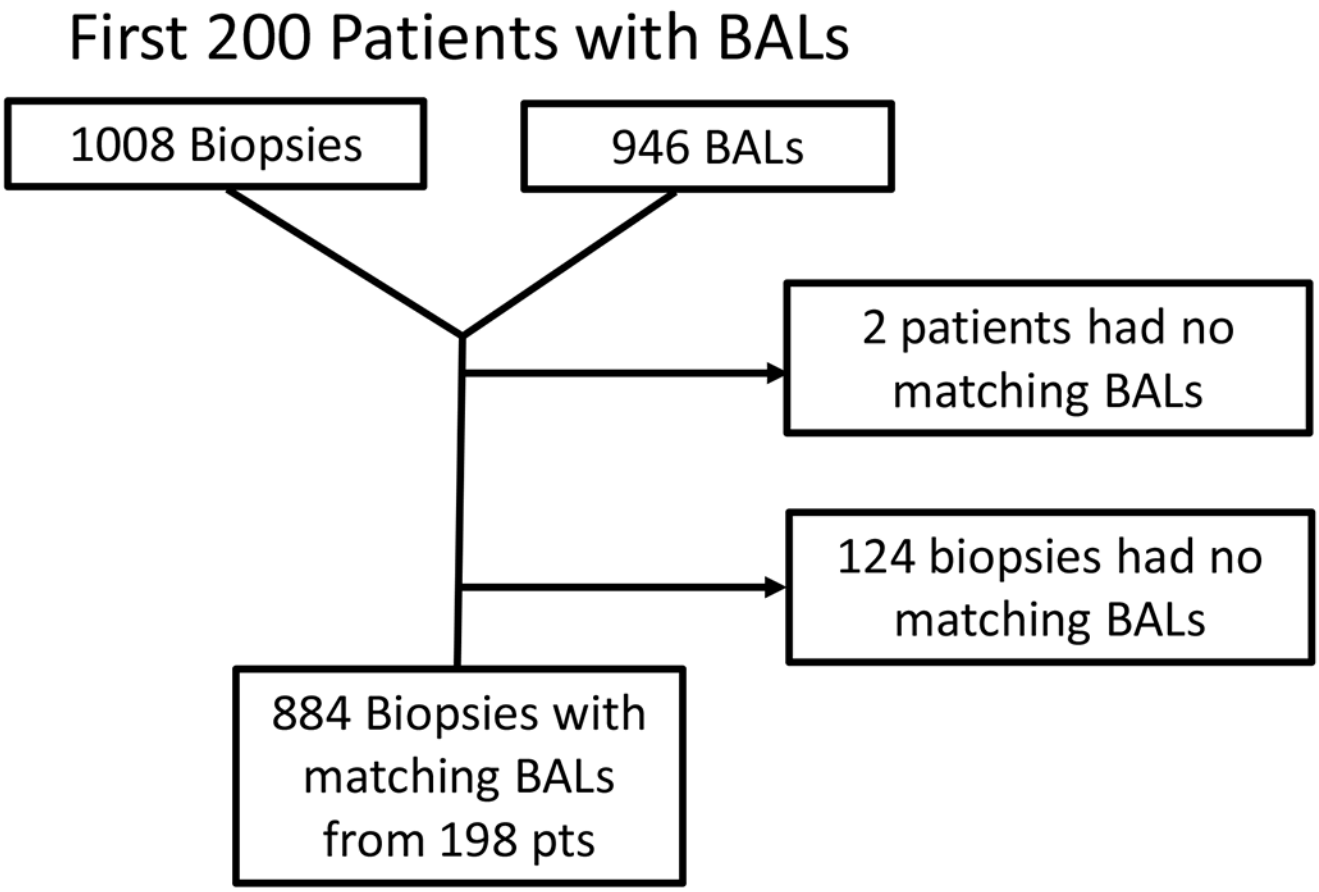

Figure 1.
Flow diagram of patient and sample size used in the analysis.

Table 1.

Baseline Patient Characteristics

	All Patients		Patients with Allograft Injury [I]		Patients without Allograft Injury [I]	
	n	%	n	%	n	%
Number of patients:	198	100%	135	68%	63	32%
Pre-transplant characteristics: Race						
White	176	89%	122	90%	54	86%
Black	15	8%	8	6%	7	11%
Asian	1	1%	1	1%	-	0%
Other	6	3%	4	3%	2	3%
Mean age at transplant (sd)	60	(14.5)	60	(14.7)	63	(14.2)
Female gender	70	35%	45	33%	25	40%
Native lung disease						
Idiopathic pulmonary fibrosis	66	33%	53	39%	13	21%
Obstructive/COPD	48	24%	29	21%	19	30%
Cystic Fibrosis	25	13%	15	11%	10	16%
Hypersensitivity pneumonitis	12	6%	9	7%	3	5%
Connective tissue associated ILD	13	7%	5	4%	7	11%
Sarcoidosis	7	4%	5	4%	2	3%
Other	27	14%	19	14%	9	14%
Bilateral lung transplant	147	74%	97	72%	50	79%
Post-transplant characteristics: Induction immunosuppression						
ATG	14	7%	9	7%	5	8%
Basiliximab	101	51%	62	46%	39	62%
None	84	42%	65	48%	19	30%
Primary Graft Dysfunction [H]						
Grade 0	24	12%	18	13%	6	10%
Grade 1	96	48%	60	44%	36	57%
Grade 2	40	20%	30	22%	10	16%
Grade 3	38	19%	27	20%	11	17%
Primary immunosuppression [HH]						
Tacrolimus	157	79%	106	79%	51	81%
Cyclosporine	42	21%	29	21%	13	21%
Secondary immunosuppression						
Mycophenolate	173	87%	120	89%	53	84%
Azathioprine	19	10%	11	8%	8	13%
Other	6	3%	4	3%	2	3%
Biopsies during first year post-transplant						
Median days to first biopsy (range)	37	(16-332)	37	(16-332)	37	(19-289)
Median # biopsies per subject (range)	5	(1-10)	5	(1-10)	4	(1-7)

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

I Allograft Injury includes the following histopathologies: acute rejection, lymphocytic bronchiolitis, organizing pneumonia and acute lung injury.

II Highest at 72 hours.

III At post-transplant discharge.

Table 2.

Median Number of Allograft Injury Events Per Patient During the First-Year Post-Transplant

	All Centers	Cleveland	Hopkins	Toronto	UCLA	Duke	p-value [I]
Number of patients:	**198**	**54**	**13**	**36**	**40**	**55**	
Median number of biopsies per patient:	**5 (3-6)**	**6 (5-7)**	**5 (4-5)**	**4 (3-5)**	**3 (1-4)**	**6 (5-6)**	**0.0001**
Patients with allograft injury [II]:	**135**	**43**	**6**	**25**	**24**	**37**	
% Patients with allograft injury	68%	80%	46%	69%	60%	67%	
Mean allograft injury events per patient	1.26	1.57	0.77	1.11	0.80	1.49	0.0073
Patients with AR:	**104**	**37**	**2**	**17**	**15**	**33**	
% Patients with AR	53%	69%	15%	47%	38%	60%	
Mean AR events per patient	0.87	1.09	0.23	0.58	0.53	1.25	0.0003
Patients with LB:	**24**	**7**	**1**	**3**	**12**	**1**	
% Patients with LB	12%	13%	8%	8%	30%	2%	
Mean LB events per patient	0.16	0.19	0.08	0.10	0.39	0.02	0.0016
Patients with OP:	**45**	**15**	**2**	**11**	**2**	**15**	
% Patients with OP	23%	28%	15%	31%	5%	27%	
Mean OP events per patient	0.26	0.37	0.23	0.33	0.05	0.27	0.0394
Patients with ALI:	**37**	**12**	**3**	**10**	**6**	**6**	
% Patients with ALI	19%	22%	23%	28%	15%	11%	
Mean ALI events per patient	0.21	0.26	0.23	0.33	0.15	0.13	0.2632
Patients with pathogen [III]:	**129**	**27**	**9**	**24**	**18**	**51**	
% Patients with pathogen	65%	50%	69%	67%	45%	93%	
Mean pathogen events per patient	1.46	0.81	2.15	1.01	0.69	2.71	0.0001
Patients with infection [IIII]:	**43**	**10**	**2**	**8**	**4**	**19**	
% Patients with infection	22%	19%	15%	22%	10%	35%	
Mean infection events per patient	0.26	0.20	0.15	0.25	0.13	0.45	0.0521

[I] P-value is from Kruskal-Wallis comparing mean number of events by study center.

[II] Allograft injury includes: AR, LB, OP or ALI.

[III] Pathogen is defined as the detection of a pathogenic organism.

[IIII] Infection is defined as pathogen detection with symptoms or radiology findings.

Table 3.

Allograft Injuries by Clinical Indication

Characteristic	Surveillance		For Cause		Total	
	n	%	n	%	n	%
Total number of biopsies	**767**	**87%**	**116**	**13%**	**884**	**100%**
Episodes of allograft injury [I]	**210**	**27%**	**39**	**34%**	**249**	**28%**
Acute cellular rejection (AR)	**148**	**19%**	**25**	**22%**	**173**	**20%**
None (A0)	561	73%	82	71%	644	73%
Minimal (A1)	108	14%	18	16%	126	14%
Mild (A2)	40	5%	6	5%	46	5%
Moderate (A3)	0	0%	1	1%	1	0%
Severe (A4)	0	0%	0	0%	0	0%
Ungradable (AX)	58	8%	9	8%	67	8%
Lymphocytic bronchiolitis (LB)	**24**	**3%**	**6**	**5%**	**30**	**3%**
None (B0)	528	69%	70	60%	599	68%
Low-grade LB (B1R)	24	3%	5	4%	29	3%
High-grade LB (B2R)	0	0%	1	1%	1	0%
Ungradable (BX)	215	28%	40	34%	255	29%
Organizing pneumonia	**41**	**5%**	**11**	**9%**	**52**	**6%**
Acute lung injury	**32**	**4%**	**10**	**9%**	**42**	**5%**
Acute	16	2%	7	6%	23	3%
Organizing	10	1%	3	3%	13	1%
Acute and organizing	6	1%	0	0%	6	1%
Pathogen [II]	**234**	**31%**	**53**	**46%**	**287**	**32%**
Infection [III]	**0**	**0%**	**52**	**45%**	**52**	**6%**

[I] Allograft injury includes: AR, LB, OP or ALI.

[II] Pathogen is defined as the detection of a pathogenic organism.

[III] Infection is defined as pathogen detection with symptoms or radiology findings.

Table 4.

Median CXCR3 Chemokine MFIs By Graft Status

	n	CXCL9	p-value [I]	CXCL10	p-value [I]
Normal	334	91.0		196.5	
Any Injury [H]	249	222.0	0.0001	470.0	0.0003
AR	173	263.0	0.0001	543.5	0.0001
LB	30	201.5	0.0009	494.0	0.0017
OP	52	119.0	0.7493	307.3	0.9628
ALI	42	333.3	0.0001	1,030.0	0.0003
Pathogen [HH]	287	361.0	0.0001	591.0	0.0013
Infection [HHH]	52	709.5	0.0001	857.0	0.0004

[I]P-values are from mixed effects model comparing each injury pattern with healthy biospsies.

[H]Any injury includes: AR, LB, OP or ALI.

[HH]Pathogen is defined as the detection of a pathogenic organism.

[HHH]Infection is defined as pathogen detection with symptoms or radiographic findings.

Table 5.

Median CXCR3 Chemokine MFIs By Acute Rejection Grade

	n	CXCL9	p-value [1]	CXCL10	p-value [1]
A0	644	145.0		290.5	
A1	126	199.5	0.0304	498.8	0.0001
≥ A2	47	315.0	0.0001	648.0	0.0129

[1] P-values are from mixed effects model comparing the two AR

Table 6.

Univariable Models for CXCR3 Chemokine MFIs

	CXCL9				CXCL10			
	Fold change	95%CI Lower	95%CI Upper	p-value [I]	Fold change	95%CI Lower	95%CI Upper	p-value [I]
Any Injury [II]	1.6	1.3	2.0	0.0001	1.4	1.1	1.8	0.0018
AR	1.7	1.4	2.2	0.0001	1.7	1.3	2.1	0.0002
LB	2.4	1.4	4.0	0.0012	1.5	0.9	2.7	0.1558
OP	1.0	0.6	1.2	0.4196	0.7	0.5	1.1	0.1719
ALI	2.0	1.3	3.1	0.0013	2.2	1.3	3.5	0.0017
Pathogen [III]	1.7	1.4	2.0	0.0001	1.3	1.1	1.7	0.0143
Infection [IIII]	2.2	1.5	3.2	0.0001	1.8	1.2	2.8	0.0055

[I] P-values are from mixed effects model comparing each allograft injury with “normal” biopsies. Fold-changes represent mean chemokine changes compared with “normal” biopsies.

[II] Any injury includes: AR, LB, OP or ALI.

[III] Pathogen is defined as the detection of a pathogenic organism.

[IIII] Infection is defined as pathogen detection with symptoms or radiographic findings.

Table 7.

Multivariable Models for CXCR3 Chemokine MFIs By Graft Status

	CXCL9				CXCL10			
	Fold change	95%CI Lower	95% CI Upper	p-value [I]	Fold change	95%CI Lower	95% CI Upper	p-value [I]
AR	1.7	1.3	2.2	0.0002	1.6	1.3	2.1	0.0002
LB	1.6	0.9	2.7	0.0992				
ALI	2.1	1.3	3.5	0.0030	2.2	1.4	3.7	0.0016
Infection [H]	3.1	1.9	4.9	0.0001	1.4	1.1	1.8	0.0088

[I] P-values are from mixed effects model comparing all allograft injuries with “normal” biopsies. Fold-changes represent mean chemokine changes compared with “normal” biopsies.

[H] Infection is defined as pathogen detection with symptoms or radiographic findings.