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THE PROGNOSTIC IMPORTANCE OF BRONCHOALVEOLAR LAVAGE FLUID CXCL9 DURING MINIMAL ACUTE REJECTION ON THE RISK OF CHRONIC LUNG ALLOGRAFT DYSFUNCTION

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Abstract

The clinical significance and treatment strategies for minimal acute rejection (grade A1), the most common form of acute rejection (AR), remains controversial. In this retrospective single-center cohort study of 441 lung transplant recipients, we formally evaluate the association between minimal AR and chronic lung allograft dysfunction (CLAD) and test a novel hypothesis using BAL CXCL9 concentration during minimal AR as a biomarker of subsequent CLAD development. In univariable and multivariable models adjusted for all histopathologic injury patterns, minimal AR was not associated with CLAD development. However, minimal AR with elevated BAL CXCL9 concentrations markedly increased CLAD risk in a dose-response manner. Minimal AR with CXCL9 concentrations greater than the 25th, 50th, and 75th percentile had an adjusted HRs for CLAD of 1.1 (95% CI 0.8–1.6), 1.6 (95% CI 1.1–2.3) and 2.2 (95% CI 1.4–3.4), respectively. Thus, we demonstrate the utility of BAL CXCL9 measurement as a prognostic biomarker that allows discrimination of recipients at increased risk of CLAD development after minimal AR. BAL CXCL9 measurement during transbronchial biopsies may provide clinically useful prognostic data and potentially guide treatment decisions for this common form of AR, as a possible strategy to minimize CLAD development.

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INTRODUCTION

Chronic lung allograft dysfunction (CLAD) is the leading cause of death after the first year and the major obstacle to improved long-term survival after lung transplantation.(1) Since there are no known effective therapies for CLAD, the identification, avoidance and treatment of known risk factors for CLAD is a crucial aspect of post-transplant management. Acute rejection (AR) remains one of the most well-studied risk factors for CLAD development. Previous studies have demonstrated a consistent association between higher grade (≥A2) acute rejection (AR) and CLAD.(2–5) However, the clinical significance and optimal treatment of minimal acute rejection (grade A1), the most common form of acute rejection, have remained controversial.

The 2006 revised working formulation for the grading of lung allograft acute rejection is based on the presence of perivascular and interstitial mononuclear infiltrates: A0 (“no AR”), A1 (“minimal AR”) – scattered, infrequent perivascular mononuclear infiltrate, A2 (“mild AR”) – more frequent perivascular mononuclear infiltrate readily recognizable at low magnification, A3 (“moderate AR”) – cuffing of venules and arterioles by dense perivascular mononuclear cell infiltrate, and A4 (“severe AR”) – diffuse perivascular, interstitial and air-space mononuclear cell infiltrate with alveolar pneumocyte damage and endothelitis.(6) Minimal AR was traditionally considered a benign pathologic finding without significant clinical sequelae.(7, 8) However, more recent studies have challenged this presumption by reporting increased CLAD risk after minimal AR.(9, 10) Furthermore, the optimal treatment of A1 rejection, including the need for augmented steroids remains unclear.

CXCL9 (MIG), CXCL10 (IP10), and CXCL11 (ITAC) are ELR- CXC chemokines which are induced by interferon-γ and signals through a G protein-coupled receptor, CXCR3. These chemokines are potent chemoattractants for Type I immune response mononuclear cells (e.g., activated T-cells and NK cells).(11–13) In animal models, we and others have demonstrated that all three chemokines and their shared receptor parallel the grade of AR as well as CLAD.(11, 12) Interestingly, CXCL9 expression was a magnitude of order greater than CXCL10 and CXCL11 expression during AR and CLAD.(11, 12) In vivo neutralization of CXCL9, with or without concurrent immunosuppressive agents, led to profound attenuation of AR as well as CLAD development.(11, 12) We have also demonstrated elevation of BAL CXCR3 chemokines during higher grade (≥ A2) AR among human lung transplant recipients, as well as the utility of serial BAL CXCR3 measurements post-transplant to predict CLAD development.(5)

The current study extends these findings by formally evaluating the association between minimal AR and CLAD risk, an association which has remained controversial. Furthermore, we evaluate the novel use of BAL CXCR3 chemokine measurement at the time of minimal AR as a prognostic marker of subsequent CLAD risk. Based on our human data as well our animal studies(5, 11, 12), we hypothesized that increased expression of CXCL9 in bronchoalveolar lavage fluid (BALF) during minimal AR, would increase the risk of subsequent CLAD development and have prognostic value as a biomarker of CLAD risk.

MATERIALS AND METHODS

With IRB approval, we conducted a retrospective cohort study of patients who received lung transplantation at UCLA between January 1, 2000 and December 31, 2010. Lung transplant recipients received a surveillance bronchoscopy with bronchoalveolar lavage and TBBX at 1, 3, 6 and 12 months post-transplant, as well as during episodes of clinical deterioration. Biopsies were interpreted by one of three pulmonary pathologists according to the International Multidisciplinary Consensus Statement on Idiopathic Interstitial Pneumonias (OP and DAD)(14), and the International Society for Heart and Lung Transplantation criteria (AR and LB).(6, 15) TBBXs with no histopathologic evidence of allograft injury were considered “healthy”.

Immunosuppression, anti-microbial prophylaxis and treatment of acute rejection were administered in accordance with UCLA protocol as previously described.(16) Higher grade (grade ≥ A2) acute rejection was treated with methylprednisolone 500 mg IV for three days followed by a prednisone taper from 0.5 mg/kg. Minimal AR was treated with a prednisone taper from 0.5 mg/kg without the methylprednisolone pulse. Treatment for DAD and OP was by discretion of the transplant pulmonologist and included: methylprednisolone, IVIG, plasmapheresis, basiliximab, ATG or no treatment. Spirometry was performed serially on at least a quarterly basis. CLAD was defined as a sustained 20% drop in the forced expiratory volume in 1 second (FEV1) from the average of the two best post-transplant FEV1 measurements.(3, 17) In a subset analysis of double lung transplant recipients, CLAD was further categorized as restrictive allograft syndrome (RAS)/restrictive CLAD (RCLAD) or bronchiolitis obliterans syndrome (BOS)/obstructive CLAD (OCLAD). RAS/RCLAD was defined as FVC/FVC baseline > 0.2 and chest CT showing pleural/septal thickening, interstitial reticulation or architectural distortion.(18, 19) Recipients with CLAD who did not fulfill RAS/RCLAD criteria were considered to have the BOS/OCLAD phenotype. Those who did not have a chest CT within 3 months of CLAD diagnosis were excluded from this subset analysis.

Recipients consented, with IRB approval, to the collection of BALF for research purposes. At the time of their bronchoscopies, 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. The supernatant was collected and stored unconcentrated at −80 °C after centrifugation. BALF CXCR3 chemokine concentrations (CXCL9, CXCL10 and CXCL11) were measured using luminex bead assays (Millipore, Billerica MA).

To evaluate the effect of minimal AR on CLAD risk, univariable proportional hazards models for time to CLAD were constructed with cumulative time-dependent counts for minimal AR. This cumulative variable started at a value of 0 for all recipients. At the first episode of minimal AR, this variable increased from 0 to 1, and increased again from 1 to 2 at the second episode of minimal AR. The multivariable model was adjusted for the other known histopathologic predictors of allograft injury (diffuse alveolar damage (DAD), organizing pneumonia (OP), lymphocytic bronchiolitis (LB) and AR≥A2) using cumulative time-dependent counts. To determine the impact of BALF CXCL9 elevation during minimal AR on subsequent CLAD risk, a time-dependent cumulative variable for minimal AR was

created using quartiles of CXCL9 concentrations observed during AR. For example, using the first quartile cutoff, the "A1 + CXCL9 25th" variable would increase from 0 to 1 at the first episode of minimal AR with BALF CXCL9 concentration greater than the 25th percentile. At the second episode of "A1 + CXCL9 25th", the variable would increase from 1 to 2. Univariable and multivariable models for CLAD were constructed using these "A1 + CXCL9" variables, multivariable models were adjusted for the other histopathologic injury patterns. ROC curves for CLAD development within 18 months of minimal AR was created for CXCL9, CXCL10 and CXCL11, as well as their principal component (PC = 0.491 log CXCL9 + 0.521 log CXCL10 + 0.273 log CXCL11).

RESULTS

The study cohort consisted of 441 lung transplant recipients with 1892 bronchoscopies with TBBXs in total. There were 114 (6%) biopsies from 96 recipients with DAD, 170 (9%) biopsies from 118 recipients with OP, 565 (30%) biopsies from 278 recipients with LB, and 393 (21%) biopsies from 232 recipients with AR. Among the AR biopsies, 198 (50%) were graded A1, 129 (33%) A2, 63 (16%) A3, and 3 (1%) A4. There were 303 biopsies that had concurrent injury patterns. AR occurred most frequently with LB (n=193), followed by OP (n=45) and DAD (n=25). Biopsies without histopathology were classified as "healthy" biopsies (n=842, 45%).

Clinical characteristics of recipients who developed grade A1 vs. grade ≥ A2 rejection were generally similar, including age at transplant, gender, race, native disease, transplant type and induction immunosuppression (Table 1). 207 (47%) recipients developed CLAD during the follow-up time. The average number of surveillance TBBXs were similar between recipients who developed CLAD compared with those who did not: 2.9 vs 2.8 (p=0.66), respectively. There were slightly more non-surveillance TBBXs among recipients who developed CLAD compared with those who did not: 2.3 vs. 2.0 (p=0.057), respectively. Stratification of the acute rejection biopsies by clinical indication showed a similar frequency of A1 and A2, but a higher frequency of ≥ A3 rejection for non-surveillance compared with surveillance biopsies: 6% vs 2% (p=0.001), respectively (Table 2). The median time to CLAD was shorter for recipients with at least one episode of A1 rejection compared to those with no episodes of A1 rejection: 1.8 vs 2.6 years, respectively (p<0.001). The median censoring time was not significantly different between recipients with A1 rejection compared to those without: 3.5 vs 4.1 years, respectively (p=0.18).

Risk of CLAD after Acute Rejection

To assess the impact of AR on CLAD risk, univariable and multivariable Cox models for CLAD were constructed with time-dependent cumulative counts for AR (grade A1, ≥A2 and ≥A3), as well as the other histopathologic injury patterns (DAD, OP and LB). In univariable analysis, DAD (HR 1.6 95% CI 1.2–2.3), OP (HR 1.5 95% CI 1.1–2.1), AR ≥ A2 (HR 1.4 95% CI 1.04–1.9) and AR ≥ A3 (HR 2.1 95% CI 1.4–3.0) were all associated with increased CLAD risk (Table 3). Minimal AR and LB were not associated with CLAD development. The three AR variables (AR = A1, AR ≥ A2 and AR ≥ A3) were then evaluated in multivariable models adjusted for other injury patterns. In the multivariable model including

DAD, OP, LB and AR ≥ A2, we found that DAD (HR 1.4 95% CI 1.02–2.0) and AR ≥ A2 (HR 1.4 95% CI 1.1–1.9) both predicted CLAD development, whereas OP and LB did not. Similarly, in the model including DAD, OP, LB and AR ≥ A3, we found that DAD (HR 1.7 95% CI 1.2–2.4) and AR ≥ A3 (HR 2.1 95% CI 1.4–3.1) predicted CLAD, whereas OP and LB did not. Minimal AR was not a significant predictor of CLAD in multivariable models adjusted for other injury patterns. We furthermore found no association between episodes of recurrent minimal AR and CLAD (data not shown).

To determine the importance of clinical indication for the biopsy on CLAD risk, episodes of minimal AR were categorized as surveillance vs. non-surveillance. Univariable and multivariable Cox models for CLAD were constructed with time-dependent cumulative counts for surveillance and non-surveillance episodes of minimal AR (Table 4). In univariable models, non-surveillance minimal AR was associated with CLAD risk (1.5 95% CI 1.02–2.2), while surveillance minimal AR was not. In the multivariable model adjusted for other injury patterns, both surveillance and non-surveillance minimal AR had no effect on CLAD risk.

BALF CXCR3 Chemokines Concentrations during Acute Rejection

We hypothesized that BALF CXCR3 ligands would be elevated during AR and that episodes of higher grade AR would have higher CXCR3 concentrations, reflecting the increased risk of CLAD development. We evaluated 1281 BALF samples from 382 recipients in total. There were 144 samples from 113 recipients with AR = A1, 119 samples from 93 recipients with AR ≥ A2, and 33 samples from 28 recipients with AR ≥ A3. 589 samples from 305 recipients did not have any histopathology and were considered “healthy” samples. Median BALF CXCL9, CXCL10 and CXCL11 concentrations were higher during all episodes of acute rejection compared with “healthy” biopsies (Table 5). CXCL9, CXCL10 and CXCL11 concentrations for AR = A1 vs. “healthy” biopsies were: 872 vs. 335 ($p=0.01$), 275 vs. 135 ($p<0.001$), and 71 vs. 62 pg/ml ($p=0.06$), respectively. There was a non-significant trend towards higher BALF CXCR3 ligand concentrations during episodes of higher grade AR. Median CXCL9 concentrations for AR ≥ A2 vs. AR ≥ A3 were: 1220 vs. 2775 pg/ml, respectively. Similarly, median CXCL10 and CXCL11 concentrations for AR ≥ A2 vs. AR ≥ A3 were: 241 vs. 433 and 67 vs. 78 pg/ml, respectively.

Similar to prior animal models of rejection by our group, the increase in BAL expression during AR was greatest for CXCL9, compared with CXCL10 and CXCL11. During minimal AR, BAL concentrations increased 3-fold for CXCL9, 2-fold for CXCL10, and just greater than 1-fold for CXCL11, compared with healthy biopsies (Table 5). Thus, for all subsequent analysis, we focused on the prognostic significance of BAL CXCL9 concentrations. The 25^{th}, 50^{th} and 75^{th} percentiles of CXCL9 during AR were: 309 pg/mL, 915 pg/mL and 3001 pg/mL, respectively, and right skewed (1.3) with a standard deviation of 2973.

Impact of BALF CXCL9 During Minimal AR on CLAD Risk

We found no association between episodes of minimal AR and CLAD development in multivariable Cox models adjusted for the other injury patterns. However, we hypothesized that minimal AR with high BALF CXCL9 concentrations would be associated with higher

CLAD risk. To test this hypothesis, time-dependent cumulative variables for minimal AR was created using quartiles of BALF CXCL9 concentrations. These variables were a cumulative count of A1 rejection only where the CXCL9 concentration was greater than the specified quartile-cutoff. Multivariable models adjusted for other histopathologic injury patterns demonstrated a strong association between CXCL9 concentrations during minimal AR and subsequent CLAD risk. The HR for an episode of minimal AR with CXCL9 concentration greater than the 25th percentile was 1.1 (95% CI 0.8–1.6). The HR increased to 1.6 (95% CI 1.1–2.3) and 2.2 (95% CI 1.4–3.4) for CXCL9 concentrations greater than the 50th and 75th percentiles, respectively (Table 6). In these multivariable models, DAD and AR ≥ A2 were also significant predictors of CLAD; However, the HRs for minimal AR with CXCL9 greater than the 75th percentile surpassed the HRs for DAD (1.5 95% CI 1.05–2.1) and AR ≥ A2 (1.4 95% CI 1.05–1.9).

We compared the prognostic performance of the three CXCR3 chemokines during minimal AR, using ROC analysis to evaluate CLAD development within a year and a half of the biopsy. As depicted in Figure 1, the prognostic performance of CXCL9 to predict CLAD development was superior to CXCL10 and CXCL11 with AUCs of: 0.76, 0.73 and 0.67, respectively. The CXCL9 optimum cutoff which maximized the correct classification rate was 3522 pg/mL. This cutoff was associated with a high negative predictive value (NPV), but low positive predictive value (PPV) for subsequent CLAD development. The sensitivity, specificity, PPV and NPV for CLAD development within a year and a half of the biopsy were as follows: 40%, 95%, 33% and 86%, respectively. Thus, low BAL CXCL9 effectively identified episodes of minimal AR with low risk of developing CLAD. With a NPV of 86%, only 14% of recipients who had minimal AR with CXCL9 < 3522 pg/mL developed CLAD in the next 18 months. On the other hand, 33% of recipients who had minimal AR with CXCL9 ≥ 3522 pg/mL developed CLAD in the next 18 months. The first principal component of the three chemokines did not significantly improve the prognostic performance. The first PC was calculated as: $PC = 0.491 \times \log(CXCL9) + 0.521 \times \log(CXCL10) + 0.273 \times \log(CXCL11)$, with an AUC for 18-month CLAD development of 0.78.

Impact of Clinical Indication for Bronchoscopy

We then explored the association between high BAL CXCL9 concentrations and subsequent CLAD development among asymptomatic and symptomatic episodes of minimal AR. Asymptomatic episodes of minimal AR observed during “surveillance” transbronchial biopsies demonstrated a similar association between higher CXCL9 concentrations and higher CLAD risk. In the multivariable model adjusted for other injury patterns, the HR for CLAD for an episode of asymptomatic minimal AR with CXCL9 concentrations greater than optimum cutoff (3522 pg/mL) was 3.9 (1.7–8.9), Table 7. Similarly, higher CXCL9 concentrations during symptomatic minimal AR observed during “non-surveillance” transbronchial biopsies were also associated with higher CLAD risk. The adjusted HR for CLAD for an episode of symptomatic minimal AR with CXCL9 concentrations greater than the optimum cutoff was 4.0 (2.1–7.7). DAD and AR ≥ A2 were also significant predictors of CLAD in this model, but the HRs for both non-surveillance and surveillance A1 with

elevated CXCL9 surpassed the HRs for DAD (HR 1.6 95% CI 1.1–2.2) and AR ≥ A2 (HR 1.4 95% CI 1.03–1.9).

Risk of BOS/OCLAD and RAS/RCLAD after Minimal AR

In a subset analysis of double lung transplant recipients, we evaluated the association between BAL CXCL9 elevation during minimal AR and the phenotypes of CLAD: BOS/OCLAD and RAS/RCLAD. Thirty-three of 106 (31%) double lung transplant recipients who developed CLAD were classified as RAS/RCLAD, whereas the remaining 73 (69%) were classified as BOS/OCLAD. Minimal A1 with CXCL9 concentrations greater than the optimum cutoff (3522 pg/mL) were associated with both BOS/OCLAD and RAS/RCLAD development with HRs: 4.5 (95% CI 1.7–11.6) and 3.7 (95% CI 1.1–12.3), respectively (Table 8). None of the allograft injury patterns (without consideration of CXCL9) increased BOS/OCLAD or RAS/RCLAD risk. However, this subset analysis of double lung transplant recipients with chest CT scans within 3 months of CLAD diagnosis was limited by sample size (n=253).

Impact of BALF CXCL9 During Other Allograft Injury Patterns

Lastly, we extended our analysis to evaluate the impact of BAL CXCL9 elevation during any allograft injury pattern (DAD, OP, LB or AR ≥ 1) on the risk of subsequent CLAD development. A time-dependent cumulative count was created for both “surveillance” and “non-surveillance” allograft injury patterns with CXCL9 concentration greater than the optimum cutoff (3522 pg/mL). In the multivariable model adjusted for the allograft injuries, CXCL9 elevation during both “surveillance” and “non-surveillance” allograft injuries were associated with CLAD: HRs 5.2 (95% CI 1.6–17.1) and 5.4 (95% CI 2.5–11.5), respectively (Table 9). DAD was the only injury pattern associated with CLAD in the multivariable model: HR 2.2 (95% CI 1.4–3.4).

DISCUSSION

CLAD remains the major factor limiting survival after lung transplantation, affecting 48% of recipients by 5 years and imparting a 3-year mortality greater than 50% after its onset.(20) Prior studies have demonstrated a consistent association between higher grade (≥A2) acute rejection and CLAD.(3, 4, 21) However, the clinical significance and treatment strategies for minimal acute rejection (grade A1), the most common form of acute rejection, remains controversial. In this analysis, we sought to formally evaluate the association between minimal AR and CLAD development and test a novel hypothesis using BAL CXCL9 concentration during minimal AR as a biomarker of subsequent CLAD development. We demonstrate for the first time the utility of BAL CXCL9 measurement as a prognostic biomarker that allows discrimination of CLAD risk after minimal AR. Low BAL CXCL9 effectively identified episodes of minimal AR with low risk of subsequent CLAD development. With a NPV of 86%, only 14% of recipients who had minimal AR with CXCL9 < 3522 pg/mL developed CLAD in the next 18 months. On the other hand, 33% of recipients who had minimal AR with CXCL9 ≥ 75th percentile developed CLAD in the next 18 months. Multivariable Cox models adjusted for other histopathologic injury patterns confirms the prognostic importance of high BAL CXCL9 (≥ 3522 pg/mL) with an adjusted

HR for CLAD of 4.0 (95% CI 2.4–6.8), surpassing the HRs of all histopathologic injury patterns. These findings suggest that BAL CXCL9 measurement at the time of TBBXs provides clinically useful prognostic data and may potentially guide treatment of this common form of AR, as a strategy to minimize subsequent CLAD development.

Prior studies have established a consistent association between high-grade AR (≥A2) and CLAD, but the association between minimal AR and CLAD, as well as the need for treatment of minimal AR, has been less clear. Minimal AR was traditionally regarded an innocuous finding without significant clinical sequelae (7, 8), but recent studies have challenged this presumption. Hopkins et al evaluated 1159 biopsies from 184 recipients and found that recurrent episodes of A1 rejection had higher CLAD risk compared to those with one or less A1 episodes: 68% vs 43%, respectively (p=0.022).(9) Another study of 259 recipients found that a single episode of A1 rejection was associated with a two-fold increase in the risk of CLAD in multivariable modeling.(10) This study also showed that treatment of minimal AR decreased the risk of subsequent CLAD development. One important difference between these studies and our study was in the treatment of asymptomatic minimal AR. While we routinely treated symptomatic and asymptomatic A1, the prior studies only treated symptomatic A1 with prednisone or methylprednisolone. This may have attenuated the association between minimal AR and CLAD in our study.

Furthermore, our work builds upon these prior studies by utilizing a biomarker obtained at the time of biopsy to further characterize episodes of minimal AR in terms of its CLAD risk. In rodent models, our group previously demonstrated the key role of CXCL9/CXCR3 biology in the pathogenesis of AR (12), through its recruitment of CXCR3 expressing mononuclear cells into the allograft. Furthermore, these models showed that the persistent elevation of CXCL9 in the allograft led to chronic rejection, while neutralization of CXCL9 attenuated its development.(12) Thus, in this study we hypothesized that elevated BALF CXCL9 concentrations during minimal AR, would significantly increase the risk of subsequent CLAD development.

Overall, minimal AR was not associated with increased CLAD risk in univariable and multivariable models adjusted for other histopathologic injury patterns. However, minimal AR with elevated BAL CXCL9 concentrations markedly increased CLAD risk in a dose-response manner. A single episode of minimal AR with CXCL9 greater than the 25^{th}, 50^{th} and 75^{th} percentile had a HR for CLAD of 1.1, 1.7 and 2.3, respectively. This association between high BAL CXCL9 and CLAD risk remained valid for both symptomatic and asymptomatic episodes of minimal AR detected during routine surveillance bronchoscopies. Furthermore, this association between elevated CXCL9 and CLAD risk remained valid for other injury patterns, including higher grade AR, DAD, OP and LB.

The major limitation of this study is the potential for confounding given the retrospective single center design. For example, patients with clinical deterioration may have received more frequent biopsies leading to a higher incidence of allograft injury and higher BALF CXCR3 ligand concentrations. Multivariable adjustment for all known risk factors for CLAD (e.g., primary graft dysfunction, donor specific antibodies, community acquired respiratory viruses) was beyond the scope of this analysis. Adjustment for these known risk

factors may have attenuated our results in multivariable models. TBBXs were interpreted by one of three pulmonary pathologists experienced in grading AR; however, several studies have demonstrated significant inter-observer variability in the grading of TBBX specimens after lung transplantation (22, 23), raising concerns about the reliability of the biopsy interpretations. Most importantly, treatments received for allograft injury were also not taken into account. At our institution, recipients routinely received augmented immunosuppression for AR but not for DAD, OP or LB. Higher grade AR, was treated with solumedrol 500 to 1000 mg IV for three days followed by a prednisone taper from 0.5 mg/kg. Minimal AR was treated with a prednisone taper from 0.5 mg/kg without the solumedrol pulse. Treatment for DAD, OP and LB was by discretion of the transplant pulmonologist and included: methylprednisolone, IVIG, plasmapheresis, basiliximab, ATG or no treatment. This may have attenuated the CLAD risk associated with higher grade AR, DAD and OP.

Despite these limitations, our results are unique in demonstrating the prognostic value of a biomarker concurrent with allograft injury, which outperforms the histopathologic finding from the biopsy. This finding may be particularly relevant for minimal AR, where the prognosis is strongly dependent on the biomarker measurement and the need for treatment remains controversial. BAL CXCL9 concentration during minimal AR can discriminate between high vs. low risk injury and identify recipients who may benefit from more aggressive treatment. This study evaluated 1892 biopsies from 441 recipients and to our knowledge, is the largest study to evaluate CLAD risk after minimal AR, and the first study to examine the prognostic significance of BAL CXCL9 concentrations during minimal AR.

In summary, we demonstrate for the first time the utility of BAL CXCL9 measurement as a prognostic biomarker that allows discrimination of a recipient's CLAD risk after minimal AR, the most common form of AR. Overall, episodes of minimal AR, without consideration of BAL CXCL9, did not increase CLAD risk in multivariable models. However, we find a marked increase in CLAD risk for episodes of minimal AR with BAL CXCL9 elevation in a dose-response manner. This association between BAL CXCL9 and CLAD risk remained valid for both surveillance and non-surveillance episodes of minimal AR. These findings suggest that BAL CXCL9 measurement at the time of TBBXs provides clinically useful prognostic data and may potentially guide treatment of this common form of AR, as a strategy to minimize subsequent CLAD development.

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Abbreviations

DAD	diffuse alveolar damage
AR	acute rejection
BAL	bronchoalveolar lavage

BOS	bronchiolitis obliterans syndrome
CLAD	chronic lung allograft dysfunction
CT	chest tomography
FEV1	forced expiratory volume in 1 second
FVC	forced vital capacity
ISHLT	International Society of Heart and Lung Transplantation
LB	lymphocytic bronchiolitis
LTR	lung transplant recipients
OCLAD	obstructive CLAD
OP	organizing pneumonia
PC	principal component
pg/ml	pictogram per milliliter
RAS	restrictive allograft syndrome
RCLAD	restrictive CLAD
SAS	Statistical Analysis Software
TBBX	transbronchial biopsy
UCLA	University of California Los Angeles

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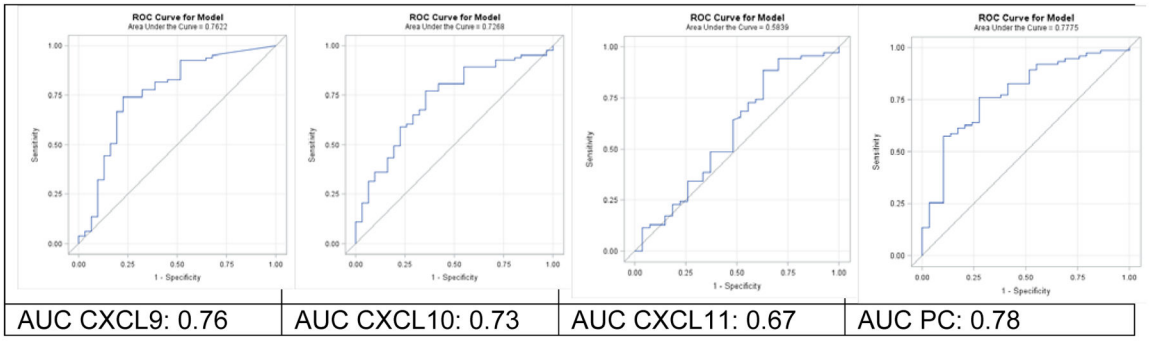

Figure 1. ROC Curves for CLAD Development within 18 Months Using BAL CXCL9 Concentrations During Minimal AR

Definition of Abbreviations: CLAD, chronic lung allograft dysfunction; BAL, bronchoalveolar lavage; AR, acute rejection; AUC, area under the curve; PC, first principal component.

Table 1

Baseline Patient Characteristics By Never/Ever Developed Acute Rejection

	All Patients		Ever Minimal AR		Ever AR ≥ A2	
	n	%	n	%	n	%
Number of patients with:	441	100%	145	33%	144	33%
Median age	60		60		60	
Male gender	259	59%	86	59%	79	55%
Single lung transplant	189	43%	56	39%	60	42%
Diagnosis						
Restrictive ILD	250	57%	83	57%	84	59%
COPD/AAT	133	30%	38	26%	37	26%
CF/bronchiectasis	27	6%	7	5%	10	7%
Other	31	7%	17	12%	12	8%
Induction						
ATG	247	56%	76	52%	72	50%
Basiliximab	192	44%	68	47%	71	50%
None	2	0%	1	1%		

Definition of abbreviations: AR = Acute rejection; A2 = grade 2 AR; ILD = interstitial lung disease; COPD = chronic obstructive pulmonary disease; AAT = alpha-1 antitrypsin deficiency; CF = cystic fibrosis; ATG = thymoglobulin.

Table 2

Acute Rejection Grades By Clinical Indication For Biopsy

	All Biopsies		Surveillance		Non-surveillance	
AR Grade	**n**	**%**	**n**	**%**	**n**	**%**
0	1490	79%	862	81%	628	77%
1	198	11%	109	10%	89	11%
2	129	7%	76	7%	53	6%
3	63	3%	20	2%	43	5%
4	3	0%	0	0%	3	0%

Definition of abbreviations: AR = Acute rejection, n= number of samples, % = percent of samples.

Table 3

Cox Proportional Hazards Model for CLAD By Acute Rejection Grade

	Univariable		Model 1†		Model 2†		Model 3†	
	HR	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
DAD	1.6**	1.2 – 2.3	1.5*	1.07 – 2.1	1.4*	1.02 – 2.0	1.7**	1.17 – 2.4
OP	1.5**	1.1 – 2.1	1.4	0.98 – 1.9	1.4	0.99 – 1.9	1.3	0.96 – 1.8
LB	1.3	0.99 – 1.8	1.2	0.9 – 1.7	1.2	0.8 – 1.6	1.1	0.8 – 1.5
AR = A1	1.2	0.9 – 1.6	1.1	0.8 – 1.5				
AR ≥ A2	1.4*	1.04 – 1.9			1.4*	1.1 – 1.9		
AR ≥ A3	2.1***	1.4 – 3.0					2.1***	1.4 – 3.1

Definition of abbreviations: CLAD = chronic lung allograft dysfunction, AR = acute rejection, HR = hazards ratio, CI = confidience interval, DAD = diffuse alveolar damage, OP = organizing pneumonia, LB = lymphocytic bronchiolitis.

†Multivariable model adjusted for variables listed.

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

Table 4

Cox Proportional Hazards Model for CLAD Surveillance vs. Non-surveillance Minimal AR

	Univariable		Multivariable[ǂ]	
	HR	**95% CI**	**HR**	**95% CI**
DAD	1.6**	1.2 – 2.3	1.5*	1.03 – 2.1
OP	1.5**	1.1 – 2.1	1.3	0.96 – 1.8
LB	1.3	0.99 – 1.8	1.1	0.8 – 1.5
AR ≥ A2	1.6**	1.2 – 2.1	1.4*	1.04 – 1.9
A1 Surveillance	1.1	0.8 – 1.5	1.1	0.8 – 1.5
A1 Non-surveillance	1.5*	1.02 – 2.2	1.3	0.8 – 1.9

Definition of abbreviations: CLAD = chronic lung allograft dysfunction, ‘AR = acute rejection, HR = hazards ratio, CI = confidience interval, DAD = diffuse alveolar damage, OP = organizing pneumonia, LB = lymphocytic bronchiolitis.

[ǂ]Multivariable model adjusted for variables listed.

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

Table 5

Median BAL CXCR3 Ligand Concentrations By Healthy Biopsies vs. Acute Rejection

		AR = A1		AR ≥ A2		AR ≥ A3	
	Healthy pg/ml	pg/ml	p-value ǂ	pg/ml	p-value ǂ	pg/ml	p-value ǂ
CXCL9	335	872	0.0122	1,220	0.0074	2,775	0.0159
CXCL10	135	275	0.0001	241	0.0004	433	0.0012
CXCL11	62	71	0.0589	67	0.0689	78	0.0420

Definition of abbreviations: BAL = bronchoalveolar lavage, AR = Acute Rejection. Pg/ml = picogram/milliliter.

ǂMixed effects model comparing AR vs healthy biopsies.

Table 6

Cox Proportional Hazards Model for CLAD Using BAL CXCL9 Concentrations During Minimal AR

	Model 1†		Model 2†		Model 3†	
	HR	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
DAD	1.5*	1.04 – 2.1	1.5*	1.04 – 2.1	1.5*	1.05 – 2.1
OP	1.3	0.97 – 1.9	1.3	0.97 – 1.9	1.3	0.9 – 1.8
LB	1.1	0.8 – 1.5	1.1	0.8 – 1.5	1.1	0.8 – 1.5
AR ≥ A2	1.4*	1.1 – 1.9	1.4*	1.1 – 1.9	1.4*	1.05 – 1.9
A1 + CXCL9 > 25th††	1.1	0.8 – 1.6				
A1 + CXCL9 > 50th††			1.6*	1.1 – 2.3		
A1 + CXCL9 > 75th††					2.2***	1.4 – 3.4

Definition of abbreviations: CLAD = chronic lung allograft dysfunction; BAL = bronchoalveolar lavage fluid, AR = acute rejection, % = percentile, HR = hazard ratio; CI = confidience interval; DAD = diffuse alveolar damage, OP = organizing pneumonia, LB = lymphocytic bronchiolitis, A2 = grade A2, A1 = grade A1.

† Multivariable model adjusted for variables listed.

†† CXCL9 > 25th: 309 pg/ml, CXCL9 > 50th: 915 pg/ml, CXCL9 > 75th: 3001 pg/ml.

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

Table 7

Cox Proportional Hazards Model for CLAD Using BAL CXCL9 Concentrations During Minimal AR By Surveillance vs Non-Surveillance Minimal AR

	CLAD[ǂ]	
	HR	**95% CI**
DAD	1.6*	1.1 – 2.2
OP	1.2	0.9 – 1.7
LB	1.1	0.8 – 1.5
AR ≥ A2	1.4*	1.03 – 1.9
Surveillance A1 + CXCL9 Opt [ǂǂ]	3.9**	1.7 – 8.9
Non-surveillance A1 + CXCL9 Opt [ǂǂ]	4.0***	2.1 – 7.7

Definition of abbreviations: CLAD = chronic lung allograft dysfunction, BAL = bronchoalveolar lavage fluid, AR = acute rejection, % = percentile, AR = acute rejection, % = percentile, HR = hazard ratio, CI = confidence interval, DAD = diffuse alveolar damage, OP = organizing pneumonia, LB = lymphocytic bronchiolitis, A2 = grade A2, A1 = grade A1.

[ǂ]Multivariable model adjusted for variables listed.

[ǂǂ]A1 rejection with CXCL9 levels > the optimum cutoff: 3522 pg/mL.

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

Table 8

Cox Proportional Hazards Model for BOS/OCLAD and RAS/RCLAD Using BAL CXCL9 Concentrations During Minimal AR

	BOS/OCLAD†		RAS/RCLAD†	
	HR	**95% CI**	**HR**	**95% CI**
DAD	1.0	0.5 – 2.1	1.7	0.7 – 4.1
OP	0.7	0.4 – 1.4	1.4	0.6 – 3.5
LB	1.3	0.7 – 2.3	1.0	0.4 – 2.5
AR ≥ A2	1.4	0.8 – 2.5	0.7	0.3 – 1.7
A1 + CXCL9 Opt ††	4.5**	1.7 – 11.6	3.7*	1.1 – 12.3

Definition of abbreviations: CLAD = chronic lung allograft dysfunction, BOS = bronchiolitis obliterans syndrom, OCLAD = obstructive CLAD, RAS = restrictive allograft syndrome, RCLAD = restrictive CLAD, BAL = bronchoalveolar lavage fluid, AR = acute rejection, HR = hazard ratio, CI = confidence interval, DAD = diffuse alveolar damage, OP = organizing pneumonia, LB = lymphocytic bronchiolitis, A2 = grade A2, A1 = grade A1.

† Multivariable model adjusted for variables listed, double lung transplant recipients only.

†† A1 rejection with CXCL9 levels > the optimum cutoff: 3522 pg/mL.

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

Table 9

Cox Proportional Hazards Model for CLAD Using BAL CXCL9 Concentrations During Any Injury By Surveillance vs Non-Surveillance Injuries

	CLAD†	
	HR	**95% CI**
DAD	2.2**	1.4 – 3.4
OP	1.0	0.6 – 1.6
LB	1.1	0.7 – 1.7
AR ≥ A1	1.0	0.6 – 1.5
Surveillance Injury + CXCL9 Opt ††	5.2**	1.6 – 17.1
Non-Surveillance Injury + CXCL9 Opt ††	5.4***	2.5 – 11.5

Definition of abbreviations: CLAD = chronic lung allograft dysfunction, BAL = bronchoalveolar lavage fluid, AR = acute rejection, % = percentile, BAL = bronchoalveolar lavage fluid, AR = acute rejection, % = percentile, HR = hazard ratio, CI = confidence interval, DAD = diffuse alveolar damage, OP = organizing pneumonia, LB = lymphocytic bronchiolitis, A1 = grade A1.

†Multivariable model adjusted for variables listed.

††A1 rejection with CXCL9 levels > the optimum cutoff: 3522 pg/mL.

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