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Impact of Allograft Injury Time-of-Onset on the Development of Chronic Lung Allograft Dysfunction After Lung Transplantation

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Abstract

The impact of allograft injury time-of-onset on the risk of chronic lung allograft dysfunction (CLAD) remains unknown. We hypothesized that episodes of *late*-onset (≥6 months) allograft injury would produce an augmented CXCR3/ligand immune response, leading to increased CLAD. In a retrospective single-center study, 1894 transbronchial biopsies from 441 lung transplant recipients were reviewed for the presence of acute rejection (AR), lymphocytic bronchiolitis (LB), diffuse alveolar damage (DAD) and organizing pneumonia (OP). The association between the time-of-onset of each injury pattern and CLAD was assessed using multivariable Cox models with time-dependent covariates. BAL CXCR3 ligand concentrations were compared between *early* and *late*-onset injury patterns using linear mixed-effects models. *Late*-onset DAD and OP were strongly associated with CLAD: adjusted HRs 2.8 95%CI 1.5-5.3 and 2.0 95%CI 1.1-3.4, respectively. The *early*-onset form of these injury patterns did not increase CLAD risk. *Late*-onset LB and AR predicted CLAD in univariable models, but lost significance after multivariable adjustment for *late* DAD and OP. AR was the only *early*-onset injury pattern associated with CLAD development. Elevated BAL CXCR3 ligand concentrations during *late*-onset allograft injury parallel the increase in CLAD risk and support our hypothesis that *late* allograft injuries result in a more profound CXCR3/ligand immune response.

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Introduction

Despite the severe impact of chronic lung allograft dysfunction (CLAD) on lung transplant survival (1), the pathogenesis is poorly understood. Since there are no effective treatments available for CLAD, the identification of risk factors is a key step towards both understanding CLAD pathogenesis and improving post-transplant outcomes. There are numerous “non-alloimmune” (e.g., respiratory infections (2-9), gastroesophageal reflux (10, 11), air pollution (12, 13), autoimmune reactivity (14, 15)) and “alloimmune” insults (e.g. acute cellular rejection (16-18)) which challenge the lung allograft. However, the histopathologic allograft response patterns are generally limited to four: diffuse alveolar damage (DAD), organizing pneumonia (OP), lymphocytic bronchiolitis (LB), and a vascular mononuclear cell infiltration consistent with acute cellular rejection (AR).

Prior studies have established AR (16-27), LB (21, 25, 28-31) and DAD (32, 33) as major risk factors for CLAD development. Several of these studies have suggested a possible propensity for higher CLAD risk after *late*-onset injury, but the results have been inconsistent.(18, 25, 27, 28, 32, 34) Importantly, these earlier studies evaluated only one or two injury patterns (e.g., AR and LB) at a time and were limited by lack of multivariable adjustment. Furthermore, the association between OP and CLAD development has not been well studied to date. In this study, we evaluated the effect of time-of-onset for all four allograft injury patterns concurrently using appropriate Cox models for CLAD with time-dependent covariates.

We previously showed in animal models and human studies that the association between allograft injury and CLAD may be mediated in part by aberrant CXCR3/ligand biology. (33, 35, 36) CXCL9 (MIG), CXCL10 (IP10), and CXCL11 (ITAC) are interfreron-γ inducible ELR-CXC chemokines (CXCR3 ligands) that signal through a shared receptor, CXCR3.(37, 38) During the first few months post-transplant, T-cells differentiate from the naïve to memory subclass depending on a number of factors (e.g., site of stimulation, antigen concentration, costimulation and cytokine milieu). (39-41) Importantly, CXCR3 ligand expression is augmented during the memory immune response and act as a potent chemoattractant for lymphocytes, particularly memory T-cells (CD4, CD8).(42-44)

Thus, we hypothesized that episodes of allograft injury occurring at later times post-transplant would produce a more profound and sustained Type I immune response, leading to increased fibroproliferation and CLAD. Given the role of CXCR3/ligand biology in perpetuating a Type I immune response (35, 36, 45), we further hypothesized that episodes of *late* allograft injury would be associated with increased bronchoalveolar lavage (BAL) CXCR3 ligand concentrations.

Methods

The study cohort consisted of all LTRs who received a first transplant at UCLA between January 1, 2000 and December 31, 2010. LTRs received bronchoscopy with bronchoalveolar lavage (BAL) and transbronchial biopsy (TBBX) at 1, 3, 6 and 12-months post-transplant, as well as during episodes of clinical deterioration. One of three pulmonary pathologists

interpreted the biopsies according to International Society for Heart and Lung Transplantation criteria (AR and LB) (46, 47) and International Multidisciplinary Consensus Statement on Idiopathic Interstitial Pneumonias (DAD and OP) (48). LB was graded as present or absent until March 1, 2009, and thereafter graded according to the revised 2007 ISHLT criteria (0, B1R, B2R or ungradable). (46) Biopsy data were coded for the presence or absence of DAD, OP, LB and AR (grade A1 or greater). TBBXs with two concurrent histopathologic findings were coded for the presence of both injury patterns.

Immunosuppression, anti-microbial prophylaxis and treatment of acute rejection were administered in accordance with the UCLA lung transplant protocols as described previously. (7) Serial spirometry was performed at least quarterly. CLAD was defined as a sustained drop of at least 20% in the forced expiratory volume in 1 second (FEV1) from the average of the two best post-transplant FEV1 measurements. (20, 49) In a subset analysis of double lung transplant recipients, CLAD was further classified as restrictive allograft syndrome (RAS) or bronchiolitis obliterans syndrome (BOS) based on Sato and colleagues's 2013 definition utilizing spirometry (50) and chest computed tomography (CT). RAS was defined as: $\Delta FVC\%/\Delta FEV1\% > 0.5$ and chest CT showing ground glass opacification, interstitial reticulation or interlobular septal thickening. Recipients with CLAD who did not fulfill RAS criteria were considered to have the BOS phenotype. Those who did not have a chest CT within 3 months of CLAD diagnosis were excluded from this subset analysis.

To explore the effect of allograft injury time-of-onset on CLAD risk, univariable Cox models for CLAD were constructed with cumulative time-dependent counts for the injury patterns. For example, a recipient was initially coded 0 for acute rejection, 1 at the first episode of acute rejection, 2 at the second episode etc. The dataset contained one observation for each recipient with multiple columns for the time and value of TBBX results. The TBBX results were entered into the Cox model as cumulative time-dependent repeated measures. Thus, both the timing and recurrence of the allograft injuries were taken into account. We began by constructing univariable Cox models with cumulative time-dependent counts for the injury patterns using various time from transplant cutoffs: ≥1-month, ≥3-month, ≥6-month, ≥9-month and ≥12-months. In this exploratory analysis, an injury pattern only increased the cumulative count when it occurred after the specified cutoff time.

In keeping with prior studies, for all subsequent analysis an injury pattern was considered “late-onset” if the time from transplant was ≥ 6 months, otherwise it was considered “early-onset”. (18, 25, 28). Univariable Cox models for CLAD were constructed with time-dependent cumulative counts for the “early” and “late-onset” forms of each allograft injury patterns. For example, a recipient was initially coded 0 for early-AR and late-AR. At the first episode of “early-onset” AR, the early-AR variable increased from 0 to 1, the late-AR variable remained at 0. At the first episode of “late-onset” AR, the late-AR variable increased from 0 to 1, the early-AR variable remained at its prior value. The final multivariable model included all significant ($p<0.05$) variables from the univariable models.

A subset of patients consented to the collection of BAL fluid 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. After centrifugation, the supernatant was collected and stored unconcentrated at -80° C. CXCR3 ligand concentrations were measured using CXCL9, CXCL10 and CXCL11 bead assays (Millipore, Billerica MA). The lower limit of detection for CXCL9, CXCL10 and CXCL11 were 19.2, 14.0 and 1.7 picogram/milliliter (pg/ml), respectively. Given the high correlation among the three CXCR3 chemokines, a principal component analysis was performed to assess them in aggregate. The first principal component (PC) of the three CXCR3 ligands was calculated as: PC = 0.357 logCXCL9 + 0.439 logCXCL10 + 0.386 logCXCL11. The PC accounted for 57% of the total chemokine variation. CXCR3 ligand concentrations were compared between *early* and *late*-onset injury patterns using linear mixed effects models to account for repeated measurements from the same individuals. Analyses were performed with SAS (v9.4).

Results

Histopathologic Findings

In total, 1894 bronchoscopies with TBBXs were evaluated from 441 lung transplant recipients. There were 114 (6%) biopsies with DAD, 169 (9%) biopsies with OP, 565 (30%) biopsies with LB and 391 (21%) biopsies with AR. The AR biopsies were graded as follows: 197 (50%) grade A1, 129 (33%) grade A2, 62 (16%) grade A3, and 3 (1%) grade A4. There were 303 TBBXs with concurrent injury patterns. AR and LB co-occurred most frequently (n=193). OP occurred with LB (n=79), AR (n=45) and DAD (n=30). DAD occurred with LB (n=49) and AR (n=25). Eight hundred and thirty-eight (44%) TBBXs from 441 (100%) recipients had no evidence of histopathology and were classified as “healthy biopsies”. Using a predetermined 6-month cutoff for *late*-onset, the number of *early* vs. *late* episodes of the “healthy”, DAD, OP, LB and AR biopsies were: 507 vs 331, 65 vs. 49, 111 vs 58, 357 vs 208 and 263 vs. 128, respectively (Figure 1). Table 1 shows demographic and clinical characteristics of recipients who had histopathologic allograft injury, stratified by whether they developed *early* vs. *late*-onset injury. Most of the demographic and clinical variables were evenly distributed between recipients who developed *early* vs *late*-onset injury. However, a higher proportion of biopsies in the *early* period was for surveillance purposes compared with the *late* period: 821 / 1181 (70%) vs. 246 / 713 (35%), respectively.

Risk of CLAD after Allograft Injury

To explore the impact of the time-of-onset of specific allograft injury patterns on CLAD risk, univariable Cox models were constructed with cumulative time-dependent counts for the injury patterns using various time from transplant cutoffs (Figure 2). Overall, episodes of allograft injury occurring at later times post-transplant were associated with increased CLAD risk. This trend was most notable for DAD and OP. The HR for an episode of DAD occurring after 1, 3, 6, 9 and 12 months was: 2.8, 4.0, 4.9, 5.2 and 5.2, respectively. Similarly, the HR for an episode of OP occurring after 1, 3, 6, 9 and 12 months was: 1.8, 2.5, 3.3, 4.3 and 5.8, respectively. The HRs for LB increased from 1.1, 1.3, 1.8, 2.4 and 2.4 during these time intervals. Compared with the other injury patterns, the HRs for AR remained relatively constant over time: 1.6, 1.5, 1.5, 2.0 and 2.1, respectively.

We further evaluated this relationship using Cox models with time-dependent counts for the each of the *early* and *late*-onset injury patterns as well as plausible demographic variables associated with poor outcomes (Table 2). In the univariable models for time to CLAD, the *late*-onset allograft injuries DAD, OP and LB were strongly predictive of CLAD development, while *early-onset* forms of these injuries were not. The unadjusted HRs for CLAD for *late* vs *early*-onset DAD, OP and LB were: 4.9 ($p<0.001$) vs 1.2 ($p=0.25$), 3.3 ($p<0.001$) vs 1.2 ($p=0.40$) and 1.8 ($p<0.001$) vs 1.2 ($p=0.18$), respectively (Table 2). *Late* and *early*-onset AR were both associated with CLAD in univariable models with HRs: 1.5 ($p=0.014$) vs 1.4 ($p=0.026$), respectively. Other demographic variables including: age > 70, gender, single lung transplant, pre-transplant diagnosis of idiopathic pulmonary fibrosis, and induction immunosuppression was not associated with CLAD development ($p<0.05$). The injury patterns significant in the univariable models were entered into the multivariable model: *late*-DAD (HR 2.8 95% CI 1.5-5.3), *late*-OP (HR 2.0 95% CI 1.1-3.4), and *early*-AR (HR 1.4 95% CI 1.0-1.8) remained significant predictors of CLAD, whereas *late*-LB and *late*-AR both lost significance.

Kaplan-Meier curves for freedom from CLAD were constructed and stratified by recipients with never vs. *late* vs. *early*-onset allograft injury. Recipients who never had an episode of allograft injury had a median time to CLAD of 6.0 years. (Figure 3) Those with an episode of *late*-onset DAD had a median time of 2.1 years, while those who had an episode of early-DAD had a median time of 3.6 years. Similarly, the median time to CLAD for those with *late* and *early*-onset OP was 1.9 and 3.3 years, respectively. The median time to CLAD for recipients with *late* and *early*-onset LB was 2.7 and 4.4 years, while the median time for those with late and early-onset AR was 2.8 and 3.7 years, respectively.

Risk of BOS and RAS after Allograft Injury

In a subset analysis among double lung transplant recipients with chest CTs within 3 months of CLAD diagnosis, we explored the association between allograft injury and the CLAD phenotypes: BOS and RAS. Of the 202 double LTRs with sufficient spirometric and radiographic data, 106 (53%) developed CLAD. 51 (48%) of these recipients met RAS criteria, while the remaining 55 (42%) were categorized BOS. The univariable models for time to RAS showed a similar pattern as time to CLAD but with higher HRs for all injury patterns. The *late*-onset injury patterns DAD, OP and LB were strongly predictive of RAS development, while the *early*-onset forms of these injuries were not. The unadjusted HRs for RAS for *late* vs *early*-onset DAD, OP and LB were: 16.0 ($p<0.001$) vs 1.8 ($p=0.087$), 6.6 ($p<0.001$) vs 1.3 ($p=0.41$) and 2.0 ($p=0.034$) vs 1.5 ($p=0.21$), respectively (Table 3). *Late* and *early*-onset AR were both associated with RAS in univariable models with HRs: 2.0 ($p=0.026$) vs. 1.8 ($p=0.040$), respectively. In the multivariable model: *late*-DAD (HR 6.2 95% CI 2.0-18.8), *late*-OP (HR 3.2 95% CI 1.1-8.8), and *early*-AR (HR 1.8 95% CI 1.0-3.2) remained significant predictors of RAS, whereas *late*-LB and *late*-AR both lost significance. The univariable models for time to BOS showed a different pattern from the time to CLAD or RAS models: Late-LB was the only injury pattern associated with time to BOS with HR 2.1 (95% CI 1.1-3.9).

BAL Chemokines Concentrations during Early and Late Allograft Injury

We previously showed that the association between allograft injury and CLAD may be mediated in part by aberrant CXCR3/ligand biology causing persistent mononuclear cell infiltration and further allograft injury. In the current study, we hypothesized that *late* episodes of allograft injury would be associated with a stronger CXCR3/ligand immune response, as measured as BAL CXCR3 ligand concentrations, corresponding to their increased CLAD risk.

779 BAL CXCR3 concentrations from 251 recipients were available for evaluation. There was no difference in CXCL9, CXCL10 or CXCL11 concentrations between the *late* and *early* "healthy" biopsies: The median CXCL9, CXCL10 and CXCL11 concentrations were: 242 vs 291 pg/ml (p=0.80), 120 vs 122 pg/ml (p=0.43), and 81 vs 80 pg/ml (p=0.80), respectively (Table 5). In contrast, *late*-onset allograft injury was associated with significantly higher BAL CXCR ligand concentrations compared to early-onset injury. The median CXCL9, CXCL10 and CXCL11 concentrations for *late* and *early*-onset injuries were: 1703 vs 623 pg/ml (p=0.0006), 340 vs 207 pg/ml (p=0.022) and 95 vs 82 pg/ml (p=0.034), respectively. The PC analysis confirmed the increased CXCR3 ligand concentrations during *late*-onset allograft injury compared with *early*-onset. The PC for the *late* and *early*-onset allograft injury was: 0.258 vs -0.056 (p=0.0024). There was no difference in the PC between the *late* and *early* healthy biopsies.

Discussion

Since the pathogenesis of CLAD remains poorly understood with no known effective therapies, the identification and avoidance of risk factors is critical. Previous studies have established AR, LB and DAD as strong risk factors for CLAD development (17, 18, 21, 32, 33). Several of these studies suggest a higher CLAD risk after late-onset injury, but the results have not been consistent (18, 25, 27, 28, 32, 34). These prior studies evaluated only one or two injury patterns (e.g., AR and LB) at a time, and the association between OP and CLAD has not been well described. In this study, using time-dependent multivariable analysis controlling for all four allograft injury patterns, we sought to evaluate the significance of time-of-onset when considering the association between histopathologic injury and CLAD. We demonstrate that *late-onset* (≥6 months from transplant) DAD and OP were strongly predictive of CLAD development, while the *early-onset* forms of these injuries have minimal effect on CLAD risk. *Late*-onset LB and AR were associated with CLAD in univariable models, but lost significance after multivariable adjustment for DAD and OP. AR was the only *early* injury pattern associated with CLAD in univariable and multivariable models.

DAD is the most severe form of acute lung allograft injury. However, the impact of DAD on CLAD risk has only been evaluated in a few studies. Fisher and colleagues found no difference in the incidence of CLAD among 291 LTRs after *early*-DAD (<7 days) compared to those without *early*-DAD. (34) In contrast, Sato and colleagues showed increased CLAD risk after both *early* (< 3 months) and *late* (≥ 3 months) onset DAD among 720 bilateral LTRs. (32) Specifically, they found that *early*-DAD increased the risk of bronchiolitis obliterans syndrome (BOS), while *late*-DAD increased the risk of restrictive allograft

syndrome (RAS). We similarly found a strong association between *late*-DAD and RAS (HR 6.2 95% CI 2.0-18.8), but no association between *early*-DAD and CLAD or BOS. Several studies have reported an association between primary graft dysfunction (PGD) and subsequent CLAD.(51, 52) The histopathology of PGD is often DAD; However, in our dataset PGD associated DAD was likely not captured due to our surveillance protocol which began at 1-month.

OP is arguably the second most severe form of acute allograft injury. However, there is a paucity of studies evaluating the association between OP and the development of CLAD. A study of 74 LTRs reported an association between OP and CLAD in univariable analysis, but found no association after multivariable adjustment. (31) A larger study involving 230 LTRs similarly did not find OP to be an independent predictor of CLAD. (28) We previously demonstrated that when time-of-onset is ignored, OP does not increase the risk of CLAD development. (33) However, when OP is stratified by time-of-onset, it is one of the strongest predictors of CLAD with HRs rivaling DAD. To our knowledge, this is the first study to show an association between OP and CLAD. The high HR of *late*-onset OP for CLAD development underscores the need for more studies to confirm this novel finding and determine treatment options to minimize the subsequent development of CLAD.

Several studies have suggested increased CLAD risk after episodes of late-onset AR and LB. A retrospective study of 259 LTRs by Hachem and colleagues reported slightly higher HRs for CLAD after *late*-onset (>6 months) minimal (A1) rejection compared with *early* rejection: HRs 2.97 vs. 2.28, respectively.(18) Similarly, a study involving 132 LTRs by Kroshus and colleagues found increased CLAD risk among recipients with *late*-onset AR (>3 months), but not *early* AR. (27) Husain and colleagues evaluated both AR and LB in a study of 134 LTRs and reported an association between both *late* (> 6 months) AR and *late* LB with CLAD. (25) In contrast to these prior studies which only evaluated one or two injury patterns at a time, we used time-dependent multivariable analysis adjusting concurrently for *all four* allograft injury patterns. While *late*-LB and *late*-AR predict CLAD in univariable models, they lost significance after multivariable adjustment for two risk factors which were significantly stronger, *late*-DAD and *late*-OP. Of note, the HRs for CLAD correlate with the severity of the allograft injury pattern with the HRs for DAD > OP > AR/LB.

The mechanism responsible for this association between time of injury onset and CLAD remains unclear. We previously demonstrated that Type I immune responses mediate in part the progression from allograft injury to CLAD.(33, 35, 36, 53) The allograft injury patterns likely represent a deleterious cycle of cell damage, Type I immune response mediated in part by CXCR3/ligands, recruitment of injurious mononuclear cells, particularly memory T-cells, into the allograft causing further cell damage, CXCR3 ligand release and eventual fibroproliferation. (33, 35, 36) Clinical studies by our group and others have shown increased BAL concentrations of the CXCR3 ligands during AR (35, 54), LB (33), DAD (33) and CLAD (33, 35, 55).

In the current study, we hypothesized that BAL CXCR3 ligand concentrations would be higher during episodes of *late* allograft injury compared with *early* injury, reflecting the

increased risk of CLAD development. We evaluated 779 BAL CXCR3 ligand concentrations from 251 recipients and found significant elevations of all three CXCR3 ligands during the allograft injury patterns compared to healthy biopsies. Furthermore, *late*-onset injuries had markedly higher CXCR3 ligand concentrations compared to *early*-onset injuries supporting our hypothesis that *late* allograft injuries result in a more profound CXCR3/ligand immune response.

Several factors may be contributing to an augmented CXCR3/ligand response after *late* allograft injury. At the time of surgery, our lung transplant recipients receive induction immunosuppression with thymoglobulin or basiliximab which may lower CXCR3 ligand levels, both agents with a duration of action between 3-6 months. (56, 57) Alternatively, during the first few months post-transplant, lymphocytes differentiate from the naïve to memory subclass depending on a number of factors (e.g., site of stimulation, antigen concentration, costimulation and cytokine milieu). (39-41) The recruitment and stimulation of memory T-cells, as opposed to naïve T-cells, during *late* allograft injury may be producing a more profound and sustained Type I immune response, leading to increased fibroproliferation and CLAD. The more vigorous immune response generated by the memory T-cell recall response, compared with naïve T-cells, has been well established. (58, 59) Future studies characterizing the population of T-cells (memory vs naive) involved during *late* and *early* allograft injury are warranted.

AR was the only *early*-onset allograft injury which was associated with CLAD development. The pathogenesis of AR however, is fundamentally different than the other injury patterns. While many cases of DAD, OP and LB are initiated by exposure related insults (e.g., aspiration, infection, pollution (13, 33, 60)), AR represents an allo-immune response. Transplant recipients with *early* AR may have had the early presence of circulating allo-responsive memory T-cells. The development of pretransplant allo-specific memory T-cells may occur due to allosensitization from prior pregnancies, blood transfusions or infection. In fact, several studies in renal transplantation have demonstrated a strong association between *pre*-transplant donor-specific memory T-cells and the development of *early* AR. (42-44, 61, 62) Further research studying the mechanism responsible for the association between acute rejection and CLAD is clearly needed.

The major limitation of this study is the potential for confounding inherent to retrospective single center studies. Patients with clinical deterioration may have received more frequent biopsies leading to a higher incidence of histopathologic findings. Our study design accounted only for allograft injuries that were captured by TBBX. Undoubtedly, there were episodes that were missed due to the infrequency of TBBX sampling and poor sensitivity for detecting the more subtle injury patterns (i.e., AR and LB). Treatments received for the allograft injury were also not taken into account. At our institution, patients routinely received augmented immunosuppression for AR but not for DAD, OP or LB. Our study design included TBBXs performed for regular surveillance as well as clinical deterioration. Since there were more non-surveillance bronchoscopies occurring later, allograft injuries occurring in the *late*-onset group were likely to be more severe and symptomatic, compared to the asymptomatic injuries observed on surveillance bronchoscopies. Unfortunately, stratification by clinical indication of the bronchoscopy was limited due to sample size.

Finally, multivariable adjustment for all known risk factors for CLAD (e.g., primary graft dysfunction, donor specific antibodies) was beyond the scope of this analysis.

This study extends our understanding of the association between CLAD and allograft injury, one of the most important risk factors associated with its development. We evaluated 1894 transbronchial biopsies from 441 lung transplant recipients and to our knowledge is the largest study to evaluate all four allograft injury patterns concurrently using appropriate Cox models for CLAD with time-dependent covariates. We demonstrate for the first time that OP is a strong predictor of CLAD when time-of-onset is taken into account, with HRs surpassing both LB and AR. Furthermore, this is one of the first studies to systematically evaluate pathophysiologic risk factors for the CLAD phenotypes: RAS and BOS. We show that the parenchymal and vascular injury patterns DAD, OP and AR predict RAS development, while the airway-centric injury pattern LB predicts BOS. Finally, this study evaluated 779 BAL CXCL9 concentrations from 251 lung transplant recipients and is the largest study to evaluate chemokine expression patterns during allograft injury.

In conclusion, we demonstrate the importance of time-of-onset when considering the association between allograft injury and CLAD. *Late*-onset DAD and OP markedly increase CLAD risk, specifically the restrictive phenotype RAS, whereas the *early*-onset form of these injury patterns do not. *Late*-onset LB increases the risk of BOS, the obstructive phenotype of CLAD, whereas *early*-onset LB does not. AR was the only *early*-onset injury pattern which predicted CLAD / RAS development. Elevated BAL CXCR3 ligand concentrations during *late*-onset allograft injury parallel the increase in CLAD risk and support our hypothesis that *late* allograft injuries result in a more profound CXCR3/ligand immune response. However, the mechanism responsible for this augmented immune response during *late* allograft injury requires further study. The identification of key events which increase CLAD risk represent unique opportunities to better understand the immunologic processes responsible for CLAD development. Given the potential importance of time-of-onset when considering the association between allograft injury and CLAD, this finding should be validated in a larger multi-center study.

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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
OP	organizing pneumonia
PC	principal component
pg/ml	pictogram per milliliter
RAS	restrictive allograft syndrome
SAS	Statistical Analysis Software
TBBX	transbronchial biopsy
UCLA	University of California Los Angeles

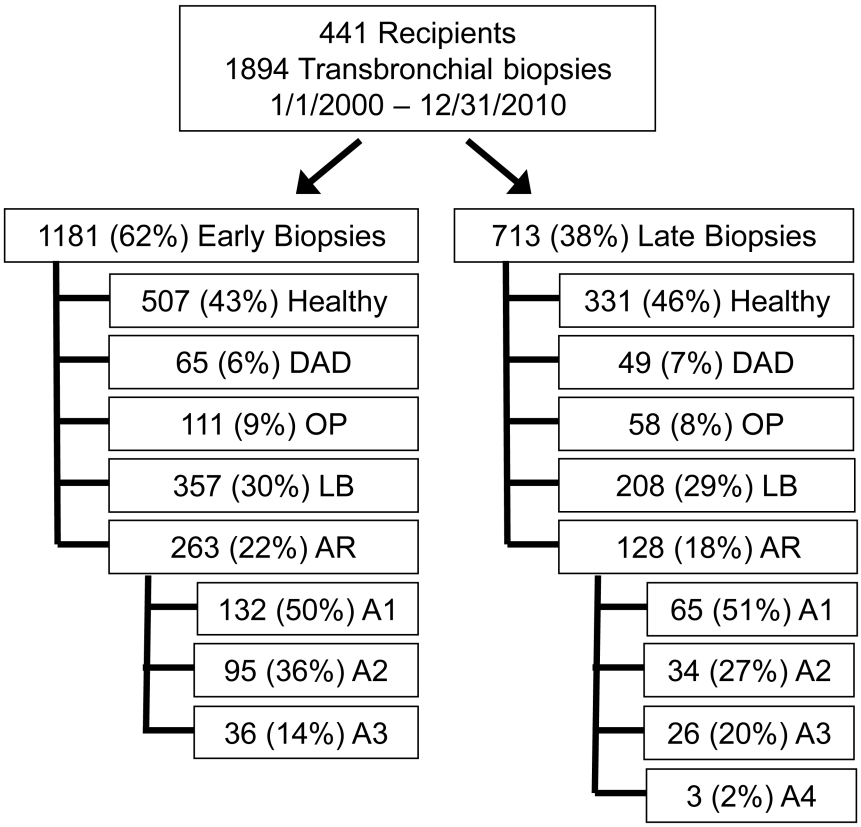

Figure 1.

Study profile. DAD = diffuse alveolar damage; OP = organizing pneumonia; LB = lymphocytic bronchiolitis; AR = Acute rejection (Grade A2 or higher); Early < 6 months, Late ≥ 6 months. B

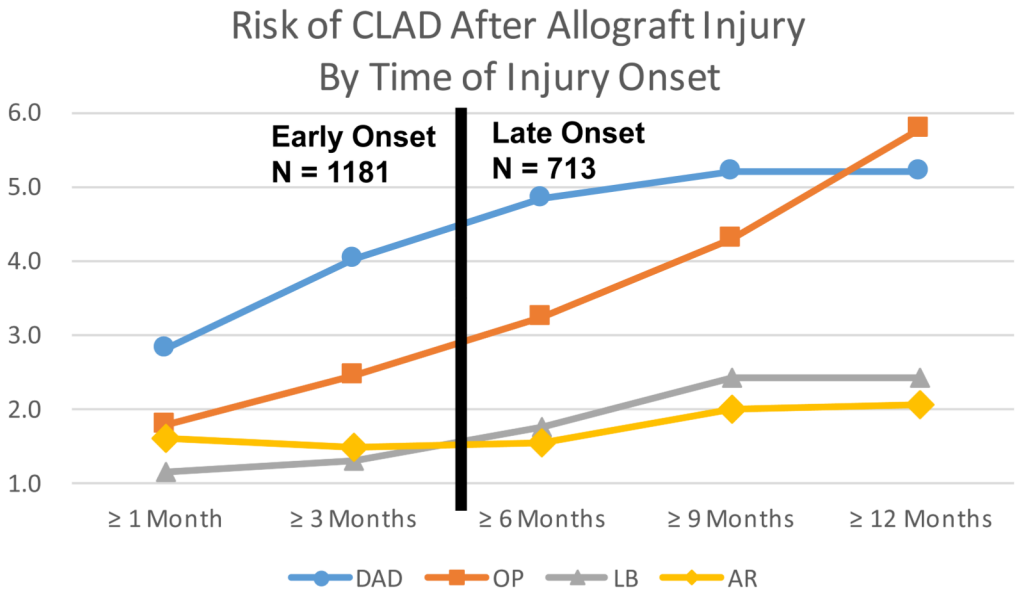

Figure 2.

Risk of CLAD after Allograft Injury by Time of Injury Onset. Early < 6 months, Late ≥ 6 months. DAD = diffuse alveolar damage; OP = organizing pneumonia; LB = lymphocytic bronchiolitis; AR = acute rejection.

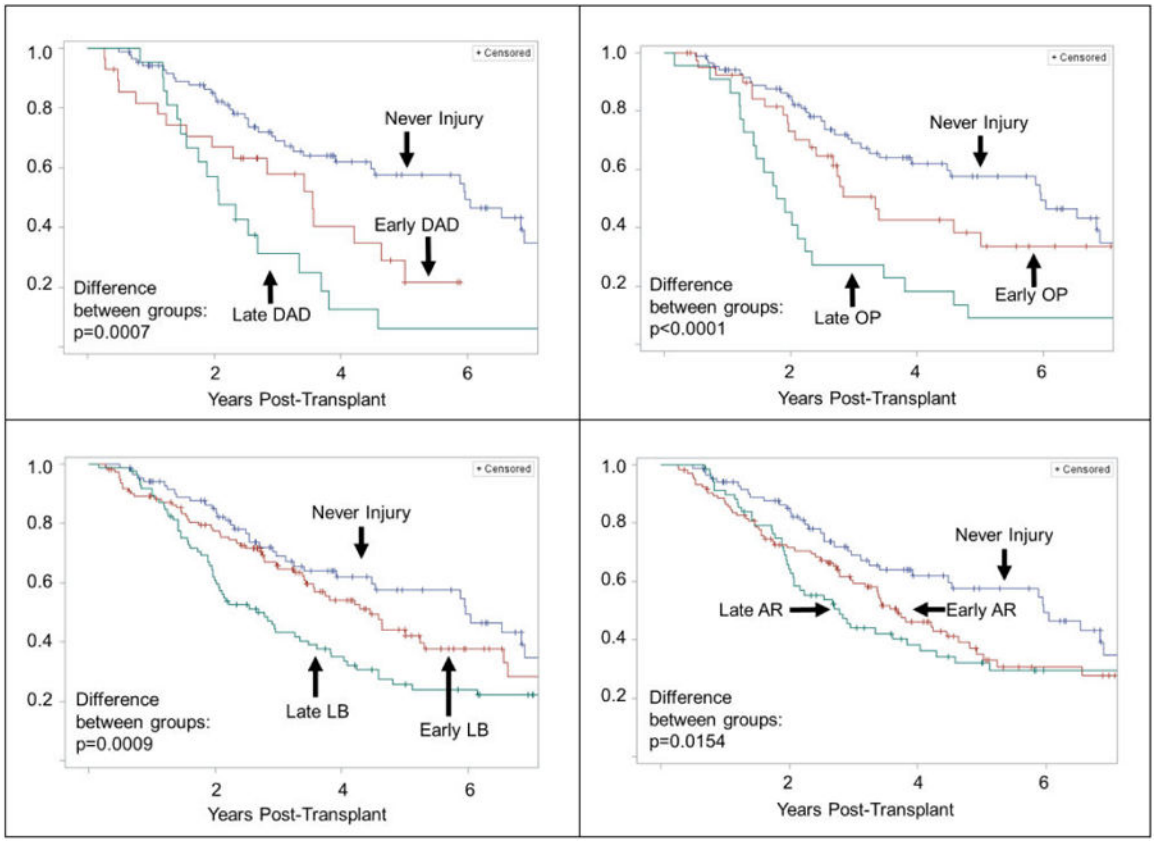

Figure 3.

Kaplan-Meier plot for time to CLAD in lung transplant recipients with never vs early vs late-onset episodes of allograft injury. Early: < 6 months from transplant, late: ≥ 6 months post-transplant.

Table 1
Baseline Patient Characteristics By Allograft Injury Status (Early vs. Late Onset)

	Early		Late	
	n (%)	%	n (%)	%
Number of patients:	316	72%	169	38%
Median age:	60		59	
Male gender:	186	59%	99	59%
Single lung transplant:	134	42%	69	41%
Diagnosis:				
Restrictive ILD	186	59%	93	55%
COPD / AAT	84	26%	52	31%
CF / bronchiectasis	21	7%	9	5%
Other	25	8%	15	9%
Induction:				
ATG	165	52%	104	61%
Basiliximab	150	47%	64	38%
None	1	1%	1	1%
Number of patients with:				
Any Injury	316	72%	169	38%
DAD	61	14%	40	9%
OP	84	19%	47	11%
LB	239	54%	126	29%
AR	183	41%	51	12%
Number of TBBX:†	2.8	(1.1)	2.4	(1.6)
Number of Surveillance TBBX:††	2.1	(0.9)	1.3	(0.5)

Definition of abbreviations: Early onset < 6 months from transplant; Late onset ≥ 6 months from transplant; ILD = interstitial lung disease; COPD = chronic obstructive pulmonary disease; AAT alpha=1 antitrypsin; CF = cystic fibrosis; ATG thymoglobulin; ALI = acute lung injury; OP = organizing pneumonia; LB = lymphocytic bronchiolitis; AR = acute cellular rejection. TBBX: transbronchial biopsies.

† Average biopsies per patient (with standard deviation).

†† Average surveillance biopsies per patient (with standard deviation).

Table 2

Cox Model for CLAD with Time-Dependent Covariates

	Univariable			Multivariable†		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
DAD < 6 months	1.2	0.9 - 1.8	0.2545			
DAD ≥ 6 months	4.9	2.8 - 8.4	0.0001	2.8	1.5 - 5.3	0.0020
OP < 6 months	1.2	0.8 - 1.7	0.4037			
OP ≥ 6 months	3.3	2.1 - 5.1	0.0001	2.0	1.1 - 3.4	0.0177
LB < 6 months	1.2	0.9 - 1.6	0.1814			
LB ≥ 6 months	1.8	1.3 - 2.4	0.0004	1.3	0.9 - 1.9	0.1839
AR < 6 months	1.4	1.0 - 1.8	0.0260	1.4	1.0 - 1.8	0.0319
AR ≥ 6 months	1.5	1.1 - 2.2	0.0137	1.2	0.8 - 1.8	0.3754
Age > 70	1.3	0.8 - 2.1	0.2965			
Single lung	1.3	1.0 - 1.7	0.0500			
Diagnosis: IPF	0.9	0.7 - 1.2	0.5075			
Induction: ATG	0.9	0.7 - 1.2	0.3705			

Definition of abbreviations: CLAD = chronic lung allograft dysfunction; HR = hazards ratio; CI = confidience interval; ALI = acute lung injury; OP = organizing pneumonia; LB = lymphocytic bronchiolitis; AR = acute rejection; Single lung = single lung transplant; IPF = idiopathic pulmonary fibrosis; ATG - thymoglobulin;

† Multivariable model includes only the variables listed.

Table 3

Cox Model for RAS with Time-Dependent Covariates

	Univariable			Multivariable †		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
DAD < 6 months	1.8	0.92 - 3.7	0.0867			
DAD ≥ 6 months	16.0	6.8 - 37.5	0.0001	6.2	2.0 - 18.8	0.0014
OP < 6 months	1.3	0.7 - 2.6	0.4088			
OP ≥ 6 months	6.6	3.2 - 13.4	0.0001	3.2	1.1 - 8.8	0.0275
LB < 6 months	1.5	0.8 - 2.6	0.2057			
LB ≥ 6 months	2.0	1.1 - 3.7	0.0336	1.0	0.5 - 2.2	0.9992
AR < 6 months	1.8	1.0 - 3.1	0.0397	1.8	1.0 - 3.2	0.0395
AR ≥ 6 months	2.0	1.1 - 3.6	0.0262	1.6	0.8 - 3.3	0.1774

Definition of abbreviations: CLAD = chronic lung allograft dysfunction; HR = hazards ratio; CI = confidience interval; ALI = acute lung injury; OP = organizing pneumonia; LB = lymphocytic bronchiolitis; AR = acute rejection;

† Multivariable model includes only the variables listed.

Table 4
Cox Model for BOS with Time-Dependent Covariates

	Univariable †		
	HR	**95% CI**	**p-value**
DAD < 6 months	1.2	0.5 - 2.6	0.7033
DAD ≥ 6 months	2.6	0.6 - 11.1	0.2012
OP < 6 months	0.6	0.3 - 1.5	0.2784
OP ≥ 6 months	1.4	0.5 - 4.2	0.5276
LB < 6 months	1.1	0.6 - 1.9	0.7887
LB ≥ 6 months	2.1	1.1 - 3.9	0.0203
AR < 6 months	1.0	0.6 - 1.7	0.9846
AR ≥ 6 months	1.4	0.8 - 2.7	0.2739

Definition of abbreviations: CLAD = chronic lung allograft dysfunction; HR = hazards ratio; CI = confidience interval; ALI = acute lung injury; OP = organizing pneumonia; LB = lymphocytic bronchiolitis; AR = acute rejection;

† Univariable model since only LB was significant.

Table 5

Median BAL CXCR3 Ligand Concentrations Stratified by Time of Injury Onset

	Healthy			Allograft Injury		
	Early pg/ml	**Late pg/ml**	**p-value †**	**Early pg/ml**	**Late pg/ml**	**p-value †**
CXCL9	291	242	0.7978	623	1,703	0.0006
CXCL10	122	120	0.4246	207	340	0.0227
CXCL11	80	81	0.7951	82	95	0.0343
PC††	(0.435)	(0.496)	0.4156	(0.056)	0.258	0.0024

Definition of abbreviations: BAL = bronchoalveolar lavage; Early < 6 months, late ≥ 6 months from transplant. Pg/ml = picogram/milliliter.

†Mixed effects model comparing early vs late biopsies.

††PC: First principal component of CXCL9, CXCL10 and CXCL11.