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Dectin-1 genetic deficiency predicts chronic lung allograft dysfunction and death

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Abstract

Background: Innate immune activation impacts lung transplant outcomes. Dectin-1 is an innate receptor important for pathogen recognition. We hypothesized that genotypes reducing dectin-1 activity would be associated with infection, graft dysfunction, and death in lung transplant recipients.

Methods: We assessed the rs16910526 CLEC7A gene polymorphism Y238X, which results in dectin-1 truncation, in 321 lung allograft recipients at a single institution and in 1,129 lung allograft recipients in the multi-center lung transplant outcomes group (LTOG) cohort. Differences in dectin-1 mRNA, cytokines, protein levels, immunophenotypes, and clinical factors were assessed.

Results: Y238X carriers had decreased dectin-1 mRNA expression (p=0.0001), decreased soluble dectin-1 protein concentrations in BAL (p=0.008) and plasma (p=0.04), and decreased monocyte surface dectin-1 (p = 0.01) compared to wild type subjects. Y238X carriers had an increased risk of fungal pathogens (HR 1.17, CI 1.0 – 1.4), an increased risk of graft dysfunction or death (HR 1.6, CI 1.0 – 2.6), as well increased mortality in the UCSF cohort (HR 1.8, CI 1.1 – 3.8) and in the LTOG cohort (HR 1.3, CI 1.1 – 1.6), compared to CLEC7A wildtype subjects.

Conclusion: Increased rates of graft dysfunction and death associated with this dectin-1 polymorphism may be amplified by immunosuppression that drives higher fungal burden from compromised pathogen recognition.

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Graphical Abstract

Bronchiole in lung transplant recipient (wildtype CLEC7A)

Bronchiole in lung transplant recipient (Y238X carrier)
Introduction

Even though lung transplantation is a life-prolonging option for many patients with advanced pulmonary disease, lung allograft recipients experience lower survival rates relative to other solid organ transplant patients (1). Chronic lung allograft dysfunction (CLAD) remains the primary driver of poor long-term survival (2). More than 50% of lung allograft recipients alive at 5 years have CLAD, manifesting as obstructive and/or restrictive defects in pulmonary function (3).

CLAD is associated with constrictive bronchiolitis and parenchymal fibrosis pathology, both of which result from the integration of multiple stressors on the allograft (4-9). Importantly, CLAD risk is increased following infection or colonization with Aspergillus and other airway pathogens (10, 11). Local tissue inflammation enhances recipient alloimmune responses leading to airway fibrosis (3, 12). Thus, increased innate immune system activation may link airway injury from infection to CLAD.

Dectin-1 is a c-type lectin innate immune receptor that recognizes β-glucans on common transplant-associated pathogens. It is expressed on macrophages, dendritic cells and epithelial cells (13). In pulmonary epithelial cells, dectin-1 activation by bacterial stimulation leads to cytokine signaling (14). Dectin-1 signals through an immunoreceptor tyrosine-based activation motif (ITAM), leading to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent cytokine production and cleavage of the dectin-1 receptor (15, 16). While dectin-1 can activate responses alone, co-stimulation with toll-like receptors further augments cytokine production through nuclear factor of activated T-cells (NFAT) and NF-κB transcription (17).

The rs16910526 or Y238X single nucleotide polymorphism (SNP) in the dectin-1 gene CLEC7A, is relatively common and leads to the presence of a premature stop codon in the carbohydrate recognition region (18). We hypothesized that lung transplant recipients with this Y238X variant would have deficient dectin-1 function resulting in increased risk for certain infections, subsequent CLAD, and impaired survival following lung transplant.
Results

Study population and polymorphism distributions

Characteristics of the included subjects are found in Table 1. In the UCSF cohort, only one subject was homozygous for the CLEC7A rs16910526 polymorphism (CC), and there were 40 subjects heterozygous for the CLEC7A polymorphism (AC). A similar distribution of CLEC7A genotypes were found in the 1,131 subjects in the LTOG validation cohort, with 7 subjects homozygous for the CLEC7A CC genotype, and 152 subjects heterozygous for the CLEC7A AC genotype.

**CLEC7A gene expression is decreased in lung tissue and peripheral blood cells of Y238X heterozygous subjects and decreased further after stimulation with zymosan.** We measured baseline CLEC7A gene expression in two cohorts. Data from GTEX demonstrated that Y238X carriers (AC, n=40; CC, n=4) had decreased dectin-1 mRNA expression in blood compared to CLEC7A wildtype subjects (Figure 1A, AA, n = 325, \( p = 0.0004 \)). A similar reduction in normalized dectin-1 mRNA expression was observed in lung tissue from subjects with Y238X variant AC (n=48) and CC genotypes (n=4) compared to wildtype CLEC7A AA genotypes (Figure 1B, n = 338, \( p = 0.0004 \)).

To assess differences in dectin-1 mRNA expression following dectin-1 receptor ligation, peripheral blood mononuclear cells (PBMCs) from lung transplant recipient Y238X carriers (AC, n = 9) and wildtype subjects (AA, n= 9) in the UCSF cohort were stimulated with the dectin-1 agonist zymosan or control media. While it does not reflect the true complexity of fungal cell walls, zymosan is widely used in vivo and in vitro to study receptor binding (13). We found no differences in baseline CLEC7A gene expression across genotypes within these 18 samples (\( p = 0.5 \)). Figure 1B demonstrates a reduction in dectin-1 gene expression in Y238X heterozygous PBMCs stimulated with zymosan as compared to PBMC treated with media alone (\( p<0.0001 \)). In comparison, there was no difference in dectin-1 mRNA expression for wildtype PBMC treated
with zymosan versus control media (p = 0.8). We also measured cytokines in the supernatant of these cell culture experiments but found no differences for Y238X heterozygotes compared to CLEC7A wildtype cells (Supplemental Figure 1).

**Soluble dectin-1 protein is decreased in bronchoalveolar lavage during CLAD and among Y238X variant subjects.** Given the observed difference in gene expression, we sought to determine if dectin-1 soluble protein concentrations differed within a nested subcohort of subjects by genotype and CLAD diagnosis (Supplemental Table 1). Dectin-1 protein differed significantly between CLAD groupings (BOS, bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome; and combined BOS and RAS subtypes), as well as across CLEC7A genotypes. BAL dectin-1 protein was significantly less in subjects with BOS (p = 0.04), RAS (p = 0.04) or CLAD (BOS and RAS, p = 0.008) compared to dectin-1 protein in subjects without CLAD (Figure 2A). By contrast, plasma dectin-1 protein concentrations did not differ between subjects with CLAD (BOS and RAS) compared to those without CLAD (p = 0.3, Figure 2B). Dectin-1 soluble protein concentrations were significantly less in BAL compared to the plasma compartment (p <0.0001) with no correlation across paired BAL and plasma samples (Figure 2D, r² = 0.2, p = 0.2). Compared to subjects with wildtype CLEC7A genotypes, Y238X heterozygotes had 2.8-fold less dectin-1 protein in BAL (p = 0.008) and 1.4-fold less dectin-1 protein in plasma (p = 0.04).

**The dectin-1 receptor is decreased on monocytes from Y238X variant subjects.** We sought to characterize cell surface dectin-1 protein following receptor ligation. As shown in Figure 3, peripheral blood monocytes were analyzed following stimulation with zymosan or control media from Y238X heterozygous subjects (AC, n=8) and wildtype subjects (AA, n=8). In both conditions, monocytes from Y238X carriers had significantly less surface dectin-1 by MFI compared to wildtype monocytes (p ≤ 0.05, Figure 3C). Within genotypes, stimulation did not affect dectin-1 receptor levels.
Because monocyte antigen presentation is closely tied to pathogen recognition, and decreases during infection, we measured HLA-DR expression changes following zymosan stimulation (21). At baseline, there was significantly less surface HLA-DR on Y238X carrier monocytes compared to CLEC7A wildtype monocytes ($p = 0.01$, Figure 3D). With zymosan stimulation, HLA-DR significantly decreased on the surface of wildtype CLEC7A AA monocytes ($p = 0.03$) but not on the surface of Y238X heterozygous monocytes ($p = 0.5$). Thus, Y238X carriers had baseline suppression of HLA-DR expression resembling the phenotype seen in wildtype subjects with stimulation.

**Y238X variants have increased risk for isolation of BAL fungal and viral pathogens.** We next sought to characterize the clinical impact of these genotypes on risk for pathogen isolation, graft dysfunction, and death in two cohorts. In the UCSF cohort, freedom from bacterial, viral, and fungal pathogen isolation in the first 90 days post-transplant were assessed in subjects presenting for bronchoscopy (Supplemental Table 2). Freedom from pathogen isolation is displayed by Kaplan-Meier plot in Figure 4. There was no difference in time to positive bacterial culture for Y238X carriers (HR 0.83, CI 0.67–1.03, $p = 0.09$) compared to wildtype (AA) subjects. In contrast, Y238X carriers (AC and CC) had an increased hazard ratio for viral airway infection, as demonstrated by positive BAL viral PCR, compared to subjects with CLEC7A wildtype subjects (HR 2.46, CI 1.08–5.63, $p = 0.03$). Similarly, fungi were identified earlier in Y238X carriers.

**The Y238X polymorphism confers increased risk for death and graft dysfunction after lung transplantation in two large cohorts.** UCSF subjects with Y238X genotypes had a median time to CLAD or death of 3.7 ± 0.4 years compared to 6.3 ± 0.2 years in subjects with wildtype CLEC7A genotypes (adjusted HR 1.8, 1.2 – 2.8, $p = 0.008$). CLAD-free survival risk was also examined within time intervals where the relative hazard was observed to be proportional. With this model, Y238X carriers had an increased risk of CLAD or death after 3 years (adjusted HR 3.9, 2.2 – 6.9, $p < 0.001$) compared to CLEC7A wildtype subjects (AA), but there was no CLAD-free survival
difference during the first 3 years following lung transplantation (adjusted HR 0.9, 0.4 – 1.8, p = 0.8).

Figure 5 shows Kaplan-Meier survival graphs demonstrating overall survival stratified by CLEC7A genotype in the UCSF (panel A) and LTOG (panel B) cohorts. Within the UCSF cohort, there was an increased risk of death among Y238X carriers with median survival of 7.5 ± 0.9 years compared to 11.8 ± 1 years in CLEC7A wildtype subjects (adjusted HR 1.8, 1.1 – 3.8, p = 0.02). A similar phenomenon was found among the 1,129 subjects included in the LTOG validation cohort with freedom from death displayed by Kaplan-Meier plot in Figure 4B. LTOG subjects with Y238X loss of function AC and CC polymorphisms had a median survival of 6.3 ± 1.1 years compared to 7.4 ± 0.7 years in CLEC7A wildtype subjects (adjusted HR 1.3, 1.1 – 1.6, p = 0.02).

While lung function and CLAD outcomes were not available within the LTOG cohort, 39 subjects underwent re-transplantation. Graft survival, defined as freedom from re-transplantation or death, was significantly decreased in Y238X carriers (AC and CC, 6 ± 0.6 years) compared to CLEC7A wildtype genotypes (AA, 7 ± 0.3 years, multivariable HR 1.3, 1.0 – 1.6, p = 0.02). Similarly, in the UCSF cohort, there was a 60% increased risk of graft failure, defined as re-transplantation or death, in Y238X carriers compared to CLEC7A wildtype subjects (multivariable HR 1.6, 1.0 – 2.6, p = 0.05).
Discussion

Our results in two large lung transplant cohorts, show that the Y238X loss of function polymorphism in the CLEC7A gene for dectin-1, a key innate immune receptor, was associated with increased risk of re-transplantation, or death. Within a single cohort, this polymorphism was also associated with increased risk for CLAD or death and the isolation of fungus or virus in BAL. These findings suggest that intact dectin-1 can be protective of chronic rejection or death by decreasing inflammation subsequent to infection that may lead to bystander activation of allo-specific immune responses.

The Y238X variant results in a premature stop codon in the last 10 amino acids of the dectin-1 carbohydrate recognition domain (18). In patients homozygous for this variant, it has been shown to result in impaired recognition of fungal beta-glucan motifs, and, subsequently, decreased cytokine production (18-20). However, our study focused primarily on heterozygous individuals, for whom we did not observe differences in cytokine production at rest or following stimulation in the presence of calcineurin inhibition. Rather, Y238X carriers had impaired dectin-1 transcription, decreased monocyte receptor expression, and decreased soluble protein levels. One possible explanation for the finding of decreased dectin-1 expression is that this variant decreases the nuclear export or stability of transcripts through conserved mechanisms of addressing transcripts with premature stop codons(21). This mechanism would be consistent with our observation that ligation of dectin-1 with zymosan, which accelerates dectin-1 shedding and turnover, resulted in further decreases in dectin-1 transcript in heterozygous individuals. At the same time, baseline monocyte HLA-DR expression was decreased in Y238X carriers. While HLA-DR is subject to negative feedback regulation, baseline HLA-DR expression is also positively correlated with innate immune function (22). Together, these findings suggest pressure of intense immunosuppression associated with lung transplantation appears to evoke a skewed phenotype of impaired innate immunity.
These findings may be applicable to other immunosuppressed patients. While heterozygosity in this SNP is a risk factor for benign mucocutaneous candidiasis in otherwise immunocompetent individuals, it has also been linked to invasive aspergillosis in hematopoietic cell transplantation recipients (18, 24, 25). The finding of decreased time to virus identified by multiplex PCR in lung transplant recipients with dectin-1 polymorphisms is novel. However, several studies have shown an association between viral and fungal infection and CLAD (10, 26, 27). Our finding of decreased CLAD-free survival may be partially explained by an increased risk for these pathogens. Consequently, the immunomodulatory effects of beta-glucans, the agonists of dectin-1, are well established: beta-glucan therapy increases mucosal immunity, decreases surgical infections, and reduces recurrent respiratory viruses in children (28-32). Mouse studies have shown dectin-1 to be crucial in activating the inflammasome and reactive oxygen species necessary for mitigating infection of parasites and fungus (23). Our findings may motivate investigation as to whether oral or intravenous beta-glucan therapy could ameliorate the risk of graft infection or inflammation in Y238X carriers.

While we postulate that increased pathogen burden secondary to impaired immunity as the mechanism linking Dectin-1 impairment with CLAD, other mechanism could also contribute. Decreased surface dectin-1 on Y238X monocytes could potentially drive the airway fibrosis more directly. For example, intact macrophage dectin-1 has been shown to promote adaptive immune suppression and tolerance of pancreatic adenocarcinoma through the ligation of the lectin galectin 9. Our subjects with decreased monocyte dectin-1 could therefore have reduced allograft tolerance. We additionally found that wildtype CLEC7A monocytes had decreased surface dectin-1 after zymosan stimulation (33). Decreased dectin-1 in BAL of CLAD subjects was not solely dependent on CLEC7A genotypes and thus may reflect dectin-1 downregulation during fungal colonization and support this mechanism of reduced tolerance and increased alloreactivity leading to chronic injury (33). It is possible that other variants in dectin-1 might result in differences in baseline function or that fibrosis or chronic infection could result in dectin-1 activation and
subsequent transcriptional inhibition. A number of genetic variants in other genes of innate immunity have also been associated with CLAD risk, further exemplifying how the stress of lung transplantation can evoke phenotypes that might otherwise be silent (34-38).

A strength of this study was the inclusion of two genotyped cohorts of lung transplant patients, representing the largest genetic association study in this field. Further, some of the 321 genotyped subjects in the UCSF cohort had additional samples to allow nested mechanistic studies. Nonetheless, the study has limitations: for example, some clinical data were not available for part of the cohort limiting statistical power for clinical analyses. This study is largely associative due to limitations in the ability to perform mechanistic studies by the scarcity of subject samples. Results should be interpreted in the setting of this reduced power. While the increased risk for CLAD or mortality among CLEC7A AC and CC genotypes in the UCSF cohort increased 3 years after transplant, this polymorphism was associated with a more constant risk in the LTOG cohort. This difference may reflect anti-fungal prophylaxis practices, which are relatively aggressive at UCSF. Variation in post-transplant protocols across LTOG centers may bias our associations to the mean. Along the same lines, our modeling of genotype effect on CLAD and survival may be incomplete as we were unable to control for some known mediators such as gastroesophageal reflux disease, induction regimen, or graft infections.

In summary, we describe an association between a common polymorphism in the innate immune receptor dectin-1 and increased risk of lung transplant allograft failure and recipient mortality. Dectin-1 dysfunction likely contributes to CLAD risk through increased pathogen susceptibility, which is augmented by the intense immunosuppression of lung transplantation. CLEC7A genotyping of lung transplant recipients may help to inform personalized surveillance and anti-microbial treatment strategies.
Methods

All mechanistic studies were performed on samples from subjects in the UCSF cohort. Quantitative tissue gene expression was available from the Genome Tissue Expression Database (GTEX) (19).

Study populations and clinical data

Genotype analysis was performed in the University of California, San Francisco (UCSF) and lung transplant outcomes group (LTOG) cohorts. For the UCSF cohort, adults who received single lung, bilateral lung, or heart-lung allografts at UCSF between 2000 and 2016 were included if they had DNA available for genotyping, survived to the first follow-up lung function study (approximately 14 days), and provided informed consent. Supplemental Figure 2 depicts the inclusion and exclusion criteria for the analyses performed as part of this study.

Bronchoalveolar lavage was performed using 5-8 aliquots of 20 ml saline, typically in the right middle lobe, followed by transbronchial biopsies as per institutional clinical management protocols. The first aliquot was sent for culture. Between 8/1/2012 and 1/1/2017, bronchoscopy was scheduled for routine surveillance at 0.5, 1, 2, 3, 6, 12, 18, and 24 months after transplantation. Additional bronchoscopy procedures were performed when clinically indicated for suspicion of acute infection or rejection.

BAL cell count and microbiologic culture results were obtained as part of routine clinical care. Data abstracted from the medical charts included information on demographics, transplant indication, bronchoscopy indication, symptoms, acute and chronic rejection, clinical culture results, management changes after bronchoscopy, and survival. Acute cellular rejection was assessed and graded in clinical transbronchial biopsies by one of two experienced thoracic pathologists using standard nomenclature (39). Chronic lung allograft dysfunction (CLAD) was defined according to established criteria as an unresolving 20% decline in FEV₁ or FVC lasting over 30 days (40) and adjudicated as previously described (41, 42). Graft failure was defined as
re-transplantation or death. Bronchiolitis obliterans syndrome (BOS) and restrictive allograft syndrome (RAS) CLAD endotypes were defined according to international criteria as a 20% decrease in post-transplant baseline values for FEV1 or FVC, respectively (40, 43, 44). CLAD-free survival was quantified as years of freedom from CLAD or death.

Standard post-transplant induction regimens for all subjects included methylprednisolone, and basiliximab. Antithymocyte globulin was used for combined heart and lung transplant recipients. Initial maintenance immunosuppressant therapy included tacrolimus, prednisone, and mycophenolate mofetil. Antifungal prophylaxis with posaconazole or voriconazole and inhaled amphotericin was initiated on post-operative day 0 and stopped after 90 days of treatment if bronchoalveolar lavage (BAL) surveillance fungal cultures were negative. Identification of pathogenic fungus on subsequent surveillance bronchoscopy in the absence of allograft dysfunction was treated with inhaled amphotericin.

A validation genotype analysis was performed in the multicenter Lung Transplant Outcomes Group. These data were prospectively collected, as previously described (45). Study subjects provided informed consent for enrollment and study protocols were approved by the institutional review boards at each of the 12 participating sites. Subjects were included in this study if complete survival data and genotyping data were available. 1,129 subjects from the LTOG cohort transplanted between July 2002 and January 2018 met inclusion criteria for the validation analysis.

**Genotyping and nomenclature**

In accordance with the Human Genome Organization Nomenclature Committee, the dectin-1 gene is denoted by *CLEC7A* (46). Single nucleotide polymorphisms (SNPs) for subjects in both cohorts were determined using the Affymetrix TxArray designed by the iGeneTRAIN consortium to target solid organ transplant-specific loci, including the *CLEC7A* SNP rs16910526 (47). Less than 1% of samples were excluded based on low call rates, mismatches between imputed and reported HLA-typing and sex. Given the low frequency of the CC dectin-1 genotype, we
dichotomized subjects as either *CLEC7A* homozygous wild-type (AA) or as carriers of the *CLEC7A* variant polymorphism (AC or CC) conferring decreased function.

**Soluble protein analysis and nomenclature**

Synchronous plasma and BAL supernatant samples were selected from a nested subcohort of 20 subjects with CLAD and 25 subjects without CLAD matched by age and time post-transplant in the UCSF cohort. Soluble dectin-1 protein (Uniprot ID: Q9BXN2) was measured using a proximity extension assay within a panel of 92 immune-related proteins (Immune Response panel, Olink Proteomics, Uppsala, Sweden). Protein abundance was quantified using real-time polymerase chain reaction cycle values and the protein quantity was expressed in normalized units on a log2 scale (48).

**Quantitative RT-PCR**

Zymosan is a glucan ligand found on the cell surface of fungi, shown to be a potent agonist of the dectin-1 receptor (17). It is an important sterile agonist used in cell culture experiments of dectin-1 (18). Following 4 hours of stimulation with the zymosan (10 ug/mL, Sigma-Aldrich, #58856-93-2) or control (R10 media), total RNA was extracted from PBMCs of dectin-1 heterozygous subjects (AC) and dectin-1 wildtype subjects (AA) using QIAzol (Qiagen Sciences) and mRNAeasy kits (Qiagen Sciences # 74104). cDNA was generated and Taqman qRT-PCR (Life Technologies) was performed to quantify dectin-1 and *ACTB* mRNA as previously described (49). ΔCt and ΔΔCt were calculated for *CLEC7A* and *ACTB* gene expression. *CLEC7A* gene expression data were also available from the Genome Tissue Expression Database (GTEX) (19).

**Monocyte immunophenotyping**

We phenotyped monocytes by flow cytometry in cryopreserved PBMC samples following 4 hours of stimulation with heat pre-treated zymosan (10 ug/mL, InvivoGen, #tlrl-zyd) to block TLR2 binding or R10 media alone. Samples were pretreated with saturating concentrations of human aggregated immunoglobulin to prevent non-specific binding and then labeled with the following antibody (Ab) combinations to define monocytes: viability dye (eBioscience, South San Francisco,
CA, #65-0863-18), Fluorescein isothiocyanate (FITC)-conjugated anti-CD14 (clone HCD14, VWR International, Radnor, PA, #325604), allophycocyanin-cyanine 7 (APC-Cy7)-conjugated anti-HLA-DR (clone L243, VWR International, Radnor, PA, #307617), R-phycoerythrin (PE)-conjugated anti-Dectin-1 (clone 259931, Fisher Scientific, Hanover Park, IL, #FAB1859P025). Monocytes were identified by forward scatter, side scatter, and size. Viable monocytes were identified by staining for CD14 and HLA-DR. After washing, samples were acquired with a Beckman Coulter cytometer and data were analyzed with the Kaluza software (Beckman Coulter), with gating strategy illustrated in Figure 3. Mean fluorescent intensity (MFI) was measured for dectin-1 and HLA-DR as HLA class II is important for pathogen recognition (50). Samples with less than 1,000 viable monocytes were excluded from statistical analysis.

**Statistics**

Paired whole genome and tissue-specific RNA sequencing data were obtained from the Genotype-Tissue Expression (GTEx) project portal on 06/05/2019. Genotype effects on lung and PBMC dectin-1 gene expression were compared using FastQTL with nominal p-values generated by testing the alternative hypothesis that the slope of a linear regression model between genotype and expression deviates from 0 (51, 52). Comparisons between groups within mechanistic studies of dectin-1 protein, gene expression, and monocyte surface markers were made using a 2-tailed Student’s t test for pairwise comparisons or ANOVA with Dunnett’s correction for studies with multiple comparisons using p value <0.05 as significant.

Subject characteristics, genotypes, and cell culture results were compared using 2-tailed Student’s t and chi-square tests for continuous and categorical variables, respectively. We used Cox proportional hazards models to determine survival and CLAD-free survival hazard ratios as a function of genotype, which included covariates for subject characteristics frequently associated with poor transplant outcomes or genetic variability: age at transplantation, recipient sex, recipient reported ethnicity, transplant diagnosis and transplant type (single, double, or heart-lung). Validation analyses were also adjusted for individual LTOG center by inclusion as a covariate.
The primary 5 principle components were included as a covariate to address LTOG population admixture. Violations of proportional hazards were assessed visually and with the Schoenfeld test. In the UCSF cohort, we also modeled the hazard ratio of these outcomes as a step function by splitting time after transplant into two sequential periods (0-3 years, and 3-10 years) as the proportionality of CLAD-free survival and overall survival risk varied with time. Cox proportional hazards models in the UCSF group were also used to determine risk for positive culture result stratified by genotype. BAL culture analyses were limited to the first 90 days after transplant to avoid the confounding effect of azole antifungal treatment, and subjects were censored once an organism was identified on culture. We visualized time to these events by Kaplan-Meier plots.

**Statistical analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria) using the “survminer,” “plyr,” “gee,” “ggplot,” “survival,” “stringr,” “multcomp,” and “ggpubr” packages.**

**Study Approval**

The UCSF institutional review board approved the single-center study components. LTOG study subjects provided informed consent for enrollment and study protocols were approved by the institutional review boards at each of the 12 participating sites.
Author Contributions

Study design: DRC, JRG, SRH, JAG, and JK; Experiments: DRC, PW, TC, DD, and JH; Analysis of results: DRC, JRG, JPS; Vital data/interpretation: LTOG investigators, DRC, JRG, JDC; Manuscript preparation: DRC and JRG with input from all authors.
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References


Figure 1. Decreased CLEC7A transcripts in Y238X variants Violin plots showing maximum and minimum values with boxes showing 25th and 75th percentile bounds median shown in bisecting line of GTEX cohort data. There is decreased CLEC7A gene expression in (A) whole blood of AC genotypes (n = 40) and CC genotypes (n = 4) compared to wildtype genotypes (AA, n = 325) as well as in (B) lung tissue of AC (n= 38) and CC (n =5) genotype subjects compared to wildtype AA (n = 330, p = 0.0004) subjects. Comparisons were made by linear regression. (C) Box and whisker plots depict decreased CLEC7A gene expression in Y238X heterozygote cells stimulated for 4 hours with zymosan relative to control media. RT-PCR CLEC7A mRNA and reference ACTB mRNA was quantified as $2^{-\Delta\Delta Ct}$. Cells in control media were normalized to a value of 1 and comparisons were made using ANOVA with Dunnett’s correction.
Figure 2. Decreased dectin-1 protein in Y238X variants and CLAD. Soluble dectin-1 protein was measured in synchronous bronchoalveolar lavage (BAL) and plasma samples from matched subjects with CLAD (n=20) and without CLAD (n=25). The two endotypes of CLAD, BOS and RAS, are displayed separately. (A) Log-normalized soluble dectin-1 protein concentrations are decreased in the BAL of subjects with BOS ($p = 0.04$), RAS ($p = 0.04$) or CLAD ($p = 0.008$) compared to BAL dectin-1 protein in subjects without CLAD. (E) In plasma, log-normalized dectin-1 protein concentrations are no different between subjects with CLAD compared to those without CLAD ($p = 0.3$). (F) BAL dectin-1 protein concentration was significantly less compared to plasma dectin-1 protein concentrations ($p<0.0001$). Subjects with Y238X AC genotypes (orange circles) had less soluble dectin-1 protein in BAL ($p = 0.008$) and plasma ($p = 0.04$) compared to AA genotypes (green circles). Differences were assessed using ANOVA with Dunnett’s correction for multiple comparisons.

Abbreviations: BAL, bronchoalveolar lavage; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; RAS, restrictive allograft syndrome
Figure 3. Decreased dectin-1 expression on monocytes from Y238X AC individuals. Following 4 hours of stimulation with control media or zymosan, monocytes were assessed by immunophenotyping from CLEC7A variants (AC, n = 8) and CLEC7A wildtype subjects (AA, n = 8). (A) monocytes were defined by forward scatter, side scatter, size, viability and staining for CD14 and HLA-DR. (B) Surface dectin-1 receptors were measured on monocytes by MFI. Box and whisker plots show dectin and HLA-DR monocyte surface expression with boxes defining the 25th and 75th percentile of data, bisecting line depicting the median value and whiskers capturing minimum and maximum values. (C) In control media, surface dectin-1 receptor MFI was increased on wildtype monocytes (AA) relative to Y238X variants (AC, p = 0.006). Following zymosan stimulation, this difference was maintained between AA and AC genotypes (p = 0.05). There was no difference in dectin-1 receptor MFI on wildtype or variant genotype monocytes in control media or following stimulation. (D) HLA-DR MFI was also measured. There was significantly decreased HLA-DR MFI on CLEC7A variant monocytes (AC) compared to wildtype monocytes (AA, p = 0.03) in control media. The monocyte HLA-DR MFI decreased on AA genotypes (p = 0.01) but not on AC genotypes following stimulation. Compared to CLEC7A wildtype genotypes, there was no difference in monocyte HLA-DR MFI on Y238X variant genotypes following zymosan stimulation. Differences were assessed using 2-tailed Student’s t test and the experiment was conducted twice.
Figure 4. Increased hazard for early viral and fungal pathogens associated with Y238X variant genotypes. Kaplan-Meier graphs show freedom from bacterial, viral, or fungal pathogen isolated from BAL. There were 220 subjects from the UCSF cohort included in this prospective analysis (AA genotype n = 192, AC&CC genotypes n = 29). There was no significant difference between the time to first positive bacterial culture result among CLEC7A genotypes (A, HR 0.83, CI 0.67-1.03, p = 0.09). Y238X variants had decreased time to identification of virus in BAL by PCR (B, HR 2.46, CI 1.08-5.63, p = 0.03) and fungus by culture (C, HR 1.17, CI 1.01-1.37, p = 0.04). Risk for pathogen isolation was assessed by Cox Proportional Hazard model.

Abbreviations: BAL, bronchoalveolar lavage; PCR, polymerase chain reaction; HR, hazard ratio; CI, confidence interval.
Figure 5: Impaired survival in lung transplant recipients with Y238X variant genotypes in derivation and validation cohorts. (A) Kaplan-Meier graph demonstrating survival in years after transplant in subjects with loss-of-function CLEC7A polymorphisms compared to those with wildtype dectin-1 genotypes (multivariable HR 1.8, 1.1 – 3.8, p = 0.02). (B) In the LTOG cohort, overall survival in subjects stratified by CLEC7A genotype was also decreased in subjects with CLEC7A AC and CC variants compared to CLEC7A wildtype genotypes (multivariable HR 1.3, 1.1 – 1.6, p = 0.02). Risk for death was determined using Cox Proportional Hazard models adjusted for subject characteristics established as important for lung transplant outcomes.
Table 1. Baseline Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>UCSF Cohort</th>
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<th>LTOG Cohort</th>
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<tbody>
<tr>
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<tr>
<td>Genotypes</td>
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<tr>
<td>Subjects N (%)†</td>
<td>280 (87)</td>
<td>41 (13)</td>
<td>970 (86)</td>
<td>159 (14)</td>
</tr>
<tr>
<td>Age at transplantation,</td>
<td>55 ± 12</td>
<td>56 ± 13</td>
<td>53 ± 13</td>
<td>53 ± 14</td>
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<tr>
<td>mean years ± SD</td>
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<tr>
<td>Male gender (%)</td>
<td>54</td>
<td>61</td>
<td>56</td>
<td>57</td>
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<tr>
<td>Male gender (%)</td>
<td>0.37</td>
<td>0.05</td>
<td>0.88</td>
<td>0.07</td>
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<tr>
<td>Transplant type: N (%)</td>
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<td></td>
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<tr>
<td>Double</td>
<td>251 (90)</td>
<td>37 (90)</td>
<td>712 (73)</td>
<td>119 (74)</td>
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<tr>
<td>Other</td>
<td>29 (10)</td>
<td>4 (10)</td>
<td>258 (27)</td>
<td>42 (26)</td>
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<tr>
<td>Race/Ethnicity: N (%)</td>
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<tr>
<td>Caucasian</td>
<td>204 (73)</td>
<td>38 (93)</td>
<td>792 (82)</td>
<td>144 (92)</td>
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<td>Other</td>
<td>76 (27)</td>
<td>3 (7)</td>
<td>178</td>
<td>17</td>
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<tr>
<td>Transplant indication: N (%)</td>
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<tr>
<td>Pulmonary Fibrosis</td>
<td>185 (66)</td>
<td>25 (61)</td>
<td>358 (39)</td>
<td>57 (37)</td>
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<tr>
<td>Other</td>
<td>95 (44)</td>
<td>16 (39)</td>
<td>612 (61)</td>
<td>102 (63)</td>
</tr>
</tbody>
</table>

† No statistically significant difference in genotype frequencies between UCSF and LTOG cohorts.