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

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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 multicenter 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 bronchoalveolar lavage ($P = 0.008$) and plasma ($P = 0.04$), and decreased monocyte surface dectin-1 ($P = 0.01$) compared with 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 with wild-type *CLEC7A* 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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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).

Conflict of interest: The authors have declared that no conflict of interest exists.

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Table 1. Baseline subject characteristics

Genotypes	UCSF Cohort			LTOG Cohort		
	AA	AC and CC	<i>P</i>	AA	AC and CC	<i>P</i>
Subjects <i>N</i> (%) ^A	280 (87)	41 (13)		970 (86)	159 (14)	
Age at transplantation, mean years ± SD	55 ± 12	56 ± 13	0.24	53 ± 13	53 ± 14	0.55
Male (%)	54	61	0.37	56	57	0.88
Transplant type: <i>N</i> (%)			0.91			0.07
Double	251 (90)	37 (90)		712 (73)	119 (74)	
Other	29 (10)	4 (10)		258 (27)	42 (26)	
Race/Ethnicity: <i>N</i> (%)			0.006			0.03
White	204 (73)	38 (93)		792 (82)	144 (92)	
Other	76 (27)	3 (7)		178	17	
Transplant indication: <i>N</i> (%)			0.52			0.54
Pulmonary Fibrosis	185 (66)	25 (61)		358 (39)	57 (37)	
Other	95 (44)	16 (39)		612 (61)	102 (63)	

^ANo statistically significant difference in genotype frequencies between UCSF and LTOG cohorts.

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–dependent (NF-κB–dependent) cytokine production and cleavage of the dectin-1 receptor (15, 16). While dectin-1 can activate responses alone, costimulation 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 1 subject was homozygous for the *CLEC7A* rs16910526 polymorphism (CC), and there were 40 subjects heterozygous for the Y238X variant (AC). A similar distribution of *CLEC7A* genotypes was found in the 1,131 subjects in the Lung Transplant Outcomes Group (LTOG) validation cohort, with 7 subjects homozygous for the *CLEC7A* CC genotype, and 152 subjects heterozygous for the *CLEC7A* Y238X variant.

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 2 cohorts. Data from the Genome Tissue Expression (GTEx) database demonstrated that Y238X carriers (AC, *n* = 40; CC, *n* = 4) had decreased dectin-1 mRNA expression in blood compared with wild-type *CLEC7A* 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 the Y238X variant AC (*n* = 48) and CC genotypes (*n* = 4) compared with wild-type *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 wild-type 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 with PBMCs treated with media alone (*P* < 0.0001). In comparison, there was no difference in dectin-1 mRNA expression for wild-type PBMCs 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

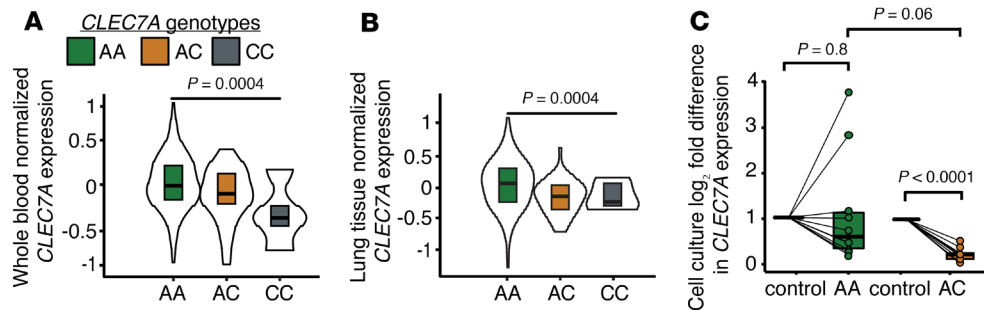


Figure 1. Decreased *CLEC7A* transcripts in Y238X variants. Violin plots showing maximum and minimum values, with boxes showing 25th and 75th percentile bounds and bisecting lines showing median values for GTEx cohort data. There is decreased *CLEC7A* gene expression in (A) whole blood of AC genotypes ($n = 40$) and CC genotypes ($n = 4$) compared with wild-type genotypes (AA, $n = 325$) as well as in (B) lung tissue of AC ($n = 38$) and CC ($n = 5$) genotype subjects compared with wild-type 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 heterozygous cells stimulated for 4 hours with zymosan relative to control media. RT-PCR *CLEC7A* mRNA and reference *ACTB* mRNA was quantified as $2^{-\Delta\Delta C_t}$. Cells in control media were normalized to a value of 1 and comparisons were made using ANOVA with Dunnett's correction.

to wild-type *CLEC7A* cells (Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.133083DS1>).

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. Bronchoalveolar lavage (BAL) dectin-1 protein was significantly lower in subjects with BOS ($P = 0.04$), RAS ($P = 0.04$), or CLAD (BOS and RAS, $P = 0.008$) compared with 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 lower in BAL compared with the plasma compartment ($P < 0.0001$), with no correlation across paired BAL and plasma samples (Figure 2C; $r^2 = 0.2$, $P = 0.2$). Compared with subjects with wild-type *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 wild-type subjects (AA, $n = 8$). In both conditions, monocytes from Y238X carriers had significantly less surface dectin-1 by mean fluorescence intensity (MFI) compared with wild-type monocytes ($P \leq 0.05$, 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 with wild-type *CLEC7A* monocytes ($P = 0.01$, Figure 3D). With zymosan stimulation, HLA-DR significantly decreased on the surface of wild-type *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 wild-type 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 2 cohorts. In the UCSF cohort, freedom from bacterial, viral, and fungal pathogen isolation in the first 90 days after 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 wild-type (AA) subjects. In contrast, Y238X carriers (AC and CC) had an increased HR for viral airway infection, as demonstrated by positive BAL viral PCR, compared with subjects with wild-type *CLEC7A* (HR 2.46, CI 1.08–5.63, $P = 0.03$). Similarly, fungi were identified earlier in Y238X carriers.

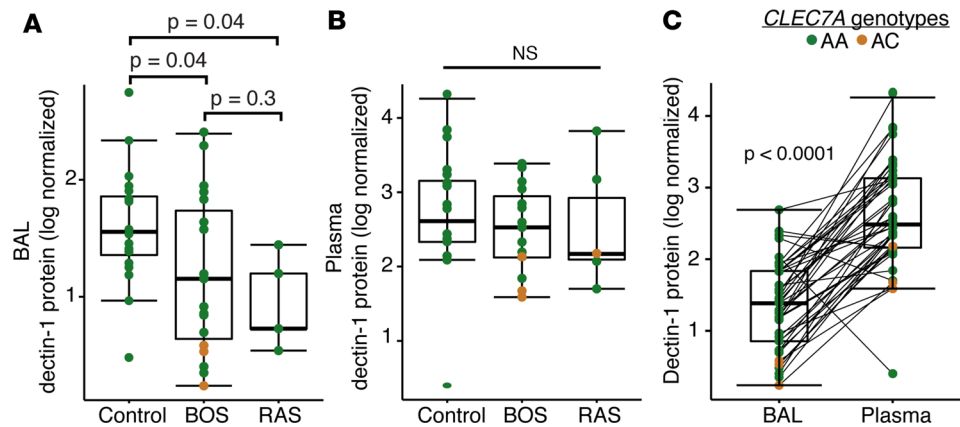


Figure 2. Decreased dectin-1 protein in Y238X variants and CLAD. Soluble dectin-1 protein was measured in synchronous bronchoalveolar lavage (BAL) and plasma samples from matched subjects with CLAD ($n = 20$) and without CLAD ($n = 25$). The 2 endotypes of CLAD, BOS and RAS, are displayed separately. **(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 with BAL dectin-1 protein in subjects without CLAD. **(B)** In plasma, log-normalized dectin-1 protein concentrations are not different between subjects with CLAD compared to those without CLAD ($P = 0.3$). **(C)** BAL dectin-1 protein concentration was significantly lower compared with 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 with AA genotypes (green circles). Differences were assessed using ANOVA with Dunnett's correction for multiple comparisons. BAL, bronchoalveolar lavage; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; RAS, restrictive allograft syndrome.

The Y238X polymorphism confers increased risk for death and graft dysfunction after lung transplantation in 2 large cohorts. UCSF subjects with Y238X genotypes had a median time to CLAD or death of 3.7 ± 0.4 years compared with 6.3 ± 0.2 years in subjects with wild-type *CLEC7A* genotypes (adjusted HR 1.8, CI 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, CI 2.2–6.9, $P < 0.001$) compared with wild-type *CLEC7A* subjects (AA), but there was no CLAD-free survival difference during the first 3 years following lung transplantation (adjusted HR 0.9, CI 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 with 11.8 ± 1 years in wild-type *CLEC7A* subjects (adjusted HR 1.8, CI 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 with 7.4 ± 0.7 years in wild-type *CLEC7A* subjects (adjusted HR 1.3, CI 1.1–1.6, $P = 0.02$).

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

Discussion

Our results in 2 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 retransplantation, 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 allospecific immune responses.

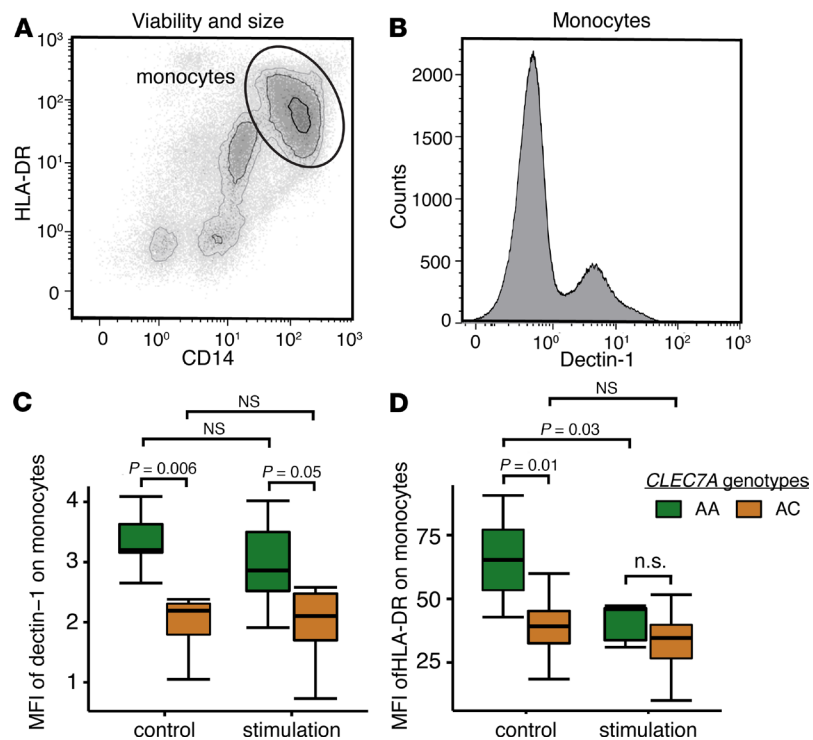


Figure 3. Decreased dectin-1 expression on monocytes from Y238X AC individuals. Following 4 hours of stimulation with control media or zymosan, monocytes were assessed by immunophenotyping those from *CLE7A* variants (AC, $n = 8$) and wild-type *CLE7A* (AA, $n = 8$). (A) Monocytes were defined by forward 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-1 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 wild-type 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 wild-type 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 *CLE7A*-variant monocytes (AC) compared with wild-type monocytes (AA, $P = 0.03$) in control media. The HLA-DR MFI decreased on AA-genotype monocytes ($P = 0.01$) but not on AC-genotype monocytes following stimulation. Compared to wild-type *CLE7A* genotypes, there was no difference in HLA-DR MFI on Y238X variant-genotype monocytes following zymosan stimulation. Differences were assessed using 2-tailed Student's *t* test and the experiment was conducted twice.

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 β -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 that pressure of intense immunosuppression associated with lung transplantation appears to evoke a skewed phenotype of impaired innate immunity.

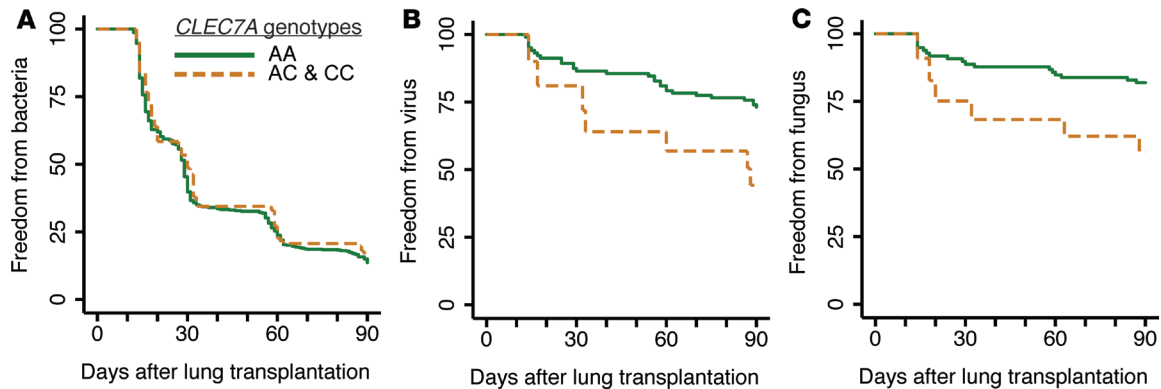


Figure 4. Increased hazard for early viral and fungal pathogens associated with Y238X variant genotypes. Kaplan-Meier graphs show freedom from bacterial, viral, or fungal pathogen isolated from BAL. There were 220 subjects from the UCSF cohort included in this prospective analysis (AA genotype $n = 192$, AC and 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 hazards models. BAL, bronchoalveolar lavage; PCR, polymerase chain reaction; HR, hazard ratio; CI, confidence interval.

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, 23, 24). The finding of decreased time to virus identified by multiplex PCR in lung transplant recipients with dectin-1 polymorphisms is potentially novel. However, several studies have shown an association between viral and fungal infection and CLAD (10, 25, 26). Our finding of decreased CLAD-free survival may be partially explained by an increased risk for these pathogens. Consequently, the immunomodulatory effects of β -glucans, the agonists of dectin-1, are well established; β -glucan therapy increases mucosal immunity, decreases surgical infections, and reduces recurrent respiratory viruses in children (27–31). 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 (32). Our findings may motivate investigation as to whether oral or intravenous β -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 mechanisms 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 wild-type *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 2 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

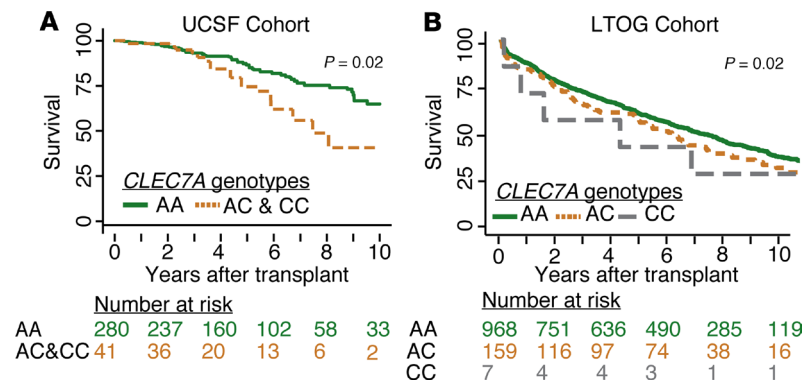


Figure 5. Impaired survival in lung transplant recipients with Y238X variant genotypes in derivation and validation cohorts. (A) Kaplan-Meier graph demonstrating survival in years after transplant in UCSF cohort subjects with loss-of-function *CLEC7A* polymorphisms compared with those with wild-type dectin-1 genotypes (multivariable HR 1.8, CI 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 with wild-type *CLEC7A* genotypes (multivariable HR 1.3, CI 1.1–1.6, $P = 0.02$). Risk for death was determined using Cox proportional hazards models adjusted for subject characteristics established as important for lung transplant outcomes.

years after transplant, this polymorphism was associated with a more constant risk in the LTOG cohort. This difference may reflect antifungal prophylaxis practices, which are relatively aggressive at UCSF. Variation in posttransplant 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 antimicrobial treatment strategies.

Methods

All mechanistic studies were performed on samples from subjects in the UCSF cohort. Quantitative tissue gene expression was available from the GTEx database (19).

Study populations and clinical data. Genotype analysis was performed in the UCSF and 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.

BAL 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 August 1, 2012 and January 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 1 of 2 experienced thoracic pathologists using standard nomenclature (39). CLAD was defined according to established criteria as an unresolving 20% decline in forced expiratory volume in 1 second (FEV_1) or forced vital capacity (FVC) lasting over 30 days (40) and adjudicated as previously described (41, 42). Graft failure was defined as retransplantation or death. BOS and RAS CLAD endotypes were defined according to international criteria as a 20% decrease in posttransplant baseline values for FEV_1 or FVC, respectively (40, 43, 44). CLAD-free survival was quantified as years of freedom from CLAD or death.

Standard posttransplant induction regimens for all subjects included methylprednisolone and basiliximab. Anti-thymocyte 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 postoperative day 0 and stopped after 90 days of treatment if 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 LTOG. 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. A total of 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). SNPs for subjects in both cohorts were determined using the Affymetrix TxArray designed by the iGeneTRAI_N 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 after 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). Protein abundance was quantified using real-time PCR 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 zymosan (10 µg/mL, Sigma-Aldrich, 58856-93-2) or control (R10 media), total RNA was extracted from PBMCs of dectin-1 heterozygous subjects (AC) and wild-type dectin-1 subjects (AA) using QIAzol (Qiagen) and mRNeasy kits (Qiagen, 74104). cDNA was generated and Taqman qRT-PCR (Life Technologies) was performed to quantify dectin-1 and *ACTB* mRNA as previously described (49). Δ Ct and $\Delta\Delta$ Ct were calculated for *CLEC7A* and *ACTB* gene expression. *CLEC7A* gene expression data were also available from the GTEx database (19).

Monocyte immunophenotyping. We phenotyped monocytes by flow cytometry in cryopreserved PBMC samples following 4 hours of stimulation with heat-pretreated zymosan (10 µg/mL, InvivoGen, tlr-zyd) to block TLR2 binding or R10 media alone. Samples were pretreated with saturating concentrations of human aggregated immunoglobulin to prevent nonspecific binding and then labeled with the following reagent and antibody combinations to define monocytes: viability dye (eBioscience, 65-0863-18), fluorescein isothiocyanate-conjugated (FITC-conjugated) anti-CD14 (clone HCD14, VWR International, 325604), allophycocyanin-cyanine 7 (APC-Cy7)-conjugated anti-HLA-DR (clone L243, VWR International, 307617), R-phycoerythrin (PE)-conjugated anti-dectin-1 (clone 259931, Thermo Fisher Scientific, 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 Kaluza software (Beckman Coulter), using the gating strategy illustrated in Figure 3. 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 GTEx project portal (<https://www.gtexportal.org/home/>) on June 5, 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 1-way ANOVA with Dunnett's correction for studies with multiple comparisons, defining *P* values less than 0.05 as significant.

Subject characteristics, genotypes, and cell culture results were compared using 2-tailed Student's *t* and χ^2 tests for continuous and categorical variables, respectively. We used Cox proportional hazards models to determine survival and CLAD-free survival HRs 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 HR of these outcomes as a step function by splitting time after transplant into 2 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) 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

DRC, JRG, SRH, JAG, and JK designed the study. DRC, PW, TC, DD, and JH performed experiments. DRC, JRG, JPS, and DT analyzed results. LTOG investigators, DRC, JRG, and JDC provided vital data and interpretation of results. DRC and JRG prepared the manuscript with input from all authors.

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