

Protein Quantitative Trait Loci Analysis Identifies Genetic Variation in the Innate Immune Regulator *TOLLIP* in Post-Lung Transplant Primary Graft Dysfunction Risk

E. Cantu^{1,*}, Y. Suzuki¹, J. M. Diamond², J. Ellis¹, J. Tiwari¹, B. Beduhn¹, J. R. Nellen¹, R. Shah², N. J. Meyer², D. J. Lederer³, S. M. Kawut^{2,4,5}, S. M. Palmer⁶, L. D. Snyder⁶, M. G. Hartwig⁷, V. N. Lama⁸, S. Bhorade⁹, M. Crespo¹⁰, E. Demissie^{2,4}, K. Wille¹¹, J. Orens¹², P. D. Shah¹², A. Weinacker¹³, D. Weill¹³, D. Wilkes¹⁴, D. Roe¹⁴, L. B. Ware¹⁵, F. Wang⁴, R. Feng⁴ and J. D. Christie^{2,4} for the Lung Transplant Outcomes Group

¹Division of Cardiovascular Surgery, University of Pennsylvania School of Medicine, Philadelphia, PA

²Pulmonary, Allergy, and Critical Care Division, University of Pennsylvania School of Medicine, Philadelphia, PA

³Division of Pulmonary, Allergy, and Critical Care Medicine, Columbia University College of Physicians and Surgeons, New York, New York

⁴Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania School of Medicine, Philadelphia, PA

⁵Penn Cardiovascular Institute, University of Pennsylvania School of Medicine, Philadelphia, PA

⁶Division of Pulmonary, Allergy, and Critical Care Medicine, Duke University, Durham, NC

⁷Division of Cardiothoracic Surgery, Duke University, Durham, NC

⁸Division of Pulmonary, Allergy, and Critical Care Medicine, University of Michigan, Ann Arbor, MI

⁹Division of Pulmonary and Critical Care Medicine, University of Chicago, Chicago, IL

¹⁰Division of Pulmonary, Allergy, and Critical Care, University of Pittsburgh, Pittsburgh, PA

¹¹Division of Pulmonary and Critical Care Medicine, University of Alabama at Birmingham, Birmingham, AL

¹²Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Johns Hopkins University Hospital, Baltimore, MD

¹³Division of Pulmonary and Critical Care Medicine, Stanford University, Palo Alto, CA

¹⁴Division of Pulmonary, Allergy, Critical Care, and Occupational Medicine, Indiana University School of Medicine, Indianapolis, IN

¹⁵Departments of Medicine and Pathology, Microbiology and Immunology, Vanderbilt University, Nashville, TN

*Corresponding author: Edward Cantu III, edward.cantu@uphs.upenn.edu

The authors previously identified plasma plasminogen activator inhibitor-1 (PAI-1) level as a quantitative lung injury biomarker in primary graft dysfunction (PGD). They hypothesized that plasma levels of PAI-1 used as a quantitative trait could facilitate discovery of genetic loci important in PGD pathogenesis. A two-stage cohort study was performed. In stage 1, they tested associations of loci with PAI-1 plasma level using linear modeling. Genotyping was performed using the Illumina CVD Bead Chip v2. Loci meeting a $p < 5 \times 10^{-4}$ cutoff were carried forward and tested in stage 2 for association with PGD. Two hundred ninety-seven enrollees were evaluated in stage 1. Six loci, associated with PAI-1, were carried forward to stage 2 and evaluated in 728 patients. rs3168046 (Toll interacting protein [*TOLLIP*]) was significantly associated with PGD ($p = 0.006$). The increased risk of PGD for carrying at least one copy of this variant was 11.7% (95% confidence interval 4.9–18.5%). The false-positive rate for individuals with this genotype who did not have PGD was 6.1%. Variants in the *TOLLIP* gene are associated with higher circulating PAI-1 plasma levels and validate for association with clinical PGD. A protein quantitative trait analysis for PGD risk prioritizes genetic variations in *TOLLIP* and supports a role for Toll-like receptors in PGD pathogenesis.

Abbreviations: AIM, ancestry informative genetic marker; ALI, acute lung injury; ISHLT, International Society of Heart and Lung Transplantation; LD, linkage disequilibrium; LTOG, Lung Transplant Outcomes Group; MAF, minor allele frequency; NOD, nucleotide binding oligomerization domain; PAI-1, plasminogen activator inhibitor-1; PGD, primary graft dysfunction; *TOLLIP*, Toll interacting protein; TLR, Toll-like receptor

Received 07 April 2015, revised 02 September 2015 and accepted for publication 03 September 2015

Introduction

As many as 30% of lung transplant recipients develop primary graft dysfunction (PGD) (1,2), making it the most common cause of death in the first 30 days and one of the more common causes of death in the first year (2–4). Despite this frequency and the association of PGD with

both short- and long-term morbidity and mortality, its mechanism remains incompletely understood (5,6).

Large-scale genetic association studies of categorically defined clinical syndromes, such as PGD, may be hampered by lack of power, increasing the risk that important variants are missed (7). Quantitative traits have been used to increase the statistical power for genetic association studies of many clinical syndromes (8,9), yet a quantitative trait has yet to be used in the study of PGD.

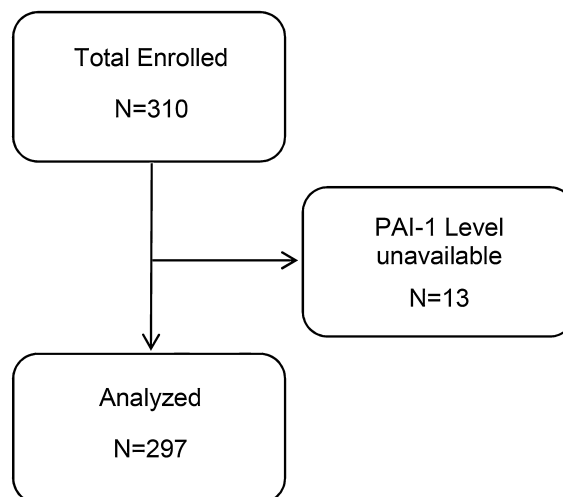
We have previously demonstrated that plasma plasminogen activator inhibitor-1 (PAI-1) levels are elevated post-transplantation in recipients with PGD and are a useful biomarker for discrimination of this syndrome (10). PAI-1 is an acute phase reactant and an endogenous inhibitor of tissue plasminogen activator that is increased in nontransplant acute lung injury (ALI) (11) with increased circulating levels being associated with mortality (12). For these reasons, PAI-1 was selected for study as a PGD quantitative trait. We hypothesized that genetic variants could be identified based on elevated plasma PAI-1 level and then subsequently validated in a separate cohort for association with clinical PGD.

Methods

Study population and data collection

Subjects were selected from the Lung Transplant Outcomes Group cohort (LTOG), which is a multicenter, prospective cohort study of lung transplant recipients (Clinical Trial NCT00457847) (2). Institutional review board approval and informed written consent were obtained before the recruitment of subjects. Consecutive subjects transplanted between May 2002 and August 2010 at 10 centers (University of Pennsylvania, Columbia University, Vanderbilt University, Stanford University, University of Alabama at Birmingham, University of Michigan, Johns Hopkins University, Duke University, University of Pittsburgh, and University of Chicago) were enrolled. A two-stage study design was used (Figure 1). Stage 1 consisted of 297 subjects enrolled between May 2002 and October 2006, in whom PAI-1 protein levels were measured as part of a previous study of biomarker discrimination (10). Stage 2 consisted mostly of LTOG subjects enrolled in a subsequent era between November 2006 and December 2010; however, 38 subjects with DNA samples not included in stage 1 due to insufficient plasma for protein analyses dating from March 2003 were also included in stage 2. Clinical data were collected prospectively as previously described (2). Subjects not included either did not provide consent for participation or did not have sufficient quantity or quality of samples for analysis. Immunosuppressive regimens were similar between institutions and included an induction agent, followed by intraoperative steroid administration with postoperative steroid, calcineurin inhibitor, and antimetabolite. PGD grade was determined by using the consensus definition of the International Society of Heart and Lung Transplantation (ISHLT) with two blinded readers and adjudication as previously described (2). We used any grade 3 PGD occurring within the first 72 hours following lung transplantation as our primary case definition. A sensitivity analysis was conducted using PGD grade 3 present at 48 or 72 hours as an alternate, more severe and restrictive outcome definition as compared to the primary definition, which includes grade 3 PGD occurring at arrival to ICU, 24, 48 or 72 hours per ISHLT guidelines (13).

Stage 1: Genetic association with PAI-1



Stage 2: Genetic association with PGD

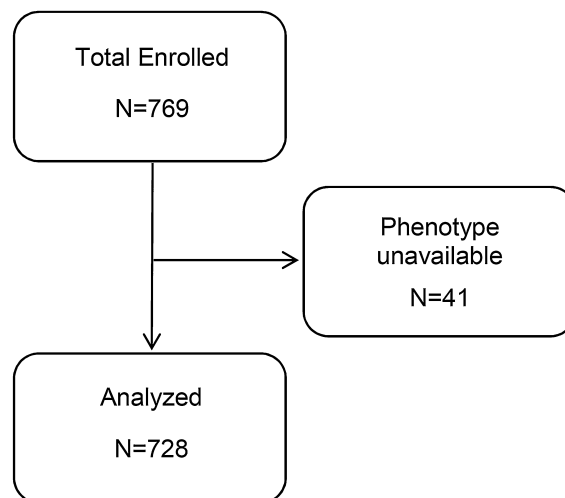


Figure 1: Study design. PAI-1, plasminogen activator inhibitor-1; PGD, primary graft dysfunction.

Collection and processing of PAI-1 samples

Plasma samples were collected in citrated tubes 24 hours after reperfusion of the lung allografts. Plasma samples were processed within 30 minutes and then stored at -80°C . PAI-1 plasma levels were measured in duplicate using a commercially available ELISA (American Diagnostica, Stamford, CT) as previously described (10). The mean coefficient of variation for the assay was 6.8%. All laboratory personnel were blinded to the PGD status of the study subjects.

Genotyping strategy

For all loci tested, we used the Human CVD Bead Chip (Illumina, San Diego, CA) (14). This platform was specifically designed with hypothesis-driven prioritized loci thought to increase risk of respiratory, cardiovascular, metabolic, and inflammatory diseases and assesses the majority of known ALI-associated genes.

General assumptions

Plasma PAI-1 levels vary after transplant according to severity of PGD (10). We hypothesized that plasma PAI-1 levels could be used to identify genotypes that are associated with these levels and, thus, that genotypes identified using this quantitative trait (plasma PAI-1 levels) would identify variants important in PGD pathogenesis.

Statistical analysis

A two-stage association study was performed (15). In stage 1, we tested genetic variant association with plasma PAI-1 protein level using linear regression models, assuming additive genetic models (meaning quantitative changes are proportional to allele dosage with a heterozygote having half the effect of a homozygote of interest) adjusted for recipient age, use of cardiopulmonary bypass, diagnosis, and population admixture (2). Body mass index and pulmonary artery pressures were not used for adjustment because their presence may be important in a genetic causal pathway of PGD pathogenesis. Population admixture can cause false associations or suppress the true associations due to population stratification because of allelic frequency differences between cases and controls caused by ancestral differences. One solution to this problem is to include the top principal components derived from the ancestry informative genetic markers (AIMs) in the regression models (16). We used 1800 AIMs on the Human CVD Bead Chip, implemented the analysis in the software package PLINK, and adjusted for the top two principal components (17). Genetic variants demonstrating chip-wide significance at 5×10^{-4} level were carried forward to stage 2, as has previously been reported (18). In stage 2, the association between PGD and selected stage 1 loci was tested using logistic models. Loci with strong linkage disequilibrium (LD) ($R^2 = 0.9$, $D' = 1.0$) (19), allele counts <10, or considered housekeeping/AIM genes were excluded. LD plots were constructed using SNAP (20). Using an additive logistic regression model, clinical factors previously shown to associate with PGD (2) were included as covariates in the association between genotype and PGD in stage 2. Genetic variants were considered significantly associated with PGD in the validation set after applying a Bonferroni-adjusted α -level to account for the number tested. A

Bonferroni-adjusted value of $p < 0.008$ was considered significant to account for the six unique regions tested. Postestimation methods were used to define the adjusted-risk difference and the false-positive rate in true negatives. We estimated that a sample size of 750 subjects in the validation set would provide 80% power to detect a locus that explains 1.1% of the gene expression at the significance level of 0.05. For loci with minor allele frequency (MAF) of 0.05, 0.1, 0.2, and 0.3, we estimated 80% power to detect a minimum relative risk of 1.5, 1.3, and 1.2 at the significance level of 0.05, respectively, given the same sample size and the estimated frequency of PGD (30%).

Results

A total of 1079 subjects were enrolled in this study, with 297 subjects tested for genetic association with PAI-1 protein level in stage 1 and 728 subjects used for association of loci with PGD in stage 2 (Figure 1). Within the stage 1 and 2 association cohorts, 79 (26.6%) of 297 and 225 (30.9%) of 728 developed grade 3 PGD within 72 hours, respectively. Baseline demographics by PGD status for each stage are summarized in Table 1. Among patients with PGD, there were significantly higher rates of cardiopulmonary bypass use and differences in recipient diagnosis; however, we did not identify significant differences in donor age, organ total ischemic time, recipient age, recipient gender, or transplant type consistent with previous reports (2).

Stage 1: Genetic association with PAI-1 plasma protein level as a quantitative trait

Genotyping was successful in typing 49 038 polymorphic variants, with 3554 markers having significant departure

Table 1: Demographic data

Variable	Stage 1			Stage 2		
	PGD (n = 79)	No PGD (n = 218)	p value	PGD (n = 225)	No PGD (n = 503)	p value
Donor						
Age	32.7 ± 14.0	32.3 ± 13.7	0.836	35.0 ± 13.8	34.9 ± 14.4	0.906
IT(min)	283.2 ± 85.5	284.5 ± 88.3	0.914	334.0 ± 94.4	328.4 ± 106.8	0.483
Recipient						
Age	52.7 ± 10.1	53.7 ± 11.6	0.511	52.7 ± 12.6	53.4 ± 13.4	0.490
Female	36 (45.6%)	103 (47.3%)	0.798	102 (45.3%)	212 (42.2%)	0.422
Diagnosis			<0.001			<0.001
COPD	26 (32.9%)	122 (56.0%)		62 (27.6%)	188 (37.4%)	
CF	3 (3.8%)	25 (11.5%)		23 (10.2%)	86 (17.1%)	
IPF	37 (46.8%)	61 (28.0%)		94 (41.8%)	185 (36.8%)	
IPAH	4 (5.6%)	4 (1.8%)		9 (4.0%)	11 (2.2%)	
Sarcoid	5 (6.3%)	1 (0.5%)		14 (6.2%)	12 (2.4%)	
Other	4 (5.1%)	5 (2.3%)		23 (10.2%)	21 (4.2%)	
BLTX	44 (55.7%)	121 (55.5%)	0.977	155 (68.9%)	346 (68.8%)	0.978
CPB	46 (42.9%)	50 (26.3%)	0.003	127 (52.5%)	152 (31.3%)	<0.001

IT, total ischemic time (time when last clamp removed); COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; IPAH, idiopathic pulmonary arterial hypertension; BLTX, bilateral lung transplant; CPB, cardiopulmonary bypass.

Data are expressed as mean ± SD, unless otherwise stated. Percent are numerators divided by the total number of subjects with PGD or without PGD with complete data. The reported p value results from unpaired t test or χ^2 as appropriate.

Table 2: Stage 1: association of loci with plasma PAI-1 levels

Locus	Gene	Counts	Mean PAI-1	p value
*rs4149338 T/C	ATP-binding cassette 1	23; 98; 106	190.2; 146.0; 97.9	0.0001
*rs30008 T/C	CD37 molecule	17; 74; 136	273.8; 122.3; 112.9	0.0001
*rs3168046 A/G	Toll interacting protein	34; 126; 67	212.6; 115.3; 109.0	0.0001
*rs866223 A/G	Mitogen-activated protein kinase kinase kinase 1	35; 93; 99	208.6; 121.7; 105.5	0.0001
*rs832573 T/C	Mitogen-activated protein kinase kinase kinase 1	20; 95; 112	245.0; 129.0; 106.3	0.0002
rs3793965 T/C	Toll interacting protein	34; 125; 68	212.6; 114.2; 111.0	0.0002
rs33328 A/G	Mitogen-activated protein kinase kinase kinase 1	34; 93; 100	208.0; 122.2; 106.2	0.0002
rs354022 G/A	CD37 molecule	17; 77; 133	273.8; 120.1; 114.0	0.0002
rs832577 G/A	Mitogen-activated protein kinase kinase kinase 1	34; 95; 97	208.0; 122.3; 105.7	0.0003
rs832552 G/T	Mitogen-activated protein kinase kinase kinase 1	41; 90; 92	194.2; 125.1; 102.8	0.0003
*rs10195871 T/C	B-cell CLL/lymphoma 11A (zinc finger protein)	34; 88; 105	83.9; 108.2; 158.9	0.0003

Locus column displays genotype categorized as heterozygous for the minor allele and major allele displayed in that order. Counts and means columns display number and mean plasma level in each genotype category (minor/minor; minor/major; major/major). Loci associated with PAI-1 plasma levels at $p < 10^{-4}$ are displayed by additive model p value risk adjusted for recipient age, use of cardiopulmonary bypass, diagnosis, and population admixture.

*Loci carried forward to stage 2. Loci not marked with an asterisk were in strong linkage disequilibrium with respective loci carried forward. We considered loci with a pairwise linkage disequilibrium $R^2 \geq 0.9$ in the CEPH HapMap population to indicate the same association signal.

from Hardy–Weinberg equilibrium ($p \leq 0.001$), 1452 loci having a missing rate $>5\%$, and 9287 loci having an MAF <0.01 were excluded from the analysis. The remaining 38 677 loci had a genotyping call rate of 98.8%. Table 2 displays the 11 gene variants with a value of $p < 5 \times 10^{-4}$, the cutoff for association with PAI-1 plasma protein level. From these 11 variants, only six distinct associations were identified given significant LD identified among loci. In each case, the lowest p-value variant was chosen to represent the LD block.

Stage 2: Genetic association with clinical PGD

These six unique loci from stage 1 were carried forward to test for association with PGD (Table 3). A single variant, rs3168046, met our Bonferroni-adjusted significance threshold for association with clinical PGD ($p < 0.008$), within the *TOLLIP* gene encoding the Toll interacting protein (TOLLIP) ($p = 0.006$, see Figure 2 for a summary of our pipeline). Additionally, the MAF did not vary across diagnosis (chronic obstructive pulmonary disease D (0.45), cystic fibrosis (0.45), idiopathic pulmonary fibrosis (0.48), and idiopathic pulmonary arterial hypertension (0.41)),

suggesting the variant had similar risk regardless of diagnosis. To define the risk this variant would pose to an individual patient, we also evaluated the genotype-attributable risk difference in a dominant model (in which a subject would need only one copy of the variant to have an effect). Using this methodology, the added absolute risk difference for a patient carrying at least one copy of the A allele (AA or AG) at rs3168046 is 11.7% (95% confidence interval 4.88–18.53) higher than the baseline risk of homozygous GG genotype (34.61% [30.69–38.54] compared with 22.91% [17.35–28.47]; $p = 0.0008$). Furthermore, the false-positive rate for this genotype among those with no PGD was low at 6.05%. This variant was in strong LD ($R^2 = 0.966$) with rs3793965 identified in stage 1 and moderately so with several other loci within *TOLLIP* (Figure 3). Additionally, rs3168046 is in marginal LD with previously reported loci associated with susceptibility to idiopathic pulmonary fibrosis (rs5743894, $R^2 = 0.29$; rs111521887, $R^2 = 0.26$; rs35705950, $R^2 = 0.09$) (21). Sensitivity analyses using a more restrictive definition of PGD (15.8% with grade 3 PGD at 48–72 hours versus 30.9% in the primary definition of grade 3 PGD at any time point) demonstrated similar magnitude and direction of risk for

Table 3: Stage 2: association of selected loci with PGD*

Locus	Risk/ref alleles	RAF no PGD	RAF PGD	OR (95% CI)	p value	Gene	Location
rs3168046	A/G	0.44	0.51	1.42 (1.11–1.83)	0.0061	TOLLIP	3'UTR
rs30008	T/C	0.24	0.22	1.21 (0.912–1.61)	0.1845	CD37	Intron
rs4149338	T/C	0.30	0.33	1.15 (0.88–1.51)	0.3178	ABCA1	3'UTR
rs832573	T/C	0.34	0.29	1.13 (0.87–1.45)	0.3583	MAP3K1	Intron
rs866223	A/G	0.43	0.38	1.09 (0.85–1.40)	0.4870	MAP3K1	Intron
rs10195871	T/C	0.33	0.33	0.96 (0.74–1.24)	0.7350	BCL11A	Intron

Note: A Bonferroni-adjusted $p < 0.008$ was considered significant to account for the six loci tested.

PGD, primary graft dysfunction; RAF, risk allele frequency; OR, odds ratio; CI, confidence interval; 3'UTR, 3' untranslated region.

*Adjusted for cardiopulmonary bypass, diagnosis, and population admixture.

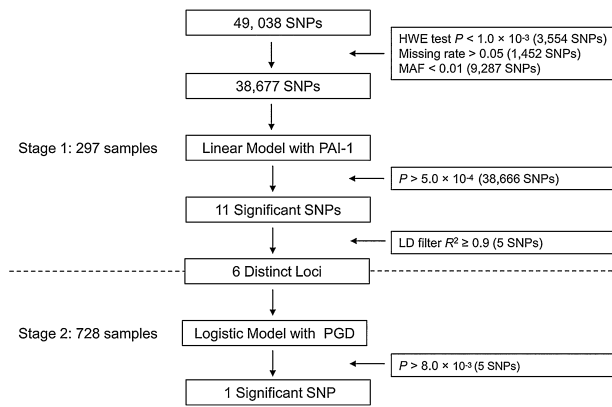


Figure 2: Procedural pipeline. HWE, Hardy–Weinberg equilibrium; PAI-1, plasminogen activator inhibitor-1; PGD, primary graft dysfunction; SNP, single nucleotide polymorphism.

rs3168046, although not reaching statistical significance likely due to smaller numbers of PGD subjects using this definition (odds ratio 1.359 [0.997–1.853]; $p = 0.052$).

Previously reported variants in *PTX3*, *PTGES2*, and *PTGER4* (rs2120243, rs2305619, rs13283456, rs11957-406, rs4434423, rs4133101) were tested for association with PGD using an additive logistic regression model adjusted for diagnosis, cardiopulmonary bypass use, and AIMs in the combined stage 1 and 2 cohorts (Table S1).

Directionality of effects and significance remained consistent with previous reports (22,23).

Discussion

In this study, we have identified significant associations of genetic loci using an established protein quantitative trait for PGD. A strength of our methodology lies in our ability to use previously validated, biologically driven quantification of PGD to improve power for selection of variants from an array optimized to cover 85% of the known ALL-associated genes (24). By using this methodology, our results suggest that *TOLLIP*, a mediator of innate immune-mediated processes, is actively involved in the pathophysiology of PGD. These results are concordant with a growing body of human and basic science studies implicating innate immune activation as a key mediator of PGD following lung transplantation (5,23,25). Additionally, the attributable risk of PGD for recipients having this variant is 11.7%, independent of other potential risks. When taking into consideration the low false-positive rate (6.05%) in non-PGD patients, given the elevated attributable risk, it may be beneficial to consider genotype-stratified populations for lung transplant clinical trials evaluating therapeutics targeted at this mechanistic axis.

The protein product TOLLIP is an attractive candidate for PGD risk. The innate immune system, once thought to be a

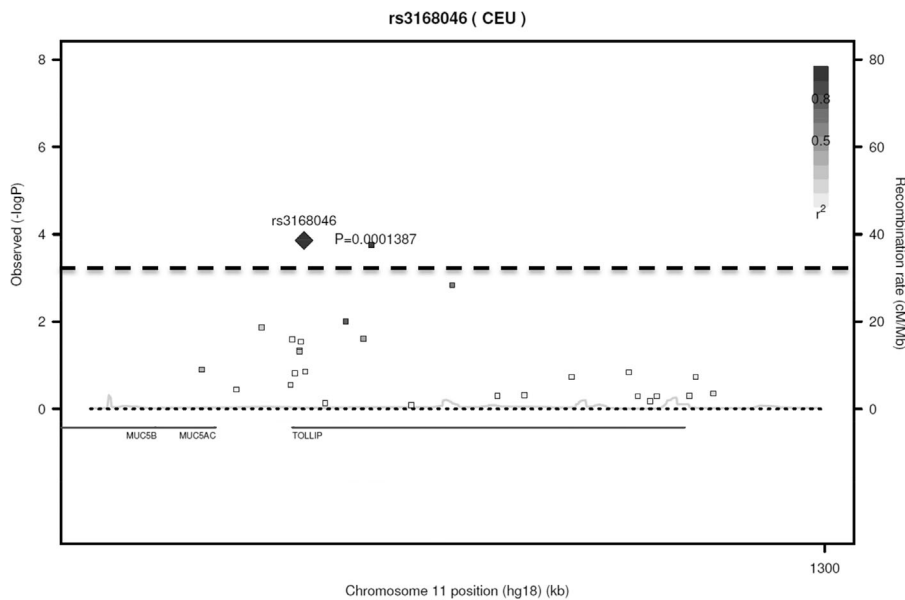


Figure 3: Regional LD plot for locus rs3168046. *MUC5B*, mucin 5B, oligomeric mucus/gel-forming; *MUC5AC*, mucin 5AC, oligomeric mucus/gel-forming. The diamond demonstrates rs3168046, which is the variant of interest and its association with PAI-1 in stage 1 (the square is rs3793965 another variant in tight LD). The remaining squares are the known variants of *TOLLIP* within the Human CVD Bead Chip. The recombinant rate is illustrated by the gray line, which demonstrates very low recombination events throughout this genetic region. The dashed line demonstrates the 5×10^{-5} cutoff.

primitive first responder to pathogens, is well conserved among vertebrates and an integral part in the initiation and regulation of host defense (26). Pattern recognition receptors, which detect nonself, pathogen-associated molecular patterns, exhibit unique properties with regard to ligand recognition and subcellular localization (27). Threats are recognized in the host by pattern recognition receptors that are either localized to the cell membrane (e.g. Toll-like receptors [TLRs]) or in the cytoplasm (e.g. Nod-like receptors) (28). TOLLIP, an intrinsic regulator of TLR signaling, is particularly relevant for TLR-induced responses to immune stimulation (27). There are four recently identified isoforms of the TOLLIP protein characterized in human macrophages (29); however, there is limited information about the role of TOLLIP in human inflammatory diseases (27). It is known that TOLLIP plays a role in inflammatory bowel disease and atopic dermatitis where dysregulation can cause chronic inflammation (30,31). Given these facts, it is tempting to speculate that variants of *TOLLIP* might alter TLR signaling and risk in posttransplantation PGD. Therefore, our findings suggest that this mechanism is a significant component of PGD pathogenesis, which warrants further investigation.

In contrast to the better characterized TLRs, genetic variation in *TOLLIP* has limited characterization and the functional significance is only recently being explored in humans (32). Recent evidence suggests *TOLLIP* polymorphisms can have significant effects with loss of function and alterations in expression (32). These changes can increase susceptibility to chronic inflammatory diseases (21,30,31) and risk for pathogen-derived infection (32). Taken together, incompletely understood implications of *TOLLIP* genetic variation and its key role in other chronic inflammatory diseases suggest it may be a key target for future therapeutics, particularly given the growing body of evidence from gene and protein studies implicating innate immune activation as a mechanistic feature of PGD (22,23,25,33). We and others are currently exploring how this pathway is targeted as part of additional studies.

We chose PAI-1 as our quantitative trait based on our prior study investigating multiple biomarkers for PGD discrimination (10). Plasma PAI-1 levels have been demonstrated to be one of the highest-performing biomarkers defining PGD (10)¹ and an independent risk factor for ALI risk and mortality in nontransplant patients (12,34). Therefore, we believe that PAI-1 protein expression provides an attractive quantification of the degree of lung injury that is concurrent with PGD development. While PAI-1 has been characterized as a marker of endothelial dysfunction, our prior study identified it as reflective of PGD when measured in plasma, and other studies have shown that the lung epithelium can produce PAI-1 (35). Although evidence is accumulating that there is significant cross-talk between endothelial injury, impaired fibrinolysis, and innate immunity (36,37), our approach may have selected variants mechanistically

specific to PAI-1 pathway itself and thus may have excluded important variants associated with PGD. For example, our quantitative trait also identified the innate immunity mediator long pentraxin 3 (*PTX3*) and the inflammatory immunomodulators prostaglandin E₂ synthase (*PTGES2*) and prostaglandin E₂ receptor subtype 4 (*PTGER4*) as associated with severe PGD, although these genes did not meet Bonferroni-adjusted significance. However, it is important to note that *PTX3*, *PTGES2*, and *PTGER4* demonstrated directionality and association with PGD consistent with previous reports (23). Despite this potential bias, variants uncovered using PAI-1 as a quantitative trait were subject to rigorous validation using a clinical definition of PGD, indicating importance of *TOLLIP* and innate immunity in clinical PGD.

There are potential limitations of this study. While we tested for quantitative trait locus associated with only PAI-1, a well-validated marker of PGD risk and mortality, there may be additional plasma markers whose genetic regulation influences PGD risk and may not have been identified in this study. A common potential issue in genetic association studies is population admixture, which can falsely attribute associations based on unbalanced genetic ancestry. We addressed this issue analytically by adjusting for genetic principal components based on ancestry. With respect to *TOLLIP*, the frequency of the variant was similar to healthy genotyped populations, suggesting that it is unlikely to be a false association. Further, rs3168046 was not enriched in patients with chronic lung diseases in our population. Additionally, it is possible that the association identified may not be a direct result of rs3168046 but rather to other nearby loci in tight LD. Future studies using regional sequencing and other techniques to determine the functional consequence of loci in this region are warranted. As in all large-scale genotyping efforts, Type I error is possible. However, we applied a conservative Bonferroni correction in the validation stage to guard against Type I error. In addition, we capitalized on the hypothesis-driven Human CVD Bead Chip with dense genotyping of covered loci and met significance thresholds using a conservative Bonferroni correction (0.0062; Bonferroni cutoff 0.008). Nonetheless, given our relative small discovery sample size, important PGD variants may have been overlooked because of insufficient power to identify smaller associations, overly restricted selection of candidate variants due to the selected biomarker, or conservative methodology for multiple-comparisons adjustment.

In summary, we have capitalized on the power of a protein quantitative trait approach to identify novel genetic variation that may be important in the PGD mechanistic pathway via the effects on PAI-1. Additionally, this methodology demonstrates a practical approach to human association studies in lung transplantation. This methodology successfully identified *TOLLIP* as an important priority locus for PGD risk, consistent with evolving evidence of the

key role of innate immune-mediated processes in the mechanism of PGD.

Acknowledgments

The National Institutes of Health, Robert Wood Johnson Foundation Harold Amos Medical Faculty Development Program, and the Thoracic Surgery Foundation for Research and Education funded this study and the views and opinions expressed therein are those of the authors and do not necessarily reflect those of the National Institute of Health, Thoracic Surgery Foundation for Research and Education or the Robert Wood Johnson Foundation. Funding sources were NIH HL087115, HL081619, HL096845, HL115354, HL114626, HL116656, HL126176, HL081332, HL121406, RWJ AMFDP70640, and NIH HL090021.

Contributors

All authors were members of the Lung Transplant Outcomes Group with responsibility for the conduct of the trial. All authors were involved in the study design and development of case report forms, analysis of data, and all aspects of writing the report. J.E., J.T., B.B., J.N., and E.D. were Clinical Research Coordinators for LTOG with specific responsibility for all data collection and oversight of the trial, and data inspection. R.F. was a statistician for study with specific responsibility for the statistical analysis of the trial. J.D.C. was a senior investigator for the study with specific responsibility for clinical oversight of the trial. E.C., Y.S., J.M.D., N.J.M., D.J.L., S.K., S.M.P., L.S., M.G.H., V.N.L., S.B., M.C., K.W., J.O., P.D.S., A.W., D. Wilkes, D. Weill, D.R., L.B.W., and R.F. were co-principal investigators for LTOG; R.F. and J.D.C. share senior authorship. E.C. was corresponding author with the final responsibility to submit for publication.

Disclosure

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

References

- Lee JC, Christie JD, Keshavjee S. Primary graft dysfunction: Definition, risk factors, short- and long-term outcomes. *Semin Respir Crit Care Med* 2010; 31: 161–171.
- Diamond JM, Lee JC, Kawut SM, et al. Clinical risk factors for primary graft dysfunction after lung transplantation. *Am J Respir Crit Care Med* 2013; 187: 527–534.
- Christie JD, Kotloff RM, Ahya VN, et al. The effect of primary graft dysfunction on survival after lung transplantation. *Am J Respir Crit Care Med* 2005; 171: 1312–1316.
- Yusen RD, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirty-First Adult Lung and Heart-Lung Transplant Report—2014; Focus Theme: Retransplantation. *J Heart Lung Transplant* 2014; 33: 1009–1024.
- Suzuki Y, Cantu E, Christie JD. Primary graft dysfunction. *Semin Respir Crit Care Med* 2013; 34: 305–319.
- Daud SA, Yusen RD, Meyers BF, et al. Impact of immediate primary lung allograft dysfunction on bronchiolitis obliterans syndrome. *Am J Respir Crit Care Med* 2007; 175: 507–513.
- Wang L, Jia P, Wolfinger RD, et al. Gene set analysis of genome-wide association studies: Methodological issues and perspectives. *Genomics* 2011; 98: 1–8.
- Schadt EE, Monks SA, Drake TA, et al. Genetics of gene expression surveyed in maize, mouse and man. *Nature* 2003; 422: 297–302.
- Schliekelman P. Statistical power of expression quantitative trait loci for mapping of complex trait loci in natural populations. *Genetics* 2008; 178: 2201–2216.
- Shah RJ, Bellamy SL, Localio AR, et al. A panel of lung injury biomarkers enhances the definition of primary graft dysfunction (PGD) after lung transplantation. *J Heart Lung Transplant* 2012; 31: 942–949.
- Horrevoets AJ. Plasminogen activator inhibitor 1 (PAI-1): In vitro activities and clinical relevance. *Br J Haematol* 2004; 125: 12–23.
- Ware LB, Matthay MA, Parsons PE, et al. Pathogenetic and prognostic significance of altered coagulation and fibrinolysis in acute lung injury/acute respiratory distress syndrome. *Crit Care Med* 2007; 35: 1821–1828.
- Shah RJ, Diamond JM, Cantu E, et al. Latent class analysis identifies distinct phenotypes of primary graft dysfunction after lung transplantation. *Chest* 2013; 144: 616–622.
- Keating BJ, Tischfield S, Murray SS, et al. Concept, design and implementation of a cardiovascular gene-centric 50 k SNP array for large-scale genomic association studies. *PLoS ONE* 2008; 3: e3583.
- Satagopan JM, Elston RC. Optimal two-stage genotyping in population-based association studies. *Genet Epidemiol* 2003; 25: 149–157.
- Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet* 2006; 38: 904–909.
- Christie JD, Wurfel MM, Feng R, et al. Genome wide association identifies PPF1A1 as a candidate gene for acute lung injury risk following major trauma. *PLoS One* 2012; 7: e28268.
- Meyer NJ, Feng R, Li M, et al. IL1RN coding variant is associated with lower risk of acute respiratory distress syndrome and increased plasma IL-1 receptor antagonist. *Am J Respir Crit Care Med* 2013; 187: 950–959.
- Carlson CS, Eberle MA, Rieder MJ, et al. Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am J Hum Genet* 2004; 74: 106–120.
- Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, de Bakker PI. SNAP: A web-based tool for identification and annotation of proxy SNPs using HapMap. *Bioinformatics* 2008; 24: 2938–2939.
- Noth I, Zhang Y, Ma SF, et al. Genetic variants associated with idiopathic pulmonary fibrosis susceptibility and mortality: A genome-wide association study. *Lancet Respir Med* 2013; 1: 309–317.
- Diamond JM, Akimova T, Kazi A, et al. Genetic variation in the prostaglandin E2 pathway is associated with primary graft dysfunction. *Am J Respir Crit Care Med* 2014; 189: 567–575.
- Diamond JM, Meyer NJ, Feng R, et al. Variation in PTX3 is associated with primary graft dysfunction after lung transplantation. *Am J Respir Crit Care Med* 2012; 186: 546–552.
- Meyer NJ, Li M, Feng R, et al. ANGPT2 genetic variant is associated with trauma-associated acute lung injury and altered

- plasma angiopoietin-2 isoform ratio. *Am J Respir Crit Care Med* 2011; 183: 1344–1353.
25. Cantu E, Lederer DJ, Meyer K, et al. Gene set enrichment analysis identifies key innate immune pathways in primary graft dysfunction after lung transplantation. *Am J Transplant* 2013; 13: 1898–1904.
 26. Jeong E, Lee JY. Intrinsic and extrinsic regulation of innate immune receptors. *Yonsei Med J* 2011; 52: 379–392.
 27. Capelluto DG. Tollip: A multitasking protein in innate immunity and protein trafficking. *Microbes Infect* 2012; 14: 140–147.
 28. Dowling JK, O'Neill LA. Biochemical regulation of the inflammasome. *Crit Rev Biochem Mol Biol* 2012; 47: 424–443.
 29. Lo YL, Beckhouse AG, Boulus SL, Wells CA. Diversification of TOLLIP isoforms in mouse and man. *Mamm Genome* 2009; 20: 305–314.
 30. Steenholdt C, Andresen L, Pedersen G, Hansen A, Brynskov J. Expression and function of toll-like receptor 8 and Tollip in colonic epithelial cells from patients with inflammatory bowel disease. *Scand J Gastroenterol* 2009; 44: 195–204.
 31. Schimming TT, Parwez Q, Petrasch-Parwez E, Nothnagel M, Eppelen JT, Hoffjan S. Association of toll-interacting protein gene polymorphisms with atopic dermatitis. *BMC Dermatol* 2007; 7: 3.
 32. Shah JA, Vary JC, Chau TT, et al. Human TOLLIP regulates TLR2 and TLR4 signaling and its polymorphisms are associated with susceptibility to tuberculosis. *J Immunol* 2012; 189: 1737–1746.
 33. Diamond JM, Lederer DJ, Kawut SM, et al. Elevated plasma long pentraxin-3 levels and primary graft dysfunction after lung transplantation for idiopathic pulmonary fibrosis. *Am J Transplant* 2011; 11: 2517–2522.
 34. Sapru A, Curley MA, Brady S, Matthay MA, Flori H. Elevated PAI-1 is associated with poor clinical outcomes in pediatric patients with acute lung injury. *Intensive Care Med* 2010; 36: 157–163.
 35. Shetty S, Padijanyayveetil J, Tucker T, et al. The fibrinolytic system and the regulation of lung epithelial cell proteolysis, signaling, and cellular viability. *Am J Physiol Lung Cell Mol Physiol* 2008; 295: L967–L975.
 36. Klarstrom Engstrom K, Khalaf H, Kalvegren H, Bengtsson T. The role of *Porphyromonas gingivalis* gingipains in platelet activation and innate immune modulation. *Mol Oral Microbiol* 2015; 30: 62–73.
 37. Katoh S, Honda S, Watanabe T, et al. Atrial endothelial impairment through Toll-like receptor 4 signaling causes atrial thrombogenesis. *Heart Vessels* 2014; 29: 263–272.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1: Adjusted association of previously reported loci with PGD.