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ORIGINAL ARTICLE

Genetic Variation in the Prostaglandin E$_2$ Pathway Is Associated with Primary Graft Dysfunction

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Abstract

Rationale: Biologic pathways with significant genetic conservation across human populations have been implicated in the pathogenesis of primary graft dysfunction (PGD). The evaluation of the role of recipient genetic variation in PGD has thus far been limited to single, candidate gene analyses.

Objectives: We sought to identify genetic variants in lung transplant recipients that are responsible for increased risk of PGD using a two-phase large-scale genotyping approach.

Methods: Phase 1 was a large-scale candidate gene association study of the multicenter, prospective Lung Transplant Outcomes Group cohort. Phase 2 included functional evaluation of selected variants and a bioinformatics screening of variants identified in phase 1.

Measurements and Main Results: After genetic data quality control, 680 lung transplant recipients were included in the analysis. In phase 1, a total of 17 variants were significantly associated with PGD, four of which were in the prostaglandin E$_2$ family of genes. Among these were a coding variant in the gene encoding prostaglandin E$_2$ synthase (*PTGES2*; $P = 9.3 \times 10^{-5}$) resulting in an arginine to histidine substitution at amino acid position 298, and three variants in a block containing the 5′ promoter and first intron of the *PTGER4* gene (encoding prostaglandin E$_2$ receptor subtype 4; all $P < 5 \times 10^{-5}$). Functional evaluation in regulatory T cells identified that rs4434423A in the *PTGER4* gene was associated with differential suppressive function of regulatory T cells.

Conclusions: Further research aimed at replication and additional functional insight into the role played by genetic variation in prostaglandin E$_2$ synthetic and signaling pathways in PGD is warranted.

Keywords: lung transplantation; genetics; prostaglandin; regulatory T cells

At A Glance Commentary

Scientific Knowledge on the Subject:
Several biologic pathways implicated in the development of primary graft dysfunction (PGD) after lung transplantation are highly genetically conserved across human populations. Large-scale gene association studies have been useful in identifying potential mechanisms for the development of acute lung injury, a syndrome closely related to PGD after lung transplantation. The influence of genetic variation on PGD risk is incompletely understood.

What This Study Adds to the Field:
We performed a large-scale candidate gene association study to detect genetic variants associated with PGD using the Lung Transplant Outcomes Group, a multicenter cohort of lung transplant recipients. We identified significant associations between polymorphisms in multiple genes in the prostaglandin E_2 pathway. Furthermore, variation in the prostaglandin EP4 receptor gene (*PTGER4*) was associated with altered T-regulatory cell suppressor function. A more complete understanding of the role played by the prostaglandin E_2 pathway family in PGD may lead to the development of new preventative therapies.

Primary graft dysfunction (PGD), a form of acute lung injury (ALI) following lung transplantation, occurs in 10–30% of cases and is a major cause of early morbidity and mortality after transplant (1–8). Recent investigations have implicated a genetic basis to ALI susceptibility and severity (9–11). The evaluation of the role of recipient genetic variation in PGD has thus far been limited to single, candidate gene analyses (12).

Several well-described biologic pathways, including airway epithelial integrity, coagulation and fibrinolytic cascades, immune activation, and endothelial cell function, have been implicated in clinical PGD pathogenesis (13–22). Many of these pathways demonstrate significant genetic conservation across human populations, between species, and also display evidence

of purifying selection (23–29). These pathways may be reasonable candidates for genetic regulation of differential response to the extreme insult of the lung transplant process. Evidence exists for a contribution of genetic variation to individual risk for acute rejection and bronchiolitis obliterans syndrome, yet genetic studies of PGD have been limited to date (30–39). A large-scale genotyping approach has not been previously performed to evaluate genetic risk in PGD.

Based on the hypothesis that there is an unidentified role for recipient genetic variation in the development of PGD, we performed a large-scale candidate gene cohort study. We sought to test the association of single-nucleotide polymorphisms (SNPs) in genes with known association with inflammatory, metabolic, and vascular phenotypes with differential risk for the development of PGD after lung transplantation (phase 1). The Illumina IBC chip (Illumina, San Diego, CA) was chosen for this evaluation rather than a genome-wide array because of its inclusion of rare variants and its dense coverage of gene targets hypothesized to be involved in pulmonary and inflammatory disease states (40). We then sought to identify the functional implications of selected candidate SNPs in three ways (phase 2): (*1*) using cell-based assays specific to implicated variants, (*2*) evaluation of selected SNPs on corresponding plasma protein levels, and (*3*) a broad-based bioinformatics approach.

Methods

See the online supplement for more detailed methods on cohort design, subject selection, PGD definition, genotyping strategy, and T-regulatory (Treg) cell suppression assays.

Cohort Design
Subjects were enrolled from the prospective, multicenter Lung Transplant Outcomes Group cohort study (clinicaltrials.gov identifier: NCT00457847). Clinical data were prospectively collected and blood samples for DNA were collected before transplant (41). All study center institutional review boards approved this study.

PGD Definition
PGD was defined as the presence of diffuse alveolar infiltrates involving the allografts

with severity determined by the $Pa_{O_2}/F_{I_{O_2}}$ ratio; grade 3 PGD was defined as $Pa_{O_2}/F_{I_{O_2}}$ less than 200 (4). The primary outcome was defined as any grade 3 PGD within 72 hours of reperfusion. Sensitivity analyses were performed with PGD defined using alternative severity definitions.

Phase 1: Study Design and Genotyping Strategy
Phase 1 was a large-scale candidate gene association study. Subjects enrolled in Lung Transplant Outcomes Group from July 2002 to July 2009 were included in the analysis. Genotyping was performed using the IBC chip (Illumina), an array designed to assay SNPs in candidate genes affecting vascular, pulmonary, and metabolic phenotypes (11, 40).

Phase 1: Statistical Analysis
Subject characteristics were compared using descriptive tests as appropriate. An odds ratio (OR) for PGD based on an additive model of genetic risk in PLINK, with significance determined by chi-square, was calculated according to genotype for all SNPs (42). Principle component analysis of the approximately 1,800 ancestry-informative markers on the chip were used to determine genetically inferred ancestry (10–12). ORs were adjusted for cardiopulmonary bypass, predisposing lung disease, and the first two principal components derived from ancestry-informative markers using logistic regression. No consensus exists for a *P* value for SNPs evaluated using hypothesis-driven candidate gene assays with redundant, linked SNPs. Similar to previous studies, we selected an adjusted *P* less than 5×10^{-4} for significance and then used functional assessments to validate the findings (43, 44).

Phase 2: Study Design
Phase 2 was a functional assessment of SNPs significantly associated with PGD in phase 1. Given the overrepresentation of prostaglandin E_2 (PGE_2) pathway family SNPs among all variants identified as significantly associated with PGD in phase 1, the four PGE_2 pathway family SNPs were assessed functionally using Treg suppression assays. Next, we evaluated the impact of variants at selected SNPs on corresponding plasma protein PGE_2 levels. To ensure generalizability of the study results, a cohort of patients was selected to

include subjects with and without PGD from eight transplant centers. We also enriched the patient population to include homozygotes for both the wild-type and recessive alleles. Finally, all significant phase 1 SNPs were evaluated with a bioinformatics approach and literature searches to identify prior associations with pulmonary disease or organ transplantation (45–47).

Phase 2: Treg Suppression Assay

Quantitative assays for Treg suppression function methods have been described previously (48). Aliquoted cryopreserved single healthy donor peripheral blood mononuclear cells were labeled with carboxyfluorescein diacetate succinimidyl ester, stimulated with CD3ε mAb-coated microbeads, and used as standardized responder cells in each assay. Tregs were isolated with $CD4^+CD25^+$ kit (Miltenyi Biotec, Auburn, CA) from 50 ml of blood taken from eight liver transplant recipients. Samples-in-hand from liver transplant recipients were selected because of the large volume of blood (>50 ml) required to isolate a sufficient number of Tregs from the subject and to ensure that subjects received standard immunosuppression, similar to lung transplant recipients. Four days later, the percent of dividing T cells was determined by carboxyfluorescein diacetate succinimidyl ester dilution. We controlled for the purity of Tregs and used standardized suppression assay conditions that differed only in the suppressive function of patients' Tregs. Suppressive function was calculated as area under the standardized suppression curve (see Figure E1 in the online supplement) (48)

Phase 2: Measurement of PGE_2 Plasma Concentration

Plasma PGE_2 concentrations were determined using a sandwich ELISA (Cayman Chemical, Ann Arbor, MI) from pretransplant and immediate post-transplant plasma samples.

Phase 2: Statistical Analysis

Quantitative Treg suppressive function was analyzed across SNP genotypes using rank-sum tests with a P less than 0.05 considered significant. Plasma PGE_2 concentrations were analyzed across PTGES2 SNP genotypes using the Kruskal-Wallis test with a P less than 0.05 considered significant. In silico gene expression results were considered significant at a P less than 0.05. Statistical analyses were performed using Stata 11.2 software (STATA Corp., College Station, TX).

Results

Patient Characteristics

After genetic quality control, 680 subjects were included in the analysis. PGD developed in 194 (29%) of 680 (95% confidence interval [CI], 25–32%) subjects. All transplant centers use a low-potassium dextran preservation solution (Perfadex; XVIVO Perfusion, Goteborg, Sweden). The characteristics of the study subjects are described in Table 1. There was no significant difference in age or sex between patients with and without PGD. Significantly more patients who developed PGD received perioperative cardiopulmonary bypass compared with those without PGD (52% vs. 29%; P < 0.001). As previously described, there were significant differences in the distribution of preoperative pulmonary diagnoses, with more idiopathic pulmonary fibrosis and less chronic obstructive pulmonary disease in the PGD group compared with the non-PGD patients (P < 0.001), and racial composition with more African American subjects in the PGD group compared with the non-PGD group (P < 0.001) (12).

Phase 1: SNP Associations with PGD

After adjustment for preoperative pulmonary diagnosis, perioperative use of cardiopulmonary bypass, and genetically inferred race, 17 SNPs met our prespecified level of significance for PGD of P less than 5×10^{-4} (Table 2). Four of the 17 SNPs were in the PGE_2 pathway genes. Of these, one SNP, rs13283456, is a coding nonsynonymous SNP in PGE synthase 2 (PTGES2, responsible for encoding a constitutive protein isoform converting PGH_2 to PGE_2). The minor allele was significantly associated with PGD (OR, 2.0; 95% CI, 1.4–2.9; P = 9.3×10^{-5}). Three other SNPs were in the gene encoding the PGE_2 receptor subtype 4 (PTGER4). Two of these SNPs, rs4434423 and rs4133101, lie in the 5′ promoter region of the gene in tight linkage disequilibrium ($r^2 = 0.92$), and the minor alleles were associated with lower risk of PGD (OR, 0.6; 95% CI, 0.4–0.8; P = 2.8×10^{-4}; and OR, 0.6; 95% CI, 0.5–0.8; P = 4.8×10^{-4}). rs11957406 lies in the second intron and the minor allele was significantly associated with PGD (OR, 1.7; 95% CI, 1.3–2.3; P = 2.2×10^{-4}). There were also three SNPs in TBC1D1, a gene linked with obesity and type 2 diabetes, all with intronic minor alleles associated with lower PGD risk (49–51).

We then performed sensitivity analyses using alternative PGD outcome definitions. When comparing patients with grade 3 PGD developing any time within the first 3 days after transplant with those with no (grade 0) or mild (grade 1) PGD, the results were similar although mildly attenuated given the smaller sample size (see Table E1). The

Table 1: Subject Characteristics

Covariate	PGD (n = 194)	Non-PGD (n = 486)	P Value
Recipient variables			
Age, mean	52	53	0.6
Female sex, n (%)	93 (48)	228 (47)	0.9
Pulmonary diagnosis, n (%)			<0.001
Chronic obstructive pulmonary disease	60 (31)	219 (45)	
Idiopathic pulmonary fibrosis	76 (39)	150 (31)	
Cystic fibrosis	14 (7)	73 (15)	
Other	44 (23)	44 (9)	
Race, n (%)			<0.001
White	149 (77)	423 (87)	
African American	33 (17)	29 (6)	
Other	12 (6)	24 (5)	
Operative variables			
Cardiopulmonary bypass use, yes, n (%)	101 (52)	141 (29)	<0.001

Definition of abbreviation: PGD = primary graft dysfunction.

Table 2: Single-Nucleotide Polymorphism Analysis for Association with PGD

rs Number	Gene	Gene Function/ Disease Association	Location	Minor Allele	Risk Allele	MAF PGD	MAF Non-PGD	Odds Ratio (95% Confidence Interval)	P Value
rs2996044	TBC1D1	Cell growth and differentiation/obesity	Intron	C	T	0.26	0.40	0.5 (0.4–0.7)	3.0×10^{-5}
rs13283456	PTGES2	Prostaglandin E$_2$ synthesis	Coding	T	T	0.21	0.16	2.0 (1.4–2.9)	9.3×10^{-5}
rs2925956	TBC1D1	Cell growth and differentiation/obesity	Intron	A	G	0.31	0.41	0.6 (0.4–0.8)	1.2×10^{-4}
rs13132184	TBC1D1	Cell growth and differentiation/obesity	Intron	C	T	0.10	0.21	0.5 (0.3–0.7)	1.6×10^{-4}
rs7973796	PMCH	Hypothalamic neurotransmitter/obesity	5′ UTR	G	A	0.40	0.54	0.6 (0.5–0.8)	1.7×10^{-4}
rs3024388	F13A1	Coagulation cascade/ venous thrombosis	Intron	T	T	0.30	0.20	1.8 (1.3–2.5)	2.0×10^{-4}
rs12452616	GAA	Glycogen degradation	Intron	A	A	0.27	0.20	1.8 (1.3–2.5)	2.1×10^{-4}
rs11957406	PTGER4	Prostaglandin E receptor (EP4)	Intron	A	A	0.56	0.47	1.7 (1.3–2.3)	2.2×10^{-4}
rs237865	CAV3	Muscle development	5′ UTR	C	C	0.51	0.40	1.7 (1.3–2.2)	2.4×10^{-4}
rs17588591	COL4A1	Extracellular matrix/ punctate palmoplantar keratoderma	Intron	G	G	0.37	0.29	1.7 (1.3–2.3)	2.6×10^{-4}
rs16836965	CASP8	Cell death and tumor regulation	Intron	T	T	0.08	0.02	3.2 (1.7–6.0)	2.7×10^{-4}
rs4434423	PTGER4	Prostaglandin E receptor (EP4)	5′ UTR	T	A	0.31	0.42	0.6 (0.4–0.8)	2.8×10^{-4}
rs260400	IRX4	Ventricular differentiation and cardiac development	5′ UTR	A	A	0.15	0.09	2.1 (1.4–3.1)	3.1×10^{-4}
rs3772843	ITGB5	Cell adhesion	Intron	A	A	0.23	0.14	1.9 (1.3–2.6)	3.2×10^{-4}
rs1881597	PRKG1	Platelet aggregation	3′ UTR	T	C	0.20	0.31	0.6 (0.4–0.8)	3.8×10^{-4}
rs4133101	PTGER4	Prostaglandin E receptor (EP4)	5′ UTR	A	G	0.32	0.42	0.6 (0.5–0.8)	4.8×10^{-4}
rs17004504	FTCD	Folate metabolism/ autoimmune hepatitis	Intron	T	T	0.08	0.03	2.9 (1.6–5.2)	4.8×10^{-4}

Definition of abbreviations: MAF = minor allele frequency; PGD = primary graft dysfunction; UTR = untranslated region.
Odds ratio and *P* value are based on an additive model. Analysis is corrected for first two principle components derived from ancestry informative markers, cardiopulmonary bypass use, and preoperative lung disease.

minor allele at the *PTGES2* coding nonsynonomous locus, rs13283456, had an OR for PGD of 1.8 (95% CI, 1.2–2.7; *P* = 0.003). The associations between risk of PGD and the minor alleles of the 5′ promoter *PTGER4* SNPs, rs4434423 (OR, 0.5; 95% CI, 0.4–0.7; *P* = 1.4×10^{-4}) and rs4133101 (OR, 0.6; 95% CI, 0.4–0.8; *P* = 3.6×10^{-4}), and the intronic *PTGER4* SNP rs11957406 (OR, 1.7; 95% CI, 1.3–2.4; *P* = 3.7×10^{-4}) were similar to the primary analysis in magnitude but slightly attenuated in significance. The results were similar when limiting the analysis to comparisons of the most extreme subjects, grade 3 versus grade 0 (*see* Table E2).

Phase 2: Functional Evaluation
SNPs in the PGE$_2$ pathway family were overrepresented in the phase 1 analysis (4 of 17 SNPs). SNPs from genes involved in the PGE$_2$ signaling pathway have been previously described to be involved in

immunomodulation and inflammation (52–54). Recently, the PG EP4 receptor was shown to regulate hematopoietic stem cell expansion and migration (55). Based on the immunomodulatory effect of PGE$_2$, the three SNPs identified in the EP4 receptor were functionally evaluated in phase 2. PGE$_2$ induces and up-regulates *FOXP3* gene expression in both CD4$^+$CD25$^+$ Tregs and CD4$^+$CD25$^-$T cells (56), and PGE$_2$ likely results in up-regulation of FOXP3 at the level of mRNA transcription (57). We therefore evaluated the suppressive function of FOXP3$^+$ Tregs against standardized CD4 and CD8 cellular populations by *PTGER4* genotype. Tregs collected from a cohort of liver transplant recipients were used for this evaluation. Tregs homozygous for the "A" risk allele at rs4434423 (n = 2) had decreased suppressor T-cell function against standardized CD4 and CD8 T-cell populations when compared with Tregs carrying the

protective "T" allele (n = 6) (Figure 1); the "T" allele at the rs4434423 locus was associated with decreased risk of PGD in phase 1.

The effect of variation at the coding, nonsynonomous SNP, rs13283456, in *PTGES2* on plasma PGE$_2$ levels was assessed in 42 lung transplant recipients, including 18 subjects without PGD and 24 subjects with PGD. All subjects had pretransplant plasma available and 40 had corresponding post-transplant plasma available. There were nine subjects homozygous for the risk "T" allele, 17 heterozygotes (CT), and 16 wild-type "CC" homozygotes. There was no significant difference in median plasma PGE$_2$ level measured before transplant (TT, 56.5 pg/ml; CT, 115.1 pg/ml; CC, 87.5 pg/ml; *P* = 0.5), immediately post-transplant (TT, 60.9 pg/ml; CT, 78.9 pg/ml; CC, 55.7 pg/ml; *P* = 0.6), or in the median change in PGE$_2$ level from pretransplant to post-transplant (TT,
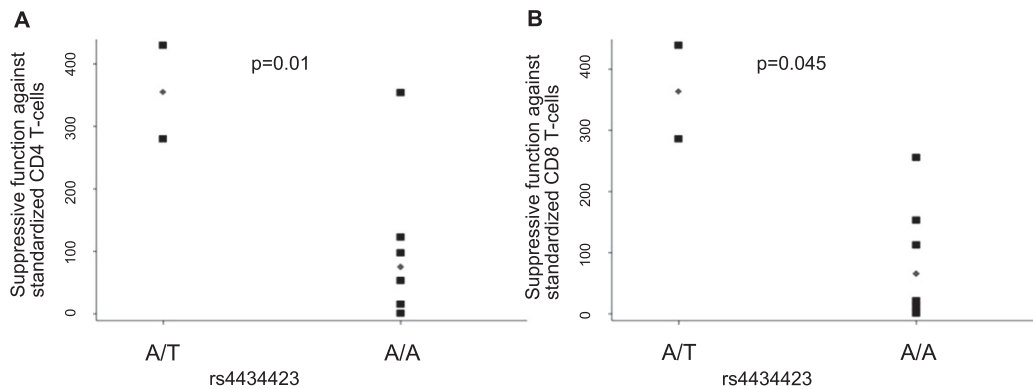
Figure 1. T-regulatory suppressor function according to genotype at the rs4434423 locus in PTGER4. (*A*) Suppressive function against standardized CD4$^+$ T cells. (*B*) Suppressive function against standardized CD8$^+$ T cells. *Squares* represent individual patient level data. *Diamonds* represent the median value. Y-axis represents area under the standardized suppression curve. *P* value is from rank-sum test.

10.8 pg/ml; CT, −21.3 pg/ml; CC, −20.1 pg/ml; $P = 0.3$) (*see* Figure E2). There was no association between genotype and pretransplant or post-transplant PGE$_2$ levels when evaluated within subjects with or without PGD (all $P > 0.1$).

Additionally, a bioinformatics evaluation of all SNPs identified as significant in phase 1 was performed. No SNPs identified in phase 1 have previously been identified to function as *cis*-eQTLs; genotype at the rs13283456 locus in *PTGES2* was nonsignificantly associated with differential gene expression in T cells ($P = 0.09$) (45, 46). *In silico* modeling of the *PTGES2* coding nonsynonomous allele, rs13283456T, leading to an arginine to histidine missense mutation at position 298, would be a benign, tolerated change, although the arginine to histidine change results in the loss of a salt bridge (47, 58–60). Three 5′ untranslated region SNPs, rs7973796 in *PMCH*, rs4434423 in *PTGER4*, and rs237865 in *CAV3*, and the intronic SNP rs17004504 in *FTCD*, are likely in promoter or regulatory regions and were predicted to have at most medium-risk effects on transcription factor binding (47).

Discussion

Using a large, multicentered cohort study of lung transplant recipients, we identified 17 SNPs in 13 genes to be prioritized for research on PGD after lung transplantation. Of these, there were four SNPs in two genes in the PGE$_2$ family, *PTGES2* and *PTGER4*, which were significantly associated with PGD. The A risk allele in *PTGER4*

rs4434423 was associated with decreased Treg suppressor cell function, indicating a potential functional consequence of the observed genetic variation, consistent with a pathogenic consequence of this variation.

The PG EP4 receptor plays a central role in immunomodulation and control of inflammation mediated by PGE$_2$ (52–54). Antagonism of the EP4 receptor results in impaired immunosuppressive responses to ultraviolet irradiation (53). Activation of the EP4 receptor inhibits the activation and proliferation of T cells (54). The documented immunosuppressive role of the EP4 receptor is consistent with our demonstration of increased Treg suppressor function in cells possessing the rs4434423T allele, which was associated with lower PGD risk. Although our bioinformatics evaluation of the identified *PTGER4* SNPs only identified the rs443443 to have a very low to medium risk of altering transcription factor binding, the recent ENCODE project highlights the tremendous diversity in genetic regulatory elements, outside of traditional transcription factor binding (61). The molecular mechanism linking the genetic variation with altered Treg cell function needs to be further elucidated.

There is no previous literature evaluating the role played by Tregs in the development of PGD. However, Treg cells play a central role in the resolution of ALI (62, 63). The adoptive transfer of Tregs into lymphocyte-deficient mice results in the resolution of fibroproliferation through reduced fibrocyte recruitment (64). In an endotracheal lipopolysaccharide-induced

model of ALI, transplantation of human umbilical cord mesenchymal stem cells results in a significant increase in the number of CD4$^+$CD25$^+$Foxp3$^+$ Tregs, an increase in the IL-10 levels, and a decrease in proinflammatory cytokines (65). A detailed evaluation of the role of Tregs in the development of PGD after lung transplantation is the focus of ongoing investigation.

Given the central role of PGE$_2$ in cancer immunology, inflammation, and autoimmunity, there is significant interest in developing therapies targeting PGE$_2$-associated pathways. Synthetic EP4 receptor agonists inhibit proinflammatory mediators and decreased mortality in a mouse liver ischemia reperfusion model (66). EP4 agonists also diminished infarction area in a rat myocardial ischemia reperfusion model (67). Pharmacologic activation of the EP4 receptor prolonged cardiac allograft survival in mouse models (68). In addition to its immunomodulatory effects, PGE$_2$ has potent vasodilatory properties, likely mediated by the EP4 receptor (69). Our findings of a genetic association between PGE-associated genetic variation and PGD, combined with the exiting knowledge on PGE$_2$ and EP4 agonism, indicate that evaluating the role of PGE$_2$ and EP4 receptor agonists in the treatment and prevention of PGD is an area ripe for future investigation.

PTGES2 encodes a constitutively expressed PG synthase distinct from cyclooxygenase-2. Although the protein crystal structure of PGES2 indicates the altered amino acid moiety encoded, by rs13283456T, is located in the protein

binding pocket and may therefore alter protein synthetic function, the function of this protein alteration is unknown at this time (70). This SNP variant has previously been associated with altered risk of type II diabetes, (71) and it is postulated that replacement of arginine, with a pKa of 12.5, to histidine, with a pKa of 6.0, may alter protein function and decrease PGE_2 production. The addition of PGE_2 results in activation of $FOXP3^+$ Treg cells and suppression of effecter T cells (56, 72). This is consistent with our finding that a variant of *PTGES2* with hypothesized decreased synthetic function is associated with the proinflammatory state leading to severe PGD after transplant. We demonstrated no association between variation in *PTGES2* and altered PGE_2 protein plasma levels in an enrichment sampled cohort of lung transplant recipients. PGE_2 is rapidly metabolized *in vivo*, with an estimated half-life of less than 15 seconds (73, 74). Rapid degradation of PGE_2 in blood may hamper our ability to adequately assess protein levels in response to genetic variation in *PTGES2*. Alternatively, this may indicate that variation in the receptors is more significant in altering the activity of this pathway, or that other PGE_2 synthase isoforms may have influence. Further evaluation of alterations in the synthetic function of PGE synthase 2 (e.g., through measurement of longer-lasting urinary metabolites or in lung tissue) is needed to further refine the role played by the rs13283456T variant.

We limited our functional evaluation to a small segment of the 17 SNPs identified in our initial screen given the abundance of PGE-associated SNPs identified in phase 1. Future investigations should focus on evaluating the potential implications of the other genes associated with PGD. The 11 genes tagged by the other 13 SNPs identified in phase 1 were not further functionally evaluated because they have not previously been associated with lung disease, organ transplantation, or ischemia-reperfusion injury (Table 2). However, one can hypothesize merit for future functional evaluation of several genes identified in phase 1. Given the newly identified association between obesity and PGD, two genes, *TBC1D1*

and *PMCH*, which have been associated with metabolism and weight, are intriguing targets for future characterization (51, 75, 76). We identified an intronic variant locus, rs3024388, in *F13A1*, the gene encoding the factor XIII A1 subunit, to be associated with PGD (Table 2). Genetic variation at the rs5985 locus in *F13A1* has previously been associated with protection against myocardial infarction (77). We also demonstrated differences at the rs1881597 locus in *PRKG1*, encoding protein kinase, cGMP-dependent, regulatory type 1, to be associated with PGD (Table 2). This protein plays an important role in inhibiting platelet aggregation. Previous studies have shown that plasma differences in plasminogen activator inhibitor-1, an inhibitor of fibrinolysis, are associated with altered risk of PGD after lung transplantation (78, 79). The association of variant *F13A1* and *PRKG1* with PGD provides support for further investigation into the possible mechanistic role of coagulation, fibrinolysis, and clot formation in the pathogenesis of PGD.

There are several limitations to our study. First, there is no available replication cohort to validate our genotyping findings. However, our cohort is multicentered and broadly generalizable. Additionally, although there is no population-based replication, we validated the findings using biologic mechanisms (80). We identified multiple SNPs in two genes in the same pathway and then demonstrated a potential functional effect of the variants in the EP4 receptor. The Treg suppressor function was evaluated in a population of liver transplant subjects, not lung transplant recipients. The genotype-specific differential Treg suppressor function identified in this population was therefore independent of PGD. The prospective assessment of Treg function in the development of PGD is an area of active research. Although the functional assessment should be interpreted cautiously because of the relatively small sample size of patients used for Treg assays, taken together the functional implications of the identified genetic differences enhance the validity of our findings. Our study does not take into

account the potential role played by donor genetic variation and the interaction between donor and recipient genotypes. We focused on the effect of recipient genetic variation and the risk of PGD. Separate evaluation of the impact of donor gene expression on the risk of PGD is ongoing (81). Next, our functional assessment focused on a single cell type, Tregs. Future studies will assess the impact of PGE_2 pathway genetics variants on other cell types, including alveolar macrophages. Finally, we did not perform a full genome-wide association study and thus may have missed the potentially important association of other genetic variation in PGD. However, the overall population of lung transplant recipients is small, thus limiting the power necessary for performing a genome-wide association study evaluation. Additionally, because of the limited study population, our study was only powered to detect relatively common variants with modest effect sizes and may therefore have missed rare variants with less substantial effect sizes. Importantly, however, our candidate gene approach focused on genes with hypothesized roles in metabolic pathways implicated in PGD, therefore increasing the potential validity of our findings.

In summary, using a targeted, large-scale candidate gene approach, we identified 17 SNPs in lung transplant recipients associated with risk of PGD after lung transplantation. These SNPs were enriched in the PGE_2 synthetic and metabolic genes *PTGES2* and *PTGER4*. Furthermore, we identified a correlation between *PTGER4* genetic variation and suppressor function of Treg cells. Taken together, our results indicate that PGE_2 pathways provide a novel target for preventing and treating severe PGD after lung transplantation. ■

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