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Anellovirus Dynamics Are Associated With Primary Graft Dysfunction in Lung Transplantation

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Background. Primary graft dysfunction (PGD) is the leading cause of early death in lung transplant. Anelloviruses are small circular DNA viruses that have been noted to be present at elevated levels in immunosuppressed patients. They have been associated with both short- and long-term outcomes in lung transplant, and we hypothesized that anellovirus dynamics might be associated with the development of PGD. **Methods.** We analyzed alphatorquevirus (ie, an anellovirus genus) levels in whole blood samples from 64 adult lung transplant recipients. **Results.** Patients with a relatively rapid rise in alphatorquevirus levels in the week following transplant were less likely to develop higher-grade PGD over the first 3 days following transplant ($P = 0.031$). **Conclusions.** This study is the first to establish an association between the development of PGD and a component of the blood virome. While it is not known whether anelloviruses directly affect outcomes in lung transplant, they may serve as a biomarker of immune status in lung transplant recipients.

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Survival in lung transplant has lagged behind other solid-organ transplants.[1] While chronic lung allograft dysfunction is considered the primary contributor to limited lung allograft survival, there is substantial morbidity and

mortality that is associated with shorter term outcomes in lung transplant.

Primary graft dysfunction (PGD) occurs within the first 72 hours after transplant. It is the leading early cause of death after lung transplant.[2] PGD is characterized by impaired oxygenation (as measured by ratio of arterial oxygen partial pressure to inhaled oxygen fraction, Pao_2/FiO_2) and chest radiograph abnormalities.[3] PGD is graded on a scale of 0–3, and higher grades of PGD are associated with a diminishing Pao_2/FiO_2 ratio. Patients with severe PGD require more prolonged mechanical ventilation following transplant, and have 7 times the risk of 30-day mortality, when compared with those without PGD.[2] While epidemiologic studies have suggested that pretransplant recipient diagnosis can impact PGD incidence,[4] the immunologic milieu associated with PGD is less well understood. Certain proinflammatory cytokines (eg, CCL2, CXCL10, interleukin-2, interferon-gamma) are characteristic in patients with PGD,[5] and the presence of preformed autoantibodies in the recipient may contribute to increased PGD risk.[6] Due to organ ischemia and relative immune suppression, solid-organ recipients have been proposed to have a distinctive microbiome at the time of transplant.[7]

The family *Anelloviridae* is composed of near-ubiquitous small circular DNA viruses[8] that have been noted to bloom in the serum and bronchoalveolar lavage fluid (BAL) of lung transplant recipients.[9,10] We focused our study of the peritransplant virome on anellovirus due to its established association with states of immunosuppression and with transplant outcomes of interest. In a recent case-control study of grade 3 PGD patients versus non-PGD patients, alphatorquevirus (a genus of anellovirus) in BAL was noted to rise less rapidly

after allograft implantation in PGD cases when compared with controls.[11] In pediatric patients, alphatorquevirus levels at 2 weeks posttransplant have already been shown to predict acute cellular rejection (ACR),[12] another key short-term adverse outcome in lung transplant recipients. In the current study, we sought to examine whether these dynamic patterns of alphatorquevirus associated with PGD and ACR in previous studies could be validated in a cohort of adult lung transplant recipients.

MATERIALS AND METHODS

Participant Description

The participants in this study were recruited as part of the lung transplant virome cohort. From July 2017 to August 2018, a total of 64 consecutive patients (19 to 75 y of age, average 58 y) were enrolled at the Washington University/Barnes-Jewish Transplant Center in Saint Louis, Missouri. Induction immunosuppression was standardized, with 62 patients receiving basiliximab and methylprednisolone, and 2 patients with evidence of donor-specific antibodies receiving thymoglobulin and methylprednisolone. Maintenance immunosuppression, including tacrolimus, mycophenolate, and prednisone, was applied per protocol, as was antiviral prophylaxis. This study was approved by the Washington University in St. Louis Institutional Review Board (ID 201706125).

Anellovirus PCR Analysis

Nucleic acid was extracted from whole blood samples using the COBAS AmpliPrep Total Nucleic Acid Isolation Kit (Roche Diagnostics), according to manufacturer's instructions. Before extraction, 425 µL of whole blood was mixed with an equal volume of the COBAS Specimen Pre-Extraction Reagent. Samples were eluted in 75 µL of buffer.

Alphatorquevirus levels in extracted samples were quantified using TaqMan quantitative real-time PCR, targeting a conserved segment of the viral untranslated region.[12,13] The 3 µL extracted sample, TaqMan Fast Advanced Master Mix (Thermo Fisher), 0.9 µM AMTS forward primer (5′ GTGCCGIAGGTGAGTTTA 3′), 0.9 µM AMTAS reverse primer (5′ AGCCCGGCCAGTCC 3′), and 250 nM AMTPTU probe (5′ TCAAGGGGCAATTCGGGCT 3′, 6-FAM/ZEN/3′IBFQ) were combined in 25 µL total reaction volume. Cycling conditions were 50°C for 2 minutes and 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second, and 60°C for 20 seconds. Betatorquevirus levels in extracted samples were likewise quantified using TaqMan quantitative real-time PCR, targeting a short segment near the ORF 2.[12,14] The 3-µL extracted sample, TaqMan Fast Advanced Master Mix, 0.5 µM forward primer (5′ AGTTTATGCCGCCAGACG 3′), 0.5 µM reverse primer (5′ CCCTAGACTTCGGTGGTTTC 3′), and 250 nM probe (5′ ACTCACCTHCGGCACCCGC 3′, 6-FAM/TAMRA). Cycling conditions were 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The ViiA 7 Real-Time PCR System (Applied Biosystems) was used with default ViiA 7 Software v1.2 settings. A plasmid control for each assay was used to generate a standard curve (alphatorquevirus: slope −3.67, R^2 0.99; betatorquevirus: slope −3.83, R^2 0.99), and the detection limit was 30 and 3 copies for alphatorquevirus and betatorquevirus,

respectively. Samples below the detection limit were assigned a value at the bottom of rank order.

Assignment of Clinical Metadata

The participants' pretransplant listing diagnoses were assigned to Group A (obstructive disease), Group B (pulmonary vascular disease), Group C (cystic fibrosis), and Group D (restrictive disease) per current United Network of Organ Sharing policy.[15] The diagnosis of PGD was based on consensus guidelines developed by The International Society for Heart & Lung Transplantation Working Group on Primary Graft Dysfunction.[3] High-grade PGD was assessed as Grade 2 or 3 disease at any time point within the first 72 hours following transplant. ACR was diagnosed using transbronchial biopsies obtained at 1, 2, and 3 months following transplant. The definition of ACR was likewise based on International Society for Heart & Lung Transplantation consensus guidelines,[16] and presence of ACR within the first 3 months (ie, "ever ACR") was defined as Grade 1 disease or above. If patients had insufficient data (ie, <2 monthly transbronchial biopsy specimens obtained within the first 3 mo), no assignment of "ever ACR" was made.

Statistical Analyses

Alphatorquevirus and betatorquevirus levels before and after transplant were compared with Spearman correlation. Pairwise comparison of PGD incidence among the pretransplant diagnoses was made with Fisher exact testing. Bivariate analyses of alphatorquevirus copy number and outcomes of interest were assessed with Mann-Whitney testing. The rate of rise of alphatorquevirus was assessed as a copy number ratio between later and earlier time points, and ratios were compared by Mann-Whitney testing. Statistical analysis was performed with GraphPad Prism version 8.

RESULTS

Study Participants

The characteristics of lung transplant virome study participants are summarized in Table 1. The most common diagnoses were Group A and Group D. High-grade PGD was more prevalent among patients with restrictive than obstructive disease ($P < 0.003$). Of the 64 patients included in the study,
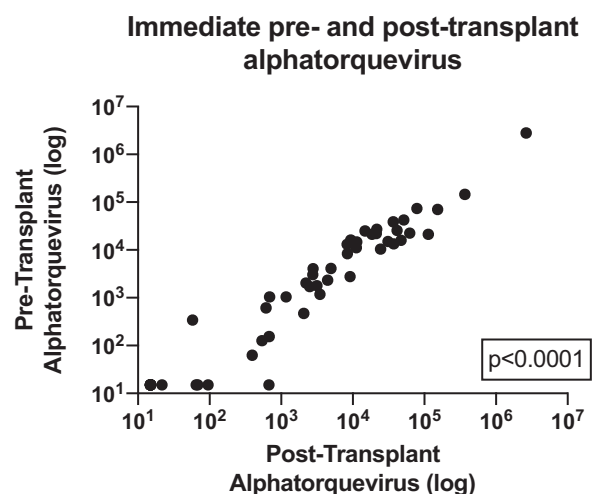

FIGURE 1. Levels of recipient blood alphatorquevirus, 1 day before transplant (pretransplant) and 1–2 days following transplant (posttransplant).

Characteristics of LTV cohort

Characteristic	Prevalence (n [%]), or mean (SD)	PGD incidence (n [%])
Female sex	35 (55%)	17 (48%)
Age at transplant	57.9 (14.0)	n/a
Ever primary graft dysfunction (PGD)	27 (42%)	n/a
Ever high-grade PGD (2 or above)	18 (28%)	n/a
Ever ACR	25 (49%)	9 (36%)
Pretransplant diagnosis		
Obstructive disease (Group A)	29 (45%)	6 (21%)
Pulmonary vascular disease (Group B)	3 (5%)	2 (67%)
Cystic fibrosis (Group C)	6 (9%)	2 (33%)
Restrictive disease (Group D)	26 (41%)	16 (62%)

ACR, acute cellular rejection; PGD, primary graft dysfunction; SD, standard deviation.

52 patients had blood samples needed for the assessment of anellovirus rise in the first week posttransplant. Of the 64 patients included in the study, 51 had sufficient data for "ever ACR" assessment. Five of 64 patients died in the first year following their transplant.

Alphatorquevirus and Betatorquevirus Levels in the First Week Following Transplant

Figure 1 shows levels of recipient blood alphatorquevirus 1 day before transplant and 1–2 days after transplant. There is a significant correlation ($P < 0.0001$) between alphatorquevirus levels at these timepoints. Betatorquevirus levels were likewise correlated ($P < 0.0001$, analysis not shown). In Figure 2, the rate of alphatorquevirus rise is expressed as a ratio of 1 week posttransplant, when compared with 1 day before transplant. Patients with "ever-high PGD" (Grade 2+) had a significantly slower rate of rise in alphatorquevirus when compared with patients without high-grade PGD ($P = 0.031$). We generated a logistic regression model to assess effect size and confounding, but when we applied this technique, there was no significant association between alphatorquevirus and PGD. The association between ever-high PGD and betatorquevirus was not significant ($P = 0.64$). There was also no association between alphatorquevirus or betatorquevirus rate of rise and the subsequent development of ACR (alphatorquevirus, $P = 0.12$; betatorquevirus, $P = 0.59$).

Alphatorquevirus Levels in the Subsequent Month Following Transplant

Because our prior study had shown an association between 2 weeks posttransplant blood alphatorquevirus levels and the subsequent development of ACR, we investigated that relationship in this cohort, but there was not a significant association ($P = 0.55$). Since there was a statistical association between immediate posttransplant alphatorquevirus rate of rise and PGD, we evaluated anellovirus expansion over the first 5 weeks following transplant, and evaluated dynamic trends. Patients with prior PGD had a nonsignificant rise in alphatorquevirus level between 1 and 3 weeks posttransplant (Figure 3). Patients with a more rapid rise in alphatorquevirus level between 1 and 5 weeks posttransplant also had a nonsignificant increase in the incidence of "ever ACR" in the first 3 months following transplant (Figure 4).

DISCUSSION

We determined that blood anellovirus levels are associated with PGD in lung transplant recipients. The finding that
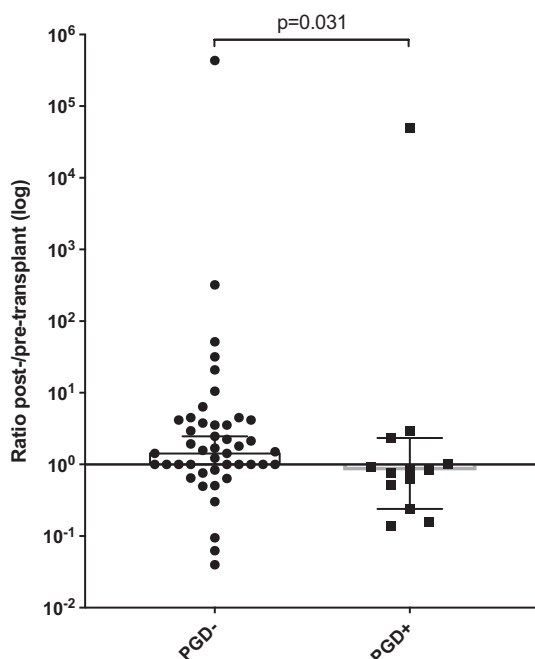

FIGURE 2. Levels of recipient blood alphatorquevirus, expressed as a ratio of 1 week posttransplant, when compared with 1 day before transplant, among patients without and with high-grade PGD (Grade 2+). PGD, primary graft dysfunction.

FIGURE 3. Levels of recipient blood alphatorquevirus, expressed as a ratio of 3 week posttransplant, when compared with 1 week after transplant, among patients without and with PGD. PGD, primary graft dysfunction.

Ratio of 5 vs. 1-wk post-transplant alphatorquevirus, by everACR status
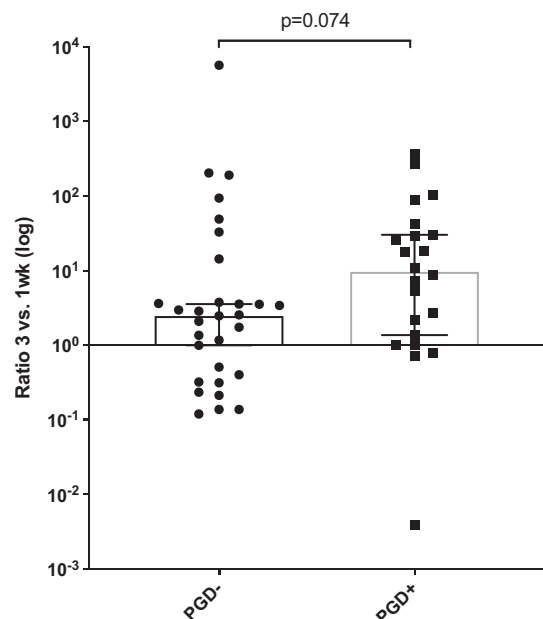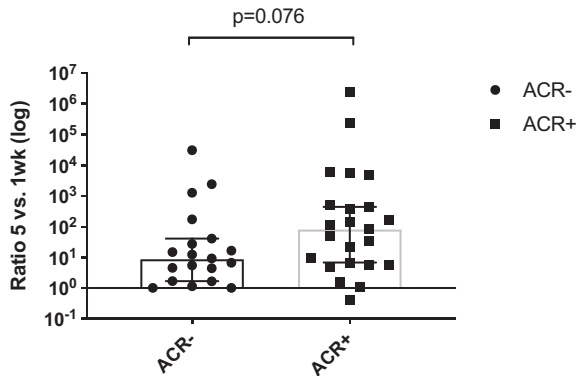

FIGURE 4. Levels of recipient blood alphatorquevirus, expressed as a ratio of 5 week posttransplant, when compared with 1 week after transplant, among patients without and with PGD. ACR, acute cellular rejection; PGD, primary graft dysfunction.

a relatively rapid rise in peritransplant alphatorquevirus is associated with protection from PGD is analogous to existing literature that has found a comparably rapid rise in BAL.[11] The relatively low rate of PGD in patients with obstructive disease has likewise been noted previously.[17] Nevertheless, this study is the first to identify a viral marker in the blood that is associated with the development of PGD in lung transplant recipients. These results need to be interpreted with caution due to small sample size, and because there is not a significant association between PGD and blood anellovirus when a multivariable analysis is applied.

We did not replicate the finding in our pediatric cohort, in whom a blood alphatorquevirus level drawn at 2 weeks following transplant could be used to predict subsequent episodes of ACR over the first 3 months following transplant.[12] While age-specific alterations in the virome are well-described in the human gut,[18] there are little data about age-dependency of the virome in the human lung. Nevertheless, anellovirus prevalence has been established to change with the age of the human host,[8] suggesting that there may be age-dependent changes in the human immune milieu that impact a host's anellovirus population. It is also possible that we did not see this alphatorquevirus association with ACR in this adult cohort due to the small sample size.

While there were not significant associations between early anellovirus levels and the subsequent development of ACR in this adult cohort, we evaluated trends in anellovirus temporal dynamics. The now established lag in anellovirus expansion after transplant,[19] rather than reflecting an uninterpretable flux until a steady state population is established, may itself hold clues as to the immune receptivity of a transplant recipient. An immediate rapid rise in anellovirus after transplant is associated with protection from PGD, while there is a nonsignificant tendency toward subsequent slowing in anellovirus expansion in ACR-free patients. The significant association between rapid anellovirus rise and the protection from PGD did not hold up in a multivariable analysis; however, so further study of anellovirus dynamics in the immediate posttransplant period may be needed.

What is not clear from the present study is whether anelloviruses have a direct effect on peritransplant host immunity, or whether they are reflective of a protectively immunosuppressed

dysbiosis. Alphatorqueviruses encode miRNAs that inhibit interferon response,[20] and some anelloviruses can inhibit IL-6 and IL-8 transcription by interfering with NF-kappaB signaling.[21] Due to the lack of understanding regarding the anellovirus life cycle, it is not yet clear why anellovirus dynamics have associations with key clinical outcomes. Nevertheless, given the importance of toll-like receptor signaling in PGD,[22] it is possible that that anellovirus levels reflect the state of host innate immunity.

There are potentially confounding factors at the time of transplant, such as blood transfusion, that add to the complexity of delineating the role of anellovirus. We were unable to assess effect size and confounding in the current study since multivariable modeling showed no association between anellovirus and PGD. Given that the association present by bivariate analysis was not present in a multivariable analysis, it is possible that a larger cohort will be needed to evaluate posttransplant anellovirus dynamics, or that there are, in fact, confounding variables limiting the applicability of these findings. The conclusions that can be drawn from this study are limited by sample size, and it is possible that a larger cohort would be needed to identify significant trends in anellovirus expansion following transplant. Nevertheless, anellovirus has now been associated with several key outcomes in lung transplant, including PGD,[11] ACR,[12] and chronic lung allograft dysfunction,[23] suggesting that it is a vital biomarker even if it does not impact human health. The development of an animal model for anellovirus infection[24] could help us to interpret host-pathogen dynamics.

ACKNOWLEDGMENTS

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