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RESPIRATORY VIRAL INFECTION IN LUNG-TRANSPLANT INDUCES EXOSOMES THAT TRIGGER CHRONIC-REJECTION

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Abstract

Background: Respiratory viral infections can increase the risk of chronic lung allograft dysfunction after lung transplantation, but the mechanisms are unknown. In this study, we determined whether symptomatic respiratory viral infections in post-lung transplantation induce circulating exosomes that contain lung-associated self-antigens, and to assess whether these exosomes activate immune responses to self-antigens.

Methods: Sera were collected from lung transplant recipients with symptomatic, lower and upper-tract respiratory viral infections and from non-symptomatic stable recipients. Exosomes were isolated via ultracentrifugation; purity was determined using sucrose cushion; presence of lung self-antigens, 20S proteasome, and viral antigens for rhinovirus, coronavirus, and respiratory syncytial virus were determined using immunoblot. Mice were immunized with circulating exosomes from each group and resulting differential immune responses and lung histology were analyzed.

Results: Exosomes containing self-antigens, 20S proteasome, and viral antigens were detected at significantly higher levels ($p<0.05$) in serum of recipients with symptomatic respiratory viral infections (n=35) as compared with stable controls (n=32). Mice immunized with exosomes from recipients with respiratory viral infections developed immune responses to self-antigens, fibrosis, small airway occlusion, and significant cellular infiltration; mice immunized with exosomes from controls did not ($p<0.05$).

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Disclosure statement

The authors have no conflicts of interest to disclose.

Conclusions: Circulating exosomes isolated from lung transplant recipients diagnosed with respiratory viral infections contained lung self-antigens, viral antigens, 20S proteasome and elicited immune responses to lung self-antigens that resulted in development of chronic lung allograft dysfunction in immunized mice.

Introduction

Lung allograft failure from chronic lung allograft dysfunction (CLAD) is the leading cause of death beyond the first year after lung transplant (LTx). Roughly 70% of lung transplant recipients (LTxRs) with CLAD have bronchiolitis obliterans syndrome (BOS)[1] and include both obstructive and restrictive phenotypes.[2] The term restrictive allograft syndrome was introduced by Sato et al[3] which was diagnosed in 30% of bilateral LTx patients with CLAD. The diagnosis was based on finding a restrictive ventilatory defect and had radiographic findings of interstitial opacities with 41% having upper zone involvement. Previously reported risk factors for CLAD include acute rejection[4–6], cytomegalovirus (CMV) pneumonitis[7], antibodies (Abs) to donor HLA[8, 9], Abs to non-HLA lung-associated self-antigens (SAgs)[10–12], primary graft dysfunction[13], and respiratory viral infections (RVI)[14–19].

The immunological mechanisms that underlie the development of CLAD remains unknown, and therapy for established CLAD is generally ineffective. RVI after LTx) has been associated with increased risk of CLAD[15, 17, 18]. Fisher *et al* conducted a large retrospective study that used systematic definitions, adjudicated assignment of CLAD by blinded reviewers, and highly sensitive and specific molecular diagnosis of RVI and found a strong and independent association between symptomatic RVI and CLAD[17]; other studies have also found an association between RVI and CLAD[20–23]. Potential mechanisms for RVI induced CLAD pathogenesis were not assessed. We recently demonstrated that LTxRs with acute and chronic rejection have circulating exosomes that contain donor mismatched HLA, lung SAgs, and immunoregulatory microRNA; exosomes from stable LTxRs do not have these same features[24]. A study by Dieude et al demonstrated that presence of 20S proteasome in exosomes increases their immunogenicity[25]. In this study, we tested the hypothesis that RVI-induced allograft injury may induce circulating exosomes that contain donor HLA, SAgs, and viral antigens, which may activate donor specific immune responses and increase the risk of CLAD.

Methods

Study population

We performed a retrospective case-control study of 35 adult LTxRs diagnosed with symptomatic upper and/or lower tract RVI (“cases”) and 32 adult LTxRs who had no RVI diagnosis (“controls”). Patients were eligible for study if they had undergone LTx at Barnes-Jewish Hospital, Washington University, St. Louis, Missouri, between 2011–2015, or at Norton Thoracic Institute, St. Joseph’s Hospital, Phoenix, Arizona between 2016–2018, and had stored serum available. Baseline patient demographics, transplant details, and laboratory data were collected from patient charts. All patients were followed for at least 6 years, with clinical and laboratory information collected. The endpoint of BOS was diagnosed

according to the guidelines from the International Society for Heart and Lung Transplantation[26].

RVI testing was performed when indicated for compatible signs and symptoms. Only patients with symptomatic RVI were included. RVI was diagnosed using the BioFire FilmArray PCR (bioMérieux, Marcy-l'Étoile, France), which detects 17 types of respiratory viruses, including adenovirus, coronavirus (types HKU1, NL63, 229E, OC43), human metapneumovirus, human rhinovirus/enterovirus, influenza (A, A/H1, A/H3, A/H1–2009, B), parainfluenza 1–4, and respiratory syncytial virus. Both upper (nasopharyngeal swabs) and lower (bronchoalveolar wash or lavage) specimens were included. Patients were considered to have lower tract infection if they had a positive lower tract specimen or upper respiratory specimen along with either lower respiratory symptoms (cough, wheezing) or decline in FEV_1. LTxRs in the control group had no evidence of symptomatic RVI during period of serum collection. Post-transplant immunosuppression comprised a triple immunosuppressive regimen of tacrolimus or cyclosporine, mycophenolate mofetil or azathioprine, and prednisone. This study was approved by the Institutional Review Boards at Washington University and at St. Joseph's Hospital. All laboratory analyses were performed by personnel blinded to clinical outcomes, and all clinical endpoints were adjudicated by personnel who were blinded to laboratory results.

Determination of Abs to lung SAgs by enzyme-linked immunosorbent assay (ELISA)

ELISA was used to analyze serum samples from LTxRs diagnosed with RVI and from stable controls for measuring Abs to two SAgs: Collagen V (Col-V) and K-alpha 1 Tubulin (Kα1T) detailed in our publication[9]. In addition to lung SAgs, we used a kidney-associated SAg, Col-IV (Meridian, A33125H), as a control. Samples were considered positive if the values were greater than the mean+2 standard deviations of the healthy controls' values. Ab concentration was calculated using a standard curve from known concentrations of Col-V and Kα1T Abs (BD Pharmingen 550513, SanJose, CA).

Exosome isolation and validation

Exosomes were isolated from sera of LTxRs with RVI and from stable by ultracentrifugation as previously described[24, 27, 28]. Exosome purity was validated using the sucrose cushion method[24, 29]. The presence of the exosome-specific marker CD9 (312102, Biolegend, SanDiego, CA) and Alix (634502, Biolegend) was assessed using immunoblot.

Determination of lung SAgs, 20S proteasome, and viral antigens using immunoblot

Immunoblot was used to detect SAgs, 20S proteasome, and viral antigens in exosomes from LTxRs diagnosed with RVI and from stable controls. Total exosome protein (3μg) was resolved in polyacrylamide gel electrophoresis, and the proteins were transferred into a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat milk in 1X PBS and was probed with exosome-specific marker CD9 (312102, Biolegend, SanDiego, CA), Col-V (ab7046, Abcam), and Kα1T (sc-12462-R, SantaCruz Biotechnology, Dallas, TX). 20S proteasome subunit α3 (sc-58414, SantaCruz Biotechnology), rhinovirus VP3 (MA5–18249, Thermo Fisher Scientific, Waltham, MA), coronavirus (NB100–64754, Novus Biologicals, Littleton, CO), RSV Glycoprotein G (7950–0980, Bio-Rad Laboratories,

Hercules, CA) were used as primary Abs; secondary Abs conjugated with HRP were used specific to primary Ab. The blots were washed with PBS Tween (Thermo Fisher Scientific), developed using chemiluminescent HRP substrate (WBKLS0500, MilliporeSigma), and exposed using Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln, NE). The band intensity of target protein was quantified using ImageJ software and normalized with CD9.

Immunization of C57BL/6 mice with exosomes from LTxRs diagnosed with RVI and stable

Exosomes (10μg/100μl) isolated from LTxRs with RVI or from stable controls were used for immunization of C57BL/6 mice (days 1,7,18, 25). Prior experiments have demonstrated that injury to the native lungs is required for Abs to lung SAgs to cause lesions[30]. Therefore, 0.1M HCL was administered intra-bronchially on both groups on day 0 prior to immunization with exosomes. Sera collected on days 10 and 30 were used to detect Abs to Col-V and Kα1T by ELISA. On day 30, the mice were sacrificed and splenocytes isolated to enumerate SAg-specific cytokines producing cells by enzyme-linked ImmunoSpot assay (ELISpot).

Detection of Abs to lung SAgs in sera from mice using ELISA.

Sera from mice immunized with exosomes of LTxRs diagnosed with RVI and from stable were used to measure Abs against Col-V and Kα1T using ELISA as described previously[24, 30]. To detect murine Abs, we used goat-anti-mouse conjugated with HRP (1:10000) as secondary Ab. The plates were developed with chemiluminescent reagent and the reactions were stopped with 0.1N hydrochloric acid. The optical density of each well was measured at a wavelength of 420nm. Serum concentration of Abs to lung SAg was calculated using the standard curve obtained with known concentration of Abs to SAgs. Samples were considered positive if the values were greater than the mean+2 standard deviations of the healthy controls' values.

ELISpot

Splenocytes were isolated from mice immunized with exosomes of LTxRs diagnosed with RVI and of stable. ELISpot was performed as described previously[31]. Cytokine-producing cells were analyzed and the spots were enumerated and subtracted from experimental control wells and reported as spots per million.

Histopathological and morphometric analysis of lungs from mice immunized with exosomes

Lungs from mice immunized with exosomes from LTxRs with RVI and from stable LTxR were histologically analyzed to detect lesions and cellular infiltration by H&E and trichrome staining, as described[31]. Lungs were fixed in 10% formaldehyde and embedded in paraffin blocks. 4–5μm-thick sections were cut and mounted on slides (Leica, Wetzlar, Germany) for H&E and trichrome staining. Images were obtained on a Leica microscope at 40X and morphometric analysis was performed using Aperio ImageScope software (Leica). Five different areas were examined for fibroproliferation, epithelial abnormalities, and cellular infiltration.

Morphometric analysis

Slides were scanned and whole slide images were analyzed using Aperio Image Scope (https://www.leicabiosystems.com/digital-pathology/manage/aperio-imagescope/) and ImageJ software (https://imagej.nih.gov/ij/). For analysis of infiltrates, manual annotation of areas with prominent as well as mild/no infiltrate was performed on whole slide images and the fraction of the total tissue area with prominent infiltrate was determined using Image Scope. For evaluation of fibrosis, whole slide images of lung sections stained with trichrome stain were exported as TIFF files. Color deconvolution of the TIFF files was performed in ImageJ using a color deconvolution plugin (https://imagej.net/Colour_Deconvolution). The extent of blue-staining collagenous fibrosis was then determined using standard tools available in the ImageJ suite.

Statistical Analysis

Data analysis was performed using Prism 6 software from GraphPad Inc. The Ab levels for lung SAgs, optical density of exosomes containing lung SAgs, and viral antigens between RVI LTxRs and controls were compared using Mann-Whitney or two-tailed Student's t-test, as indicated. Statistical data in each cohort was expressed as mean ± standard error. P-values less than 0.05 were considered statistically significant in each comparative analysis. The mean optical density of exosomes containing lung SAgs and viral antigens was calculated after normalization with exosome-specific marker CD9 and comparative analysis was performed using Mann-Whitney U test.

Results

Patient demographics

Patient demographics, age, gender, ethnicity, and HLA-mismatch status were not significantly different between groups (Table 1). Acute cellular rejection (ACR) occurred after RVI in 2 (A1, ACR) patients and in 1 (A1, ACR) stable LTxR. Acute antibody-mediated rejection occurred in 5 patients diagnosed with RVI and in none of the stable LTxRs. Donor-specific antibodies (DSA) developed during follow-up in 8/35 (23%) LTxRs with symptomatic RVI and in 4 of 32 (12.5%) stable LTxRs.

Sera from LTxRs diagnosed with symptomatic RVI demonstrated significantly elevated Ab responses to Col-V and Kα1T

Sera collected from patients diagnosed with RVI demonstrated significantly increased Ab titers to SAgs compared with stable LTxRs (Col-V: mean 54.9±15.1 vs 78.3±25.1, $p=0.0169$; Kα1T: 43.3±17.2 vs 74.7±20.6, $p=0.0145$; Figure 1). Both LTxRs with RVI and controls had similar but low levels of Abs to the control SAg, Col-IV.

Exosomes isolated from LTxRs diagnosed with RVI contain lung SAgs

Circulating exosomes in sera of both groups were found to contain exosome markers CD9 and Alix. Western blot using Abs to SAgs demonstrated that the exosomes isolated from sera samples from LTxRs with RVI contained significantly higher concentrations of Col-V (mean optical density: LTxR with RVI 1.9+0.2 vs stable LTxR 0.73±0.09, $p=0.0003$) and

Kα1T (LTxR with RVI 4.06±1.09 vs stable LTxR 0.83±0.31, *p=0.009*). Neither cohort had exosomes that contained the control kidney SAg, Col-IV (Figure 2).

Viral antigens are detectable in exosomes isolated from LTxRs diagnosed with RVI

Respiratory syncytial virus (RSV)—Exosomes from patients diagnosed with RSV were analyzed for the presence of SAgs and RSV glycoprotein G by immunoblot. 4/10 patients with RSV infection showed viral antigens; no stable LTxRs had viral antigens (Figure 3A). Furthermore, significantly increased levels of SAgs and RSV antigens (mean optical intensity: Col-V, 1.8±0.5 vs 0.5±0.1, *p=0.037*; Kα1T, 1.1±0.2 vs 0.5±0.1, *p=0.047*; RSV, 6.3±2.1 vs 1.2±0.3, *p=0.033*) were demonstrated in LTxRs diagnosed with RSV compared with stable LTxR (Figure 3 B).

Coronavirus—Immunoblot results showed that coronavirus antigens were detected in exosomes of 5/12 patients diagnosed with coronavirus compared with 0/10 stable LTxRs (Figure 3A). Levels of SAgs (mean optical intensity: Col-V, 1.37±0.19 vs 0.7±0.14, *p=0.015*; Kα1T, 1.2±0.25 vs 0.21±0.08, *p=0.003*; coronavirus, 3.78±1.05 vs 0.83±0.27, *p=0.0217*) were significantly higher in exosomes from LTxRs with coronavirus than in stable LTxR (Figure 3 B).

Rhinovirus—Twelve patients with rhinovirus infection and 10 stable LTxRs were selected to detect exosomes containing SAgs and rhinovirus antigens. Patients diagnosed with rhinovirus (6/10) showed rhinovirus antigens, but stable LTxRs did not (Figure 3 A). The mean optical density of exosomes containing SAgs (mean optical intensity: Col-V, 2.54±0.6 vs 0.92±0.2, *p=0.028*; Kα1T, 9.32±2.4 vs 1.78±0.86, *p=0.015*; rhinovirus, 5.35±1.63 vs 1.14±0.16, *p=0.030*) was significantly higher in exosomes isolated from LTxRs diagnosed with rhinovirus patients in compared to stable LTxR (Figure 3 B).

20S proteasome subunit α3 is detectable in exosomes from LTxRs with RVI

To determine 20S proteasome in exosomes isolated from stable LTxRs (n=4) and LTxRs diagnosed with RVI (n=5), we performed immunoblot using Abs to the α3 subunit of 20S proteasome. We found significantly higher levels of 20S proteasome α3 subunit in exosomes isolated from LTxRs diagnosed with RVI compared with stable LTxRs (mean optical density: LTxRs with RVI vs stable LTxRs; 1.74±0.6 vs 0.37±0.35, *p=0.0317*). Alix served as an exosome specific marker and loading control (Figure 4).

Exosomes containing lung SAgs induce Abs to lung SAgs in mouse model

Sera collected on days 10 and 30 were used to measure Abs to SAgs in mice immunized with pooled exosomes isolated from LTxRs diagnosed with RVI and from stable LTxRs. Abs to SAgs in sera collected on day 10 following immunization with the exosomes derived from RVI and stable LTxRs were not significantly different (Col-V, 18.83±6.4 vs 5.50±3.35, p=0.102; Kα1T, 120.2±39.1 vs 62.8±37.9, *p=0.323*). Serum collected on day 30 showed increased Abs to Col-V and Kα1T in mice immunized with exosomes from LTxRs diagnosed with RVI compared to stable LTxRs (Col-V, 45.9±6.5 vs 28.1±4.0, *p=0.04*; Kα1T, 604.6±140 vs 230.4±77.1, *p=0.04*; Figure 5).

Cellular immune response to exosomes of LTxRs diagnosed with RVI and stable LTxRs in a mouse model

Splenocytes isolated from mice immunized with exosomes from LTxRs with RVI and from stable LTxRs were used to enumerate the cytokine-producing cells against lung SAgs. Mice immunized with exosomes from LTxRs diagnosed with RVI had significantly increased SAg-specific IFN-γ-producing cells (Col-V: 359.3±154 vs 24.2±24, *p=0.002*; Kα1T: 696.7±202 vs 140±155, *p=0.004*) and IL-17-producing cells (Col-V: 293.3±179 vs 11±0, *p=0.010*; Kα1T: 403.3±310 vs 22±17, *p=0.002*) than mice immunized with exosomes from stable LTxRs. In contrast, IL-10-producing cells (Col-V: 36.7±83 vs 217.8±55, *p=0.010*; Kα1T: 80.7±17 vs 233.2±111, *p=0.036*) were significantly reduced in mice immunized with exosomes from LTxRs diagnosed with RVI than in those immunized with exosomes from stable LTxRs (Figure 6).

Exosomes from LTxRs diagnosed with RVI induce fibrosis in mouse model

Lungs harvested from mice immunized with exosomes from LTxRs with RVI and stable LTxRs were subjected to histopathological analysis. Mice immunized with exosomes from LTxRs diagnosed with RVI showed inflammatory cells in bronchioles and vessels. Notably, lesions involving bronchioles, cellular infiltration, and increased fibrosis were also observed (Figure 7). In contrast, no significant differences in cellular infiltration and lesions were evident in the mice immunized with exosomes from stable LTxRs (Figure 7A). These results demonstrate that circulating exosomes from LTxRs diagnosed with RVI induced cellular infiltration and alveolar lesions in the lungs of mice. Furthermore, histopathological analysis demonstrates interstitial fibrosis, which after human lung transplant is similar to the pathology seen in restrictive allograft syndrome. The morphometric data (Figure 7B) are given for the representative images.

Discussion

Studies have demonstrated an association between RVI and CLAD[15–18, 23]. Fisher *et al* applied molecular diagnostic methods to test for RVI in a large cohort of LTxRs. They not only found high rates of RVI, but also demonstrated an independent association between RVI and CLAD[17] and suggested further study to characterize the viral determinants and to define the mechanisms by which RVI increases the risk for CLAD. RVI after LTx has been shown to dysregulate the Treg cells, indicating that RVI can lead to dysregulation of tolerance to SAgs, leading to induction of immune responses to SAgs and increasing the risk of CLAD[14]. Studies by our group and others showed that Abs to lung SAgs have been shown to develop and correlate with development of CLAD in LTxRs[11, 12, 32]. Pre-existing Abs to SAgs have also been reported to increase the incidence of primary graft dysfunction, to induce pro-inflammatory cytokines, and to increase development of DSA and CLAD in post-LTx[32].

We recently demonstrated that LTxRs diagnosed with acute and chronic rejection have circulating exosomes that express mismatched donor HLA and SAgs. We proposed that the exosomes originating from transplanted lungs may contribute to the immune-pathogenesis of CLAD after LTx[24]. Based on these findings, we postulated that symptomatic RVI may

induce exosomes containing SAgs from the transplanted organ, and that persistence of circulating exosomes with SAgs can lead to immune responses resulting in increased risk of CLAD.

In this study, we determined the development of Abs to SAgs in LTxRs diagnosed with RVI. Our results, presented in Figure 1, demonstrated that Ab titers to SAgs were significantly higher in patients diagnosed with RVI than in stable LTxR. This demonstrates that RVI can induce a humoral immune response to SAgs. Circulating exosomes isolated from LTxRs diagnosed with BOS express mismatched donor HLA and SAgs (Col-V and Kα1T) confirming their source as the lung allograft and suggesting that exosomes can induce immune responses to alloantigens and SAgs, increasing the risk for CLAD[24]. Walker *et al* demonstrated that exosomes released from CMV-infected lung endothelial cells of LTxRs induces CD4 T cells responses to CMV antigens[33]. Furthermore, a human nasopharyngeal cell line transfected with Epstein-barr virus (EBV) has been shown to release exosomes containing viral peptide latent membrane protein 1 (LMP1) and FGF-2[34]. The exosomes isolated from EBV-transformed B cells contain EBV viral antigen glycoprotein 350, which can specifically bind to B cells[35]. These findings support our hypothesis that RVI have the potential to induce exosomes containing lung SAgs and viral antigens from the transplant recipient with RVI.

Our results demonstrate that circulating exosomes isolated from patients diagnosed with symptomatic RVI not only had SAgs, but also had viral antigens. In this study, we selected patients diagnosed with RSV, coronavirus, and rhinovirus and demonstrated that exosomes isolated from these patients contained specific viral antigens along with SAgs. Therefore, exosomes are induced following viral infection which contain viral and SAgs. Preliminary analysis of serial circulating exosomes containing viral antigens demonstrated that in 3/5 LTxRs there were transient presence of circulating exosomes. In contrast, 2/5 RVI LTxRs had persistence of circulating exosomes with lung SAgs and viral antigens. This interesting finding needs to be confirmed to determine the role of circulating exosomes in inducing immune responses leading to CLAD. It is likely that the exosomes with viral antigenic epitopes can activate cross-reactive T cells, which can play a role in the pathogenesis of CLAD after LTx[36, 37]. We demonstrated that exosomes isolated from symptomatic RVI patients contained increased SAgs and viral antigens; if further studies identify a useful threshold concentration, this could potentially serve as a biomarker for CLAD.

A study by Dieudé *et al* demonstrated that exosomes isolated from endothelial cells contained 20S proteasome, and therefore increased the immunogenic potential to the kidney-associated SAg, perlecan. Intravenous injection of exosomes in C57BL/6 mice led to humoral immune responses to perlecan, suggesting that presence of kidney SAgs, along with 20S proteasome, increases the immunogenicity of the exosomes[25]. In order to demonstrate that exosomes containing SAgs along with viral antigens can be immunogenic, we isolated exosomes from LTxRs with RVI, stable LTxRs and immunized into C57BL/6 mice. Mice immunized with exosomes from RVI developed increased levels of Abs to SAgs than mice immunized with exosomes from stable LTxRs. Additionally, mice immunized with exosomes from RVI LTxRs showed increased IFN-γ- and IL-17-producing cells and reduced IL-10-producing cells compared to mice injected with exosomes from stable LTxRs.

These results confirm that exosomes containing SAgs, viral antigens, and 20S proteasome are immunogenic and can induce Abs to SAgs and alter T-cell cytokine responses which can lead to CLAD.

Our study is limited in that exosomes isolated from patients diagnosed with RVI were not analyzed in mice models of obliterative airway diseases following lung transplantation. Therefore, we cannot definitively conclude that exosomes from RVI patients can increase the incidence of CLAD development. The sample size used in the mouse model were small and we have used pooled exosomes for immunization. Therefore, role of individual viruses in inducing exosomes which are immunogenic can't be concluded from the studies presented. We have shown that sera collected from lung LTxRs with RVI had increased Abs to lung SAgs and exosomes containing lung SAgs and viral antigens compared to stable LTxRs. However, viral RNA in the exosomes and its role in immune activation needs to be determined in future studies. Another limitation is that the role of individual RVI viruses to induce exosomes which can increase the risk for CLAD are not determined due to limited availability of retrospectively collected samples. Our study however demonstrated that exosomes derived from RVI LTxRs induced interstitial fibrosis and inflammatory cell infiltration by adoptive transfer of exosomes (gain of function) in mice model which suggest that RVI exosomes are sufficient to induce lesion in mice with similarities to the pathology seen in restrictive allograft syndrome in human lung transplant recipients.

We further demonstrated increased humoral and cellular immune responses to lung SAgs in mice immunized with exosomes from RVI LTxRs compared to mice immunized with exosomes from stable LTxRs. Based on these, we proposed that RVI induced exosomes containing lung SAgs and viral antigens can augment humoral and cellular immune responses to lung SAgs and alloantigens, increasing the risk of CLAD. These results strongly suggest a biologically plausible mechanistic link between RVI, induction and release of circulating exosomes with SAgs, to the development of CLAD which should be assessed in a large prospective cohort.

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References

1. Arcasoy SM, Kotloff RM Lung Transplantation. New Eng J Med 1999;340:1081–91. [PubMed: 10194239]
2. Glanville AR. Bronchoscopic monitoring after lung transplantation. Semin Respir Crit Care Med 2010;31:208–21. [PubMed: 20354933]
3. Sato M, Waddell TK, Wagnetz U, et al. Restrictive allograft syndrome (RAS): a novel form of chronic lung allograft dysfunction. J Heart Lung Transplant 2011;30:735–42. [PubMed: 21419659]
4. Witt CA, Gaut JP, Yusen RD, et al. Acute antibody-mediated rejection after lung transplantation. J Heart Lung Transplant 2013;32:1034–40. [PubMed: 23953920]

5. Stewart S, Fishbein MC, Snell GI, et al. Revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection. J Heart Lung Transplant 2007;26:1229–42. [PubMed: 18096473]
6. Arcasoy SM, Berry G, Marboe CC, et al. Pathologic interpretation of transbronchial biopsy for acute rejection of lung allograft is highly variable. Am J Transplant 2011;11:320–8. [PubMed: 21219569]
7. Almaghrabi RS, Omrani AS, and Memish ZA. Cytomegalovirus infection in lung transplant recipients. Expert Rev Respir Med 2017;11:377–83. [PubMed: 28388307]
8. Chalermskulrat W, Neuringer IP, Schmitz JL, et al. Human leukocyte antigen mismatches predispose to the severity of bronchiolitis obliterans syndrome after lung transplantation. Chest 2003;123 1825–31. [PubMed: 12796156]
9. Saini D, Weber J, Ramachandran S, et al. Alloimmunity-induced autoimmunity as a potential mechanism in the pathogenesis of chronic rejection of human lung allografts. J Heart Lung Transplant 2011;30:624–31. [PubMed: 21414808]
10. Bharat A, Saini D, Steward N, et al. Antibodies to self-antigens predispose to primary lung allograft dysfunction and chronic rejection. Ann Thorac Surg 2010;90:1094–101. [PubMed: 20868794]
11. Goers TA, Ramachandran S, Aloush A, et al. De novo production of K-alpha1 tubulin-specific antibodies: role in chronic lung allograft rejection. J Immunol 2008;180:4487–94. [PubMed: 18354170]
12. Hachem RR, Tiriveedhi V, Patterson GA, et al. Antibodies to K-alpha 1 tubulin and collagen V are associated with chronic rejection after lung transplantation. Am J Transplant 2012;12:2164–71. [PubMed: 22568593]
13. Daud SA, Yusen RD, Meyers BF, et al. Impact of immediate primary lung allograft dysfunction on bronchiolitis obliterans syndrome. American journal of respiratory and critical care medicine 2007;175:507–13. [PubMed: 17158279]
14. Bharat A, Kuo E, Saini D, et al. Respiratory virus-induced dysregulation of T-regulatory cells leads to chronic rejection. Ann Thorac Surg 2010;90:1637–44; discussion 44. [PubMed: 20971279]
15. Billings JL, Hertz MI, Savik K, et al. Respiratory viruses and chronic rejection in lung transplant recipients. The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation 2002;21:559–66.
16. Fisher CE, Mohanakumar T, and Limaye AP. Respiratory virus infections and chronic lung allograft dysfunction: Assessment of virology determinants. J Heart Lung Transplant 2016;35:946–7. [PubMed: 27235268]
17. Fisher CE, Preiksaitis CM, Lease ED, et al. Symptomatic Respiratory Virus Infection and Chronic Lung Allograft Dysfunction. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2016;62:313–9. [PubMed: 26565010]
18. Khalifah AP, Hachem RR, Chakinala MM, et al. Respiratory viral infections are a distinct risk for bronchiolitis obliterans syndrome and death. American journal of respiratory and critical care medicine 2004;170:181–7. [PubMed: 15130908]
19. Magnusson J, Westin J, Andersson LM, et al. The impact of viral respiratory tract infections on long-term morbidity and mortality following lung transplantation: a retrospective cohort study using a multiplex PCR panel. Transplantation 2013;95:383–8. [PubMed: 23444472]
20. Khalifah AP, Hachem RR, Chakinala MM, et al. Respiratory viral infections are a distinct risk for bronchiolitis obliterans syndrome and death. Am J Resp Crit Care Med 2004;170 181–7. [PubMed: 15130908]
21. Magnusson J, Westin J, Andersson LM, Brittain-Long R, Riise GC The impact of viral respiratory tract infections on long-term morbidity and mortality following lung transplantation: a retrospective cohort study using a multiplex PCR panel. Transplantation 2013;95(2):383–8. [PubMed: 23444472]
22. Billings JL, Hertz MI, Savik K, et al. Respiratory viruses and chronic rejection in lung transplant recipients. J Heart and Lung Transplantation 2002;21:559–66.
23. Bridges ND, Spray TL, Collins MH, et al. Adenovirus infection in the lung results in graft failure after lung transplantation. J Thorac Cardiovasc Surg 1998;116:617–23. [PubMed: 9766590]

24. Gunasekaran M, Xu Z, Nayak DK, et al. Donor-Derived Exosomes With Lung Self-Antigens in Human Lung Allograft Rejection. Am J Transplant 2017;17:474–84. [PubMed: 27278097]
25. Dieude M, Bell C, Turgeon J, et al. The 20S proteasome core, active within apoptotic exosome-like vesicles, induces autoantibody production and accelerates rejection. Science translational medicine 2015;7:318ra200.
26. Verleden GM, Raghu G, Meyer KC, et al. A new classification system for chronic lung allograft dysfunction. J Heart Lung Transplant 2014;33:127–33. [PubMed: 24374027]
27. Gunasekaran M, Sharma M, Hachem R, Bremner R, Smith MA, Mohanakumar T Circulating exosomes with distinct properties during chronic lung allograft rejection. J Immunol 2018;200(8):2535–41. [PubMed: 29491008]
28. Sharma M, Liu W, Perincheri S, Gunasekaran M, Mohanakumar T Exosomes expressing the self-antigens myosin and vimentin play an important role in syngeneic cardiac transplant rejection induced by antibodies to cardiac myosin. Amer J Transplant 2018;18:1626–35. [PubMed: 29316217]
29. Thery C, Amigorena S, Raposo G, et al. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 2006; Chapter 3: Unit 3 22.
30. Subramanian V, Ramachandran S, Banan B, et al. Immune response to tissue-restricted self-antigens induces airway inflammation and fibrosis following murine lung transplantation. Am J Transplant 2014;14:2359–66. [PubMed: 25220332]
31. Fukami N, Ramachandran S, Saini D, et al. Antibodies to MHC class I induce autoimmunity: role in the pathogenesis of chronic rejection. J Immunol 2009;182:309–18. [PubMed: 19109162]
32. Tiriveedhi V, Gautam B, Sarma NJ, et al. Pre-transplant antibodies to Kalpha1 tubulin and collagen-V in lung transplantation: clinical correlations. J Heart Lung Transplant 2013;32:807–14. [PubMed: 23856218]
33. Walker JD, Maier CL, and Pober JS. Cytomegalovirus-infected human endothelial cells can stimulate allogeneic CD4+ memory T cells by releasing antigenic exosomes. Journal of immunology 2009;182:1548–59.
34. Ceccarelli S, Visco V, Raffa S, et al. Epstein-Barr virus latent membrane protein 1 promotes concentration in multivesicular bodies of fibroblast growth factor 2 and its release through exosomes. International journal of cancer 2007;121:1494–506. [PubMed: 17546597]
35. Vallhov H, Gutzeit C, Johansson SM, et al. Exosomes containing glycoprotein 350 released by EBV-transformed B cells selectively target B cells through CD21 and block EBV infection in vitro. Journal of immunology 2011;186:73–82.
36. Fleming A, Sampey G, Chung M, Bailey C, van Hoek ML, Kashanchi F, Hakami RM The carrying pigeons of the cell: exosomes and their role in infectious diseases caused by human pathogens. Pathogens and Disease 2014;71:109–20. [PubMed: 24449527]
37. Yao Z, Qiao Y, Li X, Chen J, Ding J, Bai L, Shen F, Shi B, Liu J, Peng L, Li J, Yuan Z Exosomes exploit the virus entry machinery and pathway to transmit IFN-alpha-induced antiviral activity. J Virol 2018.

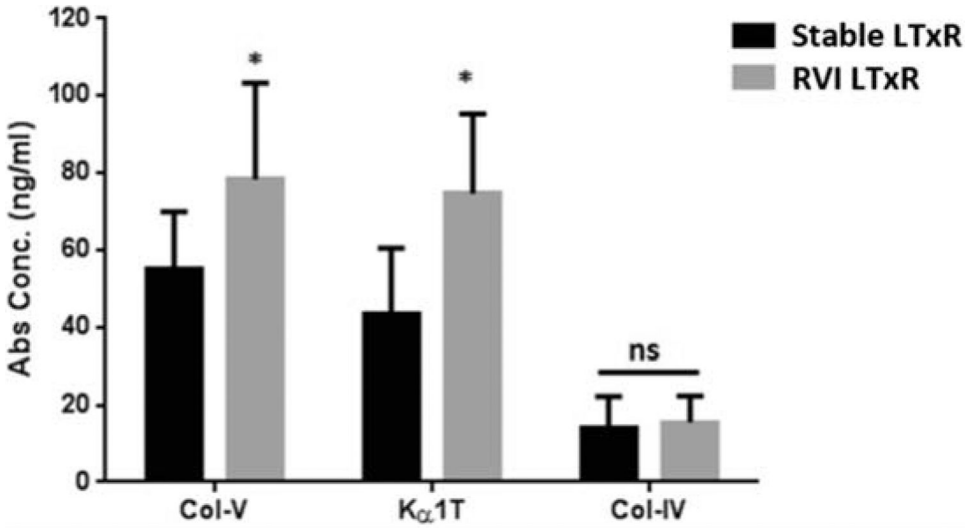

Figure 1: Abs to lung-associated SAgs in LTxRs diagnosed with RVI and stable LTxRs:

Sera collected from LTxRs diagnosed with RVI (n=35) and from stable LTxRs (n=32) were used to measure Abs to lung-associated SAgs (Col-V and Kα1T). Abs to Col-V (78.3±25.1 vs 54.9±15.1, *p=0.0169*) and Kα1T (74.7±20.6 vs 43.3±17.2, *p=0.0145*) were significantly higher in RVI LTxRs compared with stable LTxRs. Further, Abs to kidney associated antigen Col-IV were not significantly different between stable LTxRs and LTxRs with RVI (14.2±8 vs 15.6±6.7, *p=0.248)*. The antibody development to lung SAgs compared between the stable and RVI LTxR using Mann-Whitney test.

RVI, respiratory viral infection; LTxRs, lung transplant recipients; SAgs, self-antigens; Col-IV, Collagen-IV; Col-V, Collagen-V; Kα1T, K alpha 1 tubulin; *, statistically significant.

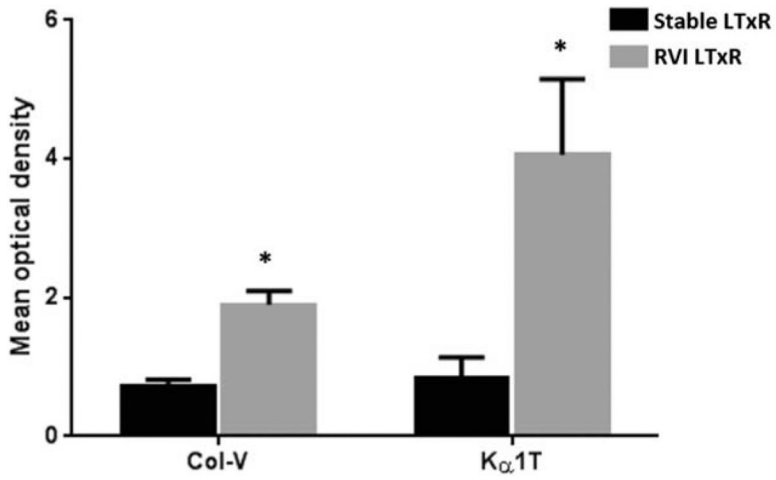

Figure 2: Exosomes containing lung-associated SAgs in LTxRs diagnosed with RVIs and stable LTxRs:

Exosomes isolated from sera of LTxRs with RVI (n=34) and stable LTxR (n=30) were analyzed for the presence of lung-associated SAgs (Col-V and Kα1T) by immunoblot. The mean relative optical densities of Col-V (1.9±0.2 vs 0.73±0.09, *p=0.0003*) and Kα1T (4.06±1.09 vs 0.83±0.31, *p=0.009*) were significantly higher in RVI LTxRs than stable LTxRs. Optical density was measured using ImageJ software and the OD value of SAgs were calculated in RVI LTxRs and stable LTxRs after normalization with CD9 OD value. CD9 also served as loading control and exosome-specific markers. The lung SAgs presence in the exosomes compared between the cohorts using Mann-Whitney test.

RVI, respiratory viral infection; SAgs, self-antigens; Col-V, collagen-V; Kα1T, k alpha 1 tubulin; LTxR, lung transplant recipients; OD, optical density: *, statistically significant.

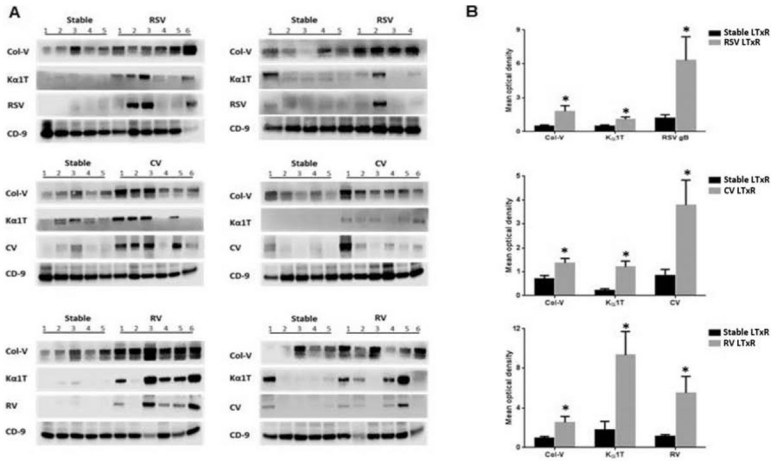

Figure 3: Lung-associated SAgs and viral antigens were demonstrable in exosomes isolated from RVI patients:

Exosomes isolated from sera of RVI patients and from stable LTxRs were used to detect the presence of lung-associated SAgs and viral antigens using immunoblot. The results showed a significant increase in lung-associated antigens and viral antigens RSV (n=10), coronavirus (n=12), and rhinovirus (n=12) in respective viral infection patients compared to stable LTxRs (n=30) (A). Graphical representation shows the optical density of lung-associated SAgs and viral antigens measured in RVI and stable LTxRs using ImageJ software (B). Optical density of lung SAgs and viral antigens were normalized with exosomes specific marker CD9. The lung SAgs and viral antigens presence in the exosomes compared between the cohorts using Mann-Whitney test.

RVI, respiratory viral infection; RSV, respiratory syncytial virus; CV, coronavirus; RV, rhinovirus; Col-V, Collagen-V; Kα1T, K alpha 1 tubulin; *, statistically significant.

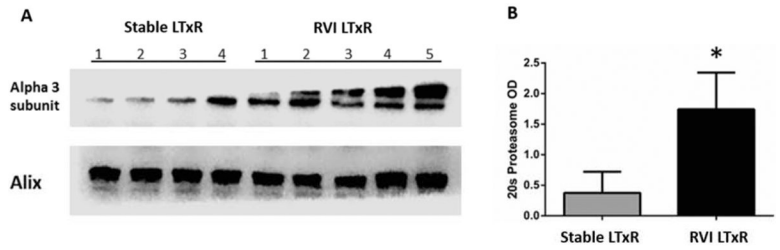

Figure 4: Exosomes containing 20S proteasome core in LTxRs with RVIs and stable LTxRs:
Circulatory exosomes isolated from LTxRs with RVI (n=5) and stable LTxRs (n=4) were used to detect the presence of 20S proteasome subunit α3 using immunoblot. The exosomes isolated from patients with RVI showed a significant increase in 20S proteasome compared with exosomes from stable LTxRs (Mean optical density: 1.74± 0.6 vs 0.37±0.35, $p=0.0317$) (A). Alix served as loading control and exosomes specific marker. Graphical representation shows optical intensity of 20S proteasome α3 subunit abundance in LTxRs with viral infection and stable LTxRs (B). The presence of 20S proteasome was compared between stable and RVI LTxR using student T test.
RVI, respiratory viral infection; LTxR, lung transplant recipients.

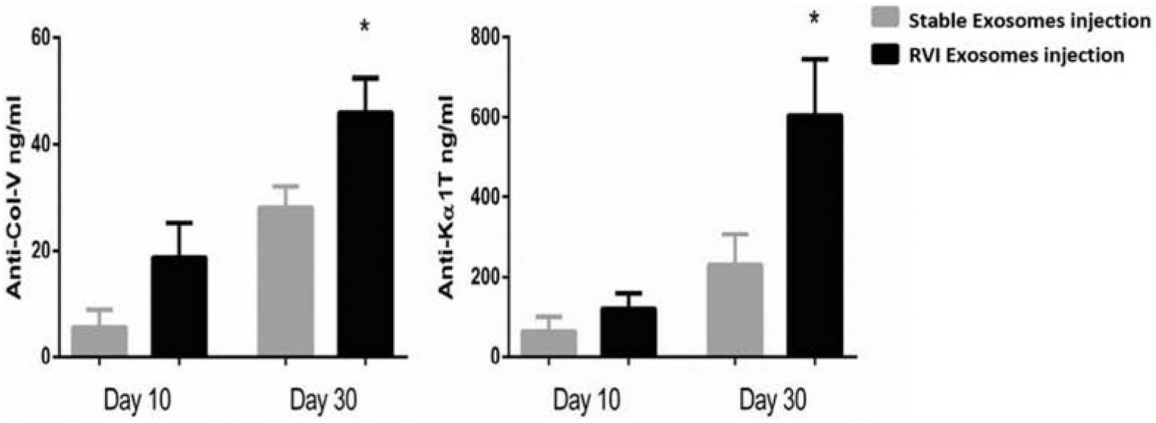

Figure 5: Exosomes from LTxRs with RVIs induce a humoral immune response to lung SAgs:
Sera collected on days 10 and 30 from C57BL/6 mice immunized with exosomes isolated from LTxRs with RVI (n=5) and from stable LTxRs (n=5) were utilized to measure Abs to lung SAgs by ELISA. Sera collected on day 30 from mice immunized with exosomes from LTxRs with RVI showed significantly increased Abs to SAgs (Col-V, 28.1±4.0 vs 45.9±6.5, p=0.04; Kα1T, 230.4±77.1 vs 604.6±140, p=0.04) stable LTxRs exosome injected mice. The antibody development was compared between the cohorts using student T test.
RVI, respiratory viral infection; SAgs, self-antigens; Col-V, collagen-V; Kα1T, k alpha 1 tubulin; *, statistically significant.

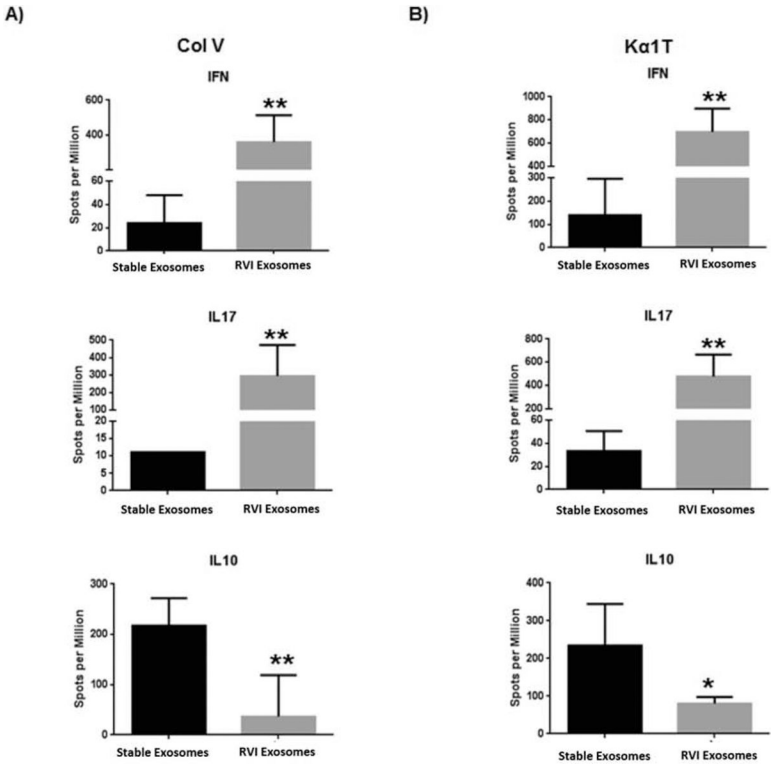

Figure 6: Exosomes from LTxRs with RVIs induce cytokines producing T cells to lung SAgs:
Spleen were collected on day 30 from C57BL/6 mice immunized with exosomes of LTxRs with RVI (n=5) and from stable LTxRs (n=5) were used to measure cytokine producing T cells against lung SAgs by ELISPOT. Mice immunized with exosomes of RVI showed significant increase in T cells producing IL-17 and IFN-γ to SAgs. Mice injected with exosomes isolated from stable LTxRs showed increased frequency of IL-10-producing T cells compared to mice immunized with RVI exosomes. The cytokine levels were compared between the cohorts using Mann-Whitney test.
RVI, respiratory viral infection; SAgs, self-antigens; ** statistically significant.

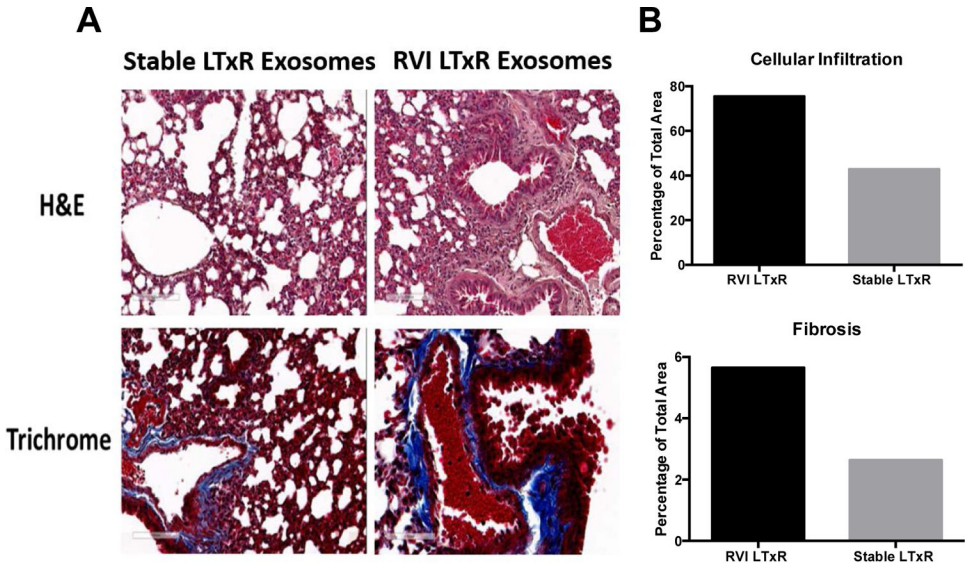

Figure 7: Fibrosis and cellular infiltration were demonstrable in mice injected with exosomes isolated from LTxRs with RVIs:

Mice were sacrificed on day 30 and their lungs were collected and analyzed using hematoxylin and eosin and trichrome staining. Interstitial and inflammatory infiltrates and fibrosis was more prominent in mice injected with LTxRs with RVI compared to mice injected with exosomes from stable LTxRs (A). Images were obtained on a Leica microscope at 40X and morphometric analysis was performed using Aperio ImageScope software (Leica). The morphometric data (B) was given for the representative images. LTxRs, lung transplant recipients; RVIs, respiratory viral infections

Table 1:

Patient Demographics

Variable	LTxRs with RVI (n=35)	Stable LTxRs (n=32)
Mean recipient age at transplant, years	58.6±12.8	63.5±6.4
Recipient gender (male/female)	19/16	25/7
Race, n (%)		
White	27 (77)	30 (94)
African American	1 (3)	0 (0)
American Indian	1 (3)	2 (6)
Hispanic	6 (17)	0 (0)
Cause of ESLD, n (%)		
COPD	14 (40)	14 (44)
IPF	5 (14)	10 (31)
CF	3 (9)	1 (3)
Pulmonary Fibrosis	5 (14)	6 (19)
Others	8 (23)	1 (3)
HLA mismatch		
A	1.8±0.4	1.7±0.5
B	1.8±0.4	1.8±0.4
C	1.7±0.5	1.6±0.6
Prior acute rejection, n (%)		
ACR	2 (8)	1 (3)
AMR	5 (21)	0
Prior chronic rejection, n (%)		
BOS	2 (8)	NA
Infection, n (%)		
RSV	10 (29)	0
Rhinovirus	12 (34)	0
Coronavirus	12 (34)	0
Parainfluenza	1 (3)	0
Mean sample collection, years (p<0.05)	1.7±1.6	3.4±1.4

*Values presented as n (%), unless otherwise noted. Abbreviations: ESLD, end-stage lung disease; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; BOS, bronchiolitis obliterans syndrome; CF, cystic fibrosis; AMR, antibody-mediated rejection; ACR, acute cellular rejection; RSV, respiratory syncytial virus; LTxRs, lung transplant recipients; N/A, not applicable.