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ORIGINAL CLINICAL SCIENCE

Circulating exosomes with lung self-antigens as a biomarker for chronic lung allograft dysfunction: A retrospective analysis

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KEYWORDS:
circulating exosomes;
biomarker;
lung self-antigens;
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human lung transplant

BACKGROUND: Exosomes isolated from plasma of lung transplant recipients (LTxRs) with bronchiolitis obliterans syndrome (BOS) contain human leukocyte antigens and lung self-antigens (SAgs), K-alpha 1 tubulin (Kα1T) and collagen type V (Col-V). The aim was to determine the use of circulating exosomes with lung SAgs as a biomarker for BOS.

METHODS: Circulating exosomes were isolated retrospectively from plasma from LTxRs at diagnosis of BOS and at 6 and 12 months before the diagnosis ($n = 41$) and from stable time-matched controls ($n = 30$) at 2 transplant centers by ultracentrifugation. Exosomes were validated using Nanosight, and lung SAgs (Kα1T and Col-V) were detected by immunoblot and semiquantitated using ImageJ software.

RESULTS: Circulating exosomes from BOS and stable LTxRs demonstrated 61- to 181-nm vesicles with markers Alix and CD9. Exosomes from LTxRs with BOS ($n = 21$) showed increased levels of lung SAgs compared with stable ($n = 10$). A validation study using 2 separate cohorts of LTxRs with BOS and stable time-matched controls from 2 centers also demonstrated significantly increased lung SAgs−containing exosomes at 6 and 12 months before BOS.

CONCLUSIONS: Circulating exosomes isolated from LTxRs with BOS demonstrated increased levels of lung SAgs (Kα1T and Col-V) 12 months before the diagnosis (100% specificity and 90% sensitivity), indicating that circulating exosomes with lung SAgs can be used as a non-invasive biomarker for identifying LTxRs at risk for BOS.

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The development of chronic rejection following solid organ transplantation is a major barrier for continued function of the transplanted organ. Once chronic rejection develops, there are no treatment options available to reverse the process. Among transplanted organs, chronic rejection is most common following human lung transplantation (LTx), where the 5-year incidence is approximately 50% and nearly 90% develop chronic rejection within 10 years.[1] Lung allograft failure because of chronic lung allograft dysfunction (CLAD) is the leading cause of death beyond the first year after transplantation.[2] Approximately 70% of patients with CLAD have bronchiolitis obliterans syndrome (BOS),[3,4] a fibrotic obliteration of respiratory and membranous bronchioles.[5–7] Histological confirmation of BOS is often difficult because surgical lung biopsy is invasive and carries unacceptable risk. In addition, the sensitivity of transbronchial lung biopsy is poor because of the limited sample size and the patchy involvement of respiratory and membranous bronchioles.[8] Therefore, BOS, diagnosed and staged according to changes in spirometry, was developed as a clinical surrogate for obliterative bronchiolitis.[6,7] Nonetheless, it is recognized that changes in spirometry are downstream of the pathogenic injury that results in the small airway fibrosis. Although a decrement in small airway forced expiratory flow (25%−75%) may presage the decrement in forced expiratory volume in 1 second (FEV_1), this lacks specificity for BOS.[7,9] It has been reported that bronchoalveolar lavage fluid (BAL) and its cell transcriptome may serve as biomarker for BOS.[10,11] Thus, there is a clinical need for a biomarker that predicts the development of BOS at an early stage to enhance monitoring and provide an opportunity for intervention.

Our laboratory demonstrated that circulating exosomes isolated from plasma and BAL from LTx recipients (LTxRs) with BOS are from the donor and have donor human leukocyte antigens (HLAs) and expressed K-alpha 1 tubulin (Kα1T) and collagen type V (Col-V), the prototypic lung self-antigens (SAgs).[12,13] Another report has shown the presence of exosomes in BAL of LTxRs with acute and chronic rejection.[14] Therefore, we hypothesized that detection of circulating exosomes containing lung SAgs before the diagnosis of BOS would serve as a biomarker for identifying LTxRs at risk for BOS.

Methods

Sample collection

A total of 71 LTxRs were selected from Washington University School of Medicine (WUSM) and University of Washington, Seattle (UW). This is a retrospective study based on collected plasma samples and clinical information. We obtained institutional review board approval from all centers and consent from all subjects for this study. Among these, 41 were diagnosed with BOS and 30 did not have BOS (control/stable group). For the discovery cohorts, 21 samples with BOS and 10 stable time-matched controls were used. For the validation cohorts, plasma from 20 LTxRs with BOS and 20 stable/control LTxRs from WUSM and UW (10 from each) was collected at 6 and 12 months and at the time of BOS after LTx. All of the plasma collected for LTxRs with BOS were time matched with the stable/control LTxRs.

We diagnosed BOS according to the standard International Society for Heart and Lung Transplantation criteria.[15,16] Specifically, our clinical approach to allograft dysfunction includes a thorough evaluation of potential causes (e.g., acute cellular rejection, infection, antibody-mediated rejection, and bronchostenosis) including a history and physical exam, imaging studies, nasopharyngeal swab for the detection of respiratory viruses, donor-specific antibody (DSA) test, and bronchoscopy with BAL and transbronchial lung biopsies. We then confirm the diagnosis of CLAD in cases where there is a persistent ≥20% decline in FEV_1 without an alternative explanation for more than 3 months. CLAD phenotype was based on forced vital capacity (FVC), FEV_1, and the presence or absence of radiographic infiltrates on imaging. In this cohort study, we did not have sufficient total lung capacity measurements as this has not been part of our routine follow-up of patients. Thus, we excluded restrictive allograft syndrome by using FVC, FEV_1/FVC ratio, and chest imaging. We treated cases where we identified an alternate explanation for allograft dysfunction (e.g., appropriate antibiotics for a bacterial bronchitis) and only made a diagnosis of CLAD if there was a persistent ≥20% decline in FEV_1 in spite of appropriate therapy for more than 3 months.

Isolation of exosomes

Circulating exosomes were isolated from plasma using ultracentrifugation as described previously.[12] Plasma (1 ml) was centrifuged at 2,000 g for 30 minutes followed by 10,000 g for 40 min at 4°C; supernatant was diluted with phosphate-buffered saline (PBS) and centrifuged at 100,000 g for 120 minutes at 4°C. When plasma was <100 μl, a total exosomes isolation kit was used as described by manufacturer (Invitrogen, Thermo Fisher Scientific, Waltham, MA) with modifications including passing through a 0.2-μm filter. Comparison of these 2 approaches provided similar results in Nanosight and lung SAg measurements. Exosome pellet was suspended in PBS and concentration of protein was analyzed using bicinchoninic acid. Isolated exosomes were subjected to Nanosight NS300 instrument (Malvern Instruments, Malvern, United Kingdom) to analyze size distribution. Exosomes were diluted in PBS (1:50 dilution), their size was verified, and the exosomes used in this study had a range between 40 and 200 nm.

Western blot analysis

To analyze the presence of lung SAgs in exosomes, we performed western blot. A total of 10 μg of protein was used for sodium dodecyl sulfate−polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Antibodies (Abs) to SAgs, anti-rabbit Col-V (Abcam, Cambridge, United Kingdom) and anti-rabbit Kα1T (Santa Cruz Biotechnology, Dallas, TX) IgG, were used to detect protein. Goat anti-rabbit IgG conjugated with horseradish peroxidase was used as secondary Ab. Blot was developed using enhanced chemiluminescent detection kit. J Image Software (National Institutes of Health, Bethesda, MD) was used for densitometry of the signal band.

Receiver operating curve (ROC) analysis

Results obtained for the fold change from the western blot analysis for lung SAgs−containing exosomes, ROC determination was

performed by GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, CA). The cut off values were determined from the discovery cohort for lung SAgs with 21 patients with BOS and 10 stable controls (time-matched control LTxRs) at 6 and 12 months before clinical diagnosis of BOS. The validation cohort was from 20 LTxRs with BOS and 20 control LTxRs from WUSM and UW (10 from each center) at 6 and 12 months before development of BOS after LTx.

Statistical analysis

Optical density (OD) of exosomes containing lung SAgs was quantitated using ImageJ software. OD of SAgs was normalized with exosome-specific markers Alix and CD9 and expressed as mean ± SD. The relative OD values for SAgs between BOS and stable LTxRs were compared using unpaired and paired non-parametric Mann-Whitney test. The two-sided Wilcoxon rank-sum test was used to compare the normalized level of each antigen between patients diagnosed with chronic rejection and patients with stable/control condition at 6 and 12 months. Bonferroni correction was utilized to adjust for multiple comparisons, so a significance level of 0.0125 was used for each test (2 antigens and 2 time points). The same procedure was repeated for the validation data. GraphPad Prism version 7 for Windows (GraphPad Software) was used to perform the analysis.

Table 1 Discovery Studies: Demographic of Lung Transplant Recipients

Characteristics	Stable 2000–2015	BOS 2002–2015
Number	10	21
Sex, *n* (%)		
Male	7 (70%)	15 (71.4%)
Female	3 (30%)	6 (28.6%)
Age, years, mean ± SD	53.8 ± 8.0	50.7 ± 14.1
Ethnicity, *n* (%)		
Caucasian	10 (100%)	21 (100%)
Black	0	0
Bilateral transplant	10	21
Disease, *n* (%)		
Cystic fibrosis	2 (10%)	8 (31%)
IPF	3 (50%)	6 (28.6%)
COPD	4 (30%)	5 (23.8%)
BOS	0	1 (4.8%)
Interstitial lung disease	0	1 (4.8%)
MCTD	1 (10%)	0

Abbreviations: BOS, bronchiolitis obliterans syndrome; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; MCTD, mixed connective tissue disease.

Results

Clinical data of LTxRs

LTxR demographics and laboratory data of the discovery cohort ($n = 31$) were collected from patient charts. BOS was diagnosed according to International Society for Heart and Lung Transplantation guidelines.[17] Patient demographics including age, sex, ethnicity, and underlying diagnosis were not significantly different between LTxRs with BOS and stable time-matched controls (Table 1).

Size distribution and characterization of exosomes

Size distribution of isolated exosomes from LTxRs was carried out using Nanosight NS300. As shown in Figure 1a, the size of the vesicles used in this study ranged from 61 to 181 nm, compatible with exosomes as described in a position statement of the International Society for Extracellular Vesicles.[18] Western blot analysis also showed the presence of exosome markers Alix and CD9 (Figure 1b). These results confirm that the isolated vesicles are exosomes.

Increased levels of circulating exosomes with lung SAgs in LTxRs diagnosed with BOS

Previous studies demonstrated that exosomes isolated from LTxRs with BOS contained lung SAgs (Col-V and Kα1T).[12,19] To confirm our earlier findings, we isolated exosomes from plasma of LTxRs with BOS from a different LTx center (UW) and observed similar results, that is, circulating exosomes from LTxRs with BOS contained increased levels of Col-V (2.09 ± 1.06 vs 1.17 ± 0.66, $p = 0.0096$; 1.79-fold) and Kα1T (2.10 ± 1.16 vs 1.19 ± 0.67, $p = 0.0096$; 1.76-fold) in comparison to stable time-matched controls (Figure 1d). Western blots from 5 BOS and 5 stable controls are given in Figure 1c. This result corroborates our previous findings.[12,19]

Detection of circulating exosomes with lung SAg Col-V 12 months before the diagnosis of BOS

To determine whether circulating exosomes were detectable before the clinical diagnosis of BOS, we isolated exosomes from plasma collected from 21 LTxRs with BOS and 10 stable time-matched control LTxRs at 6 and 12 months before the diagnosis of BOS (discovery cohort). Western blot results shown in Supplementary Figure S1a, available online at www.jhltonline.org, demonstrate that exosomes from LTxRs contained significantly higher levels of Col-V at 6 and 12 months before BOS. Semiquantitation by densitometry demonstrated significantly higher levels of lung SAgs at 6 months (1.79 ± 0.59 vs 0.49 ± 0.27, $p < 0.0001$; 3.65-fold) and 12 months (2.06 ± 0.65 vs 0.56 ± 0.26, $p < 0.0001$; 3.67-fold) in the exosomes isolated from LTxRs with BOS than matched stable controls (Figure 2a). We also assessed the amount of Kα1T in exosomes using western blot (Supplementary Figure S1b online). Results of semiquantitation of the gels demonstrate that exosomes from LTxRs with BOS contained significantly increased levels of Kα1T (Figure 2b) compared with stable controls, both at 6 months (1.20 ± 0.55 vs 0.56 ± 0.34, $p = 0.0049$; 2.14-fold) and 12 months (1.41 ± 1.02 vs 0.71 ± 0.37, $p = 0.0348$; 1.99-fold) before BOS. These results demonstrate that circulating exosomes containing significantly increased levels of both lung SAgs (Kα1T and Col-V) are present in LTxRs with BOS

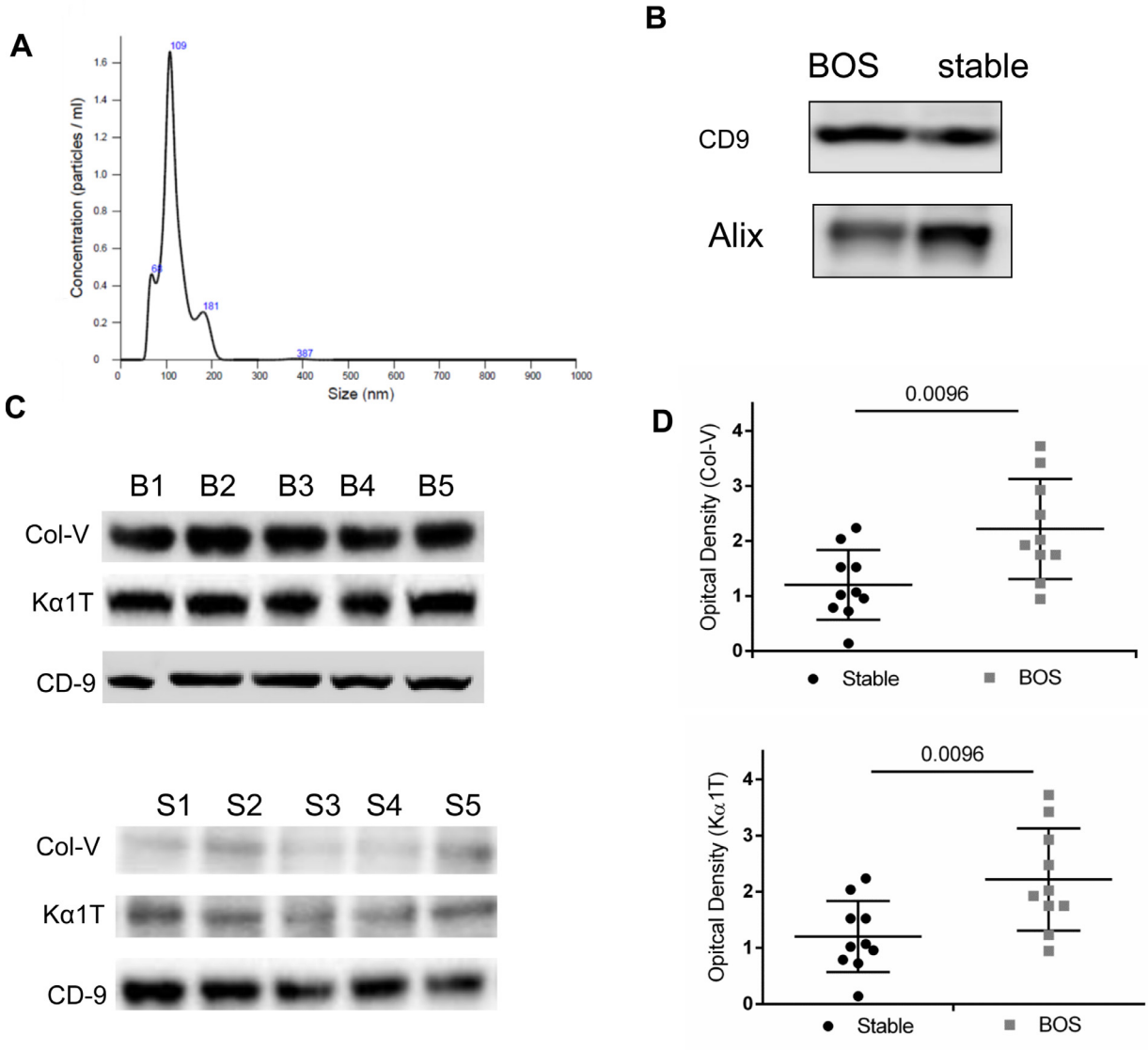

Figure 1 Characterization of exosomes containing lung SAgs at the time of BOS. (**a**) Exosomes were isolated from plasma by ultracentrifugation, diluted in PBS, and subjected to Nanosight NS300 analysis, and results demonstrated that the extracellular vesicles have a size of 60 to 200 nM. Similar results were also obtained using the modified kit method. (**b**) Western blot analysis showed the presence of exosome markers CD9 and Alix in exosomes isolated from plasma of stable controls and patients with BOS. (**c**) Exosomes isolated from plasma from a different center (UW) at the time of BOS diagnosis contain Col-V and Kα1T. Exosomes were isolated from plasma of LTxRs from UW (different center) diagnosed with BOS ($n = 10$) and stable time-match controls ($n = 10$) and subjected to western blot analysis for the detection of lung self-antigens. Western blot analysis of representative 5 patients with BOS and 5 stable controls showed an increased amount of Col-V and Kα1T in exosomes derived from LTxRs with BOS but not in stable controls. Representation depicts 5 out of 10 LTxRs with BOS (top) and stable (bottom). (**d**) Semiquantification by densitometry showed a significant increase in OD for Col-V (top) and Kα1T (bottom) in LTxRs with BOS when compared with stable controls. BOS, bronchiolitis obliterans syndrome; Col-V, collagen type V; Kα1T, K-alpha 1 tubulin; LTxR, lung transplant recipient; OD, optical density; PBS, phosphate-buffered saline; SAgs, self-antigens; UW, University of Washington, Seattle.

compared with stable time-matched controls up to 12 months before the diagnosis of BOS.

Validation using a different cohort of patients with BOS

To validate the results obtained in the preliminary analysis indicating that circulating exosomes with increased levels of SAgs could identify patients at increased risk for BOS, we analyzed circulating exosomes from independent cohorts of LTxRs consisting of 10 with BOS and 10 stable controls from WUSM. Demographics of LTxRs used in this study are given in Table 2. Plasma collected at 6 and 12 months before BOS and time-matched samples from stable LTxRs without BOS. In agreement with our preliminary results, western blot results of exosomes isolated from patients with BOS demonstrated significantly higher levels of lung SAgs than stable LTxRs (Supplementary Figure S2). Semiquantitation by densitometry corroborated western results (6 months [OD]: Col-V, 1.24 ± 1.06 vs 0.13 ± 0.07, $p < 0.0001$, 9.54-fold; Kα1T, 0.80 ± 0.64 vs 0.18 ± 0.07,

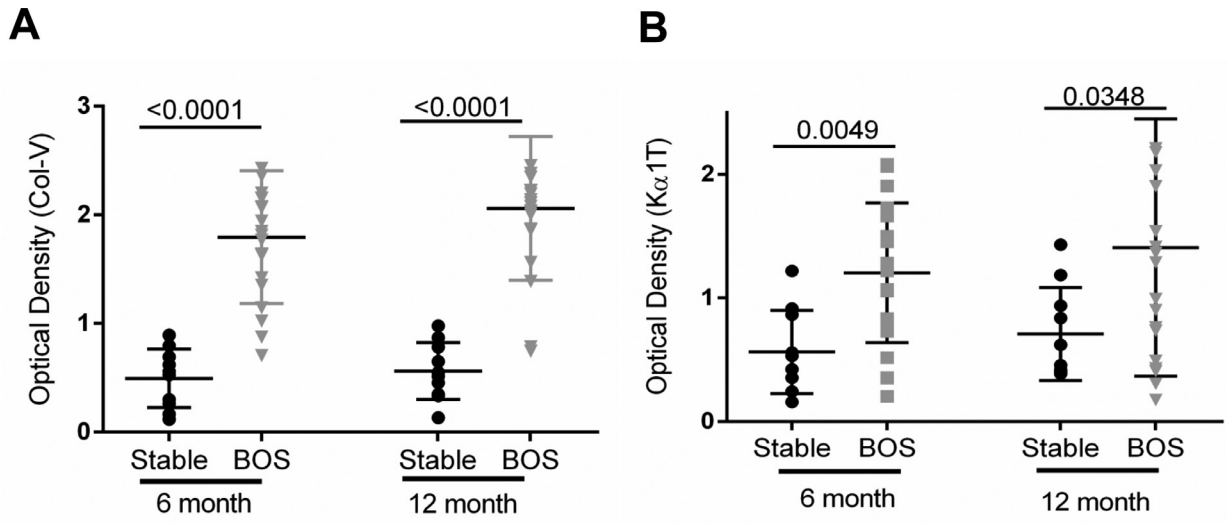

Figure 2 Detection of circulating exosomes with lung SAgs at 6 and 12 months prior to diagnosis of BOS. (**a**) Analysis of exosomes isolated from circulation of 21 LTxRs with BOS and 10 time-matched stable LTxRs (discovery cohort). Semiquantification by densitometry revealed a significant increase in Col-V OD in comparison to stable controls, 6 month (1.79 ± 0.59 vs 0.49 ± 0.27, $p < 0.0001$) and 12 month (2.06 ± 0.65 vs 0.56 ± 0.26, $p < 0.0001$). (**b**) Densitometry analysis showed significantly increased Kα1T OD when compared with stable controls, 6 month (1.20 ± 0.55 vs 0.56 ± 0.34, $p = 0.0049$) and 12 month (1.41 ± 1.02 vs 0.71 ± 0.37, $p = 0.0348$). Western blot data are presented in Supplementary Figure S1. BOS, bronchiolitis obliterans syndrome; Col-V, collagen type V; Kα1T, K-alpha 1 tubulin; LTxR, lung transplant recipient; OD, optical density; SAgs, self-antigens.

$p < 0.0001$, 4.44-fold; 12 months [OD]: Col-V, 1.18 ± 1.02 vs 0.12 ± 0.05, $p < 0.0001$, 9.83-fold; Kα1T 0.94 ± 0.59 vs 0.21 ± 0.09, $p < 0.0001$, 4.48-fold) (Figure 3a and b).

Further, to revalidate our findings, we isolated circulating exosomes from plasma in 10 LTxRs with BOS and 10 stable time-matched controls from UW. Demographics of LTxRs used for this study from UW are given in Table 2. Plasma was collected at 6 and 12 months before BOS. We observed similar results in western blot for lung SAgs; exosomes containing lung SAgs were present 12 months before the diagnosis of BOS compared with stable (Supplementary Figure S3). Semiquantitation of the western blot showed Col-V (6 months: 3.8 ± 2.6 vs 1.09 ± 0.84, $p = 0.0116$, 3.21-fold; 12 months: 4.39 ± 2.79 vs 1.57 ± 1.39, $p = 0.0089$, 2.8-fold) and Kα1T (6 months: 2.00 ± 0.49 vs 0.080 ± 0.37,

Table 2 Validation Studies: Demographic of a Different Cohort of Lung Transplant Recipients from WUSM and University of Washington

Characteristic	WUSM Samples		UW Samples	
	Stable 2008–2013	BOS 2003–2013	Stable 2008–2012	BOS 2007–2012
Number	10	10	10	10
Sex, *n* (%)				
Male	6 (60%)	7 (70%)	7 (70%)	3 (30%)
Female	4 (40%)	3 (30%)	3 (30%)	7 (70%)
Age, years, mean ± SD	51.3 ± 10.2	54.3 ± 15.4	53.8 ± 13.8	50.7 ± 11.3
Ethnicity, *n* (%)				
Caucasian	8 (80%)	10 (100%)	9 (90%)	9 (90%)
Black	2 (20%)	0	1 (10%)	1 (10%)
Bilateral transplant	10	10	10	10
Disease, *n* (%)				
Cystic fibrosis	2 (20%)	1 (10%)	1 (10%)	3 (30%)
IPF	3 (30%)	3 (30%)	1 (10%)	5 (50%)
COPD	3 (30%)	3 (30%)	6 (60%)	2 (20%)
Alpha 1	1 (10%)	0	1 (10%)	0
Sarcoidosis	0	2 (20%)	0	0
Scleroderma	0	1 (10%)	0	0
PCH	1 (10%)	0	0	0
Interstitial lung disease	0	0	1 (10%)	0

Abbreviations: BOS, bronchiolitis obliterans syndrome; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; PCH, pulmonary capillary hemangiomatosis; UW, University of Washington, Seattle; WUSM, Washington University School of Medicine.

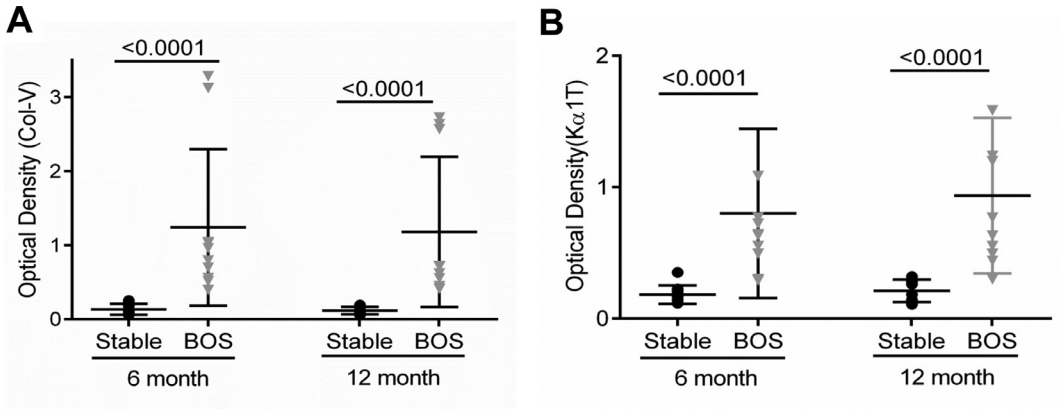

Figure 3 Analysis of circulating exosomes for lung SAgs from a different LTxR center (WUSM) (validation cohort) consisting of 10 patients with BOS and 10 stable controls demonstrating increased levels of lung SAgs−containing exosomes 6 and 12 months before clinical diagnosis of BOS. Densitometry analysis demonstrated that exosomes isolated from patients with BOS has increased lung self-antigens. (**a**) OD, Col-V: 6 months, 1.24 ± 1.06 vs 0.13 ± 0.07, $p < 0.0001$; 12 months, 1.18 ± 1.02 vs 0.12 ± 0.05, $p < 0.0001$. (**b**) OD, Kα1T: 6 months, 0.80 ± 0.64 vs 0.18 ± 0.07, $p < 0.0001$; 12 months, 0.94 ± 0.59 vs 0.21 ± 0.09, $p < 0.0001$. Western blot results are presented in Supplementary Figure S2. BOS, bronchiolitis obliterans syndrome; Col-V, collagen type V; Kα1T, K-alpha 1 tubulin; LTxR, lung transplant recipient; OD, optical density; SAg, self-antigen; WUSM, Washington University School of Medicine.

$p = 0.025$, 1.25-fold; 12 months: 2.48 ± 1.92 vs 0.98 ± 0.19, $p = 0.0042$, 2.53-fold) (Figure 4a and b). These results validate the results from the discovery cohort, using 2 separate set of LTxRs from 2 different centers, and provide evidence that circulating exosomes containing increased lung SAgs can be detected in plasma up to 12 months before the diagnosis of BOS.

Sensitivity and specificity analysis

ROC analysis of lung SAgs (Col-V and Kα1T) for the discovery cohort (Figure 5a and b) and validation cohort (Figure 5c) were performed at 6 and 12 months before BOS. In the discovery cohort, Col-V levels at 6 months (area under curve [AUC] = 0.99) showed a sensitivity of 85.71% and a specificity of 100% at a cutoff fold change of >1.09. Col-V levels at 12 months (AUC = 0.98) showed a sensitivity of 90.48% and a specificity of 100% at a cutoff fold change of >1.18. Kα1T levels at 6 months (AUC = 0.81) and 12 months (AUC = 0.74) showed a sensitivity of 61.9% (6 months) and 57.14% (12 months) and specificity of 90% (6 months); 80% (12 months) at cutoff of >1.06 for 6 months and >1.09 for 12 months respectively. The cutoff values for validation cohort was determined based on the discovery cohort. The validation cohort revealed that Col-V levels at 6 months (AUC = 0.87) showed sensitivity of 70% and specificity of 80% at cutoff of >0.99. Col-V levels at 12 months (AUC = 0.82) showed sensitivity of 60% and a specificity of 75% at cutoff of >0.99. Kα1T levels at 6 months (AUC = 0.85) and 12 months (AUC = 0.82)

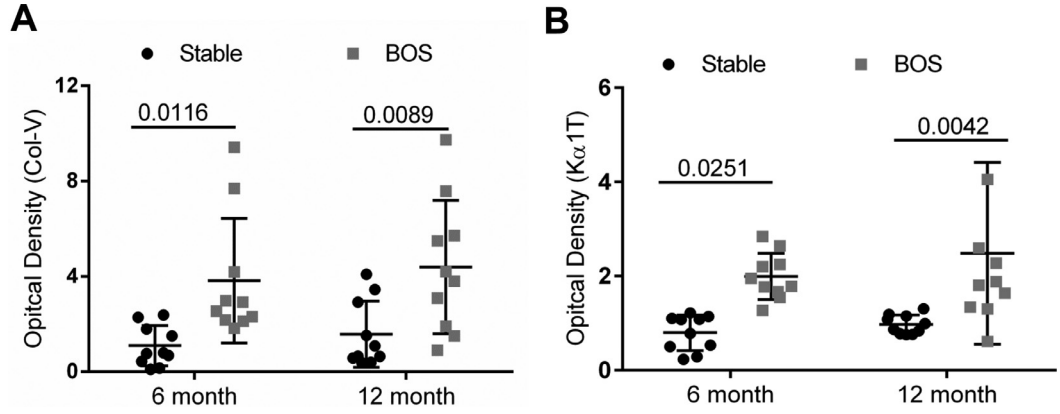

Figure 4 Analysis of circulating exosomes for lung SAgs from a different LTxR center (UW) (validation cohort) consisting of 10 patients with BOS and 10 stable controls demonstrating increased levels of lung SAgs−containing exosomes 6 and 12 months before clinical diagnosis of BOS. Exosomes isolated from plasma of patients with BOS had significantly increased levels of lung SAgs in comparison with stable controls. (**a, b**) Semiquantification analysis of OD. (**a**) Col-V. 6 months: 3.8 ± 2.6 vs 1.09 ± 0.84, $p = 0.0116$; 12 months: 4.39 ± 2.79 vs 1.57 ± 1.39, $p = 0.0089$. (**b**) Kα1T. 6 months: 2.00 ± 0.49 vs 0.080 ± 0.37, $p = 0.0251$; 12 months: 2.48 ± 1.92 vs 0.98 ± 0.19, $p = 0.0042$. Western blot results are presented in Supplementary Figure S3. BOS, bronchiolitis obliterans syndrome; Col-V, collagen type V; Kα1T, K-alpha 1 tubulin; LTxR, lung transplant recipient; OD, optical density; SAg, self-antigen; UW, University of Washington, Seattle.

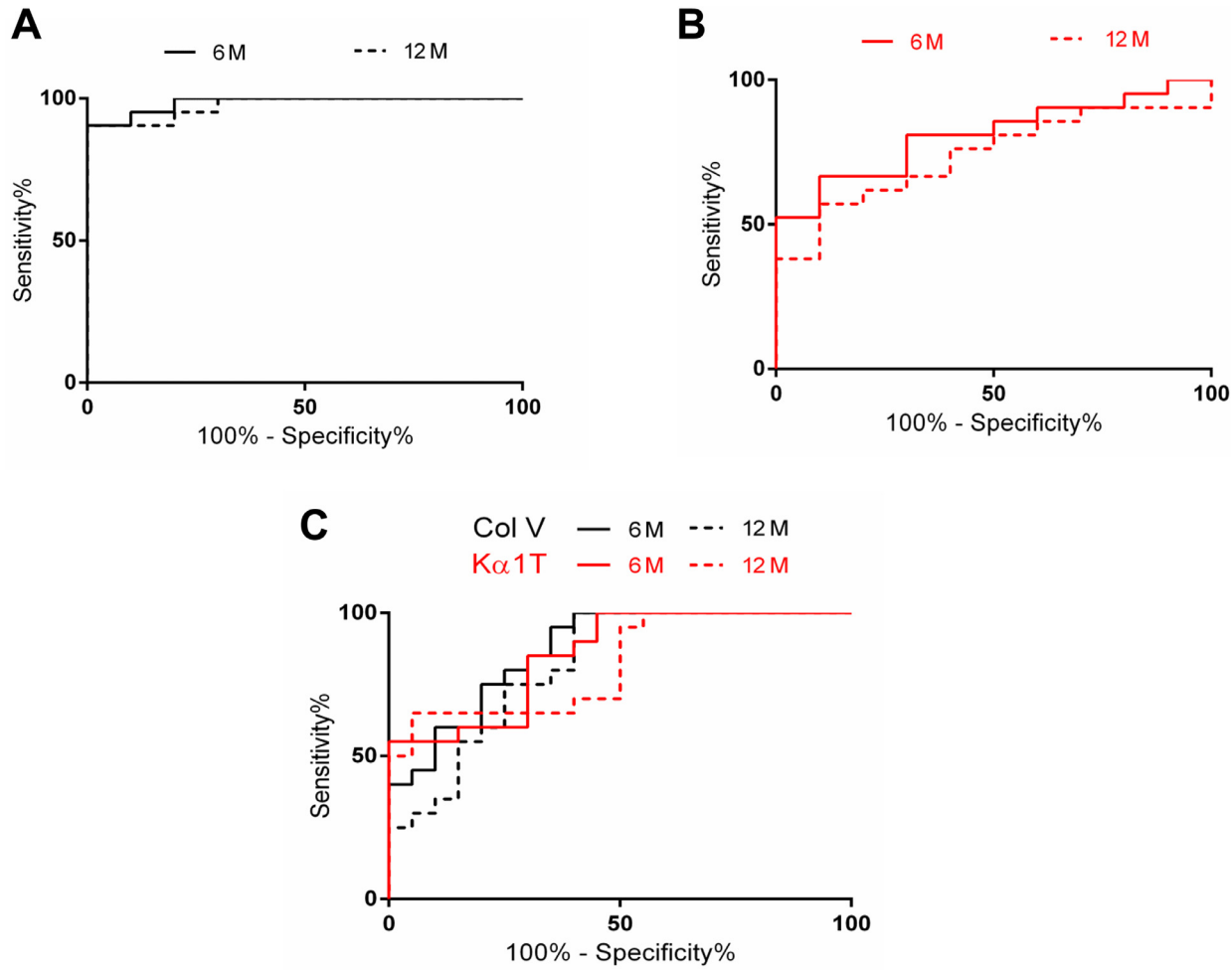

Figure 5 ROC analysis of discovery and validation cohorts. (**a**) ROCs were calculated for circulating exosomes with lung SAg Col-V at 2 time points (6 and 12 months) in the discovery cohort to determine optimum threshold values. Col-V levels at 6 months (– – –), AUC = 0.99; 12 month (—), AUC = 0.98. (**b**) ROC was calculated for circulating exosomes with lung SAg Kα1T at 2 time points (6 and 12 months) in the discovery cohort to determine optimum threshold values. Kα1T levels had an AUC at 6 months of 0.81 and at 12 months of 0.74. (**c**) Validation cohort: ROCs were calculated for circulating exosomes with lung SAgs at 2 time points (6 and 12 months) for the combined validation cohorts from both centers (WUSM and UW). The validation cohort revealed that Col-V levels at 6 months had an AUC of 0.87 and at 12 months had an AUC of 0.82 (black). Kα1T levels at 6 months had an AUC of 0.85 and at 12 months had an AUC of 0.82 (red). AUC, area under curve; Col-V, collagen type V; Kα1T, K-alpha 1 tubulin; ROC, receiver operating curve; SAg, self-antigen; UW, University of Washington, Seattle; WUSM, Washington University School of Medicine.

showed a sensitivity of 60% (6 months) and 65% (12 months) and a specificity of 80% (6 months) and 80% (12 months) at a cutoff of >1.07 for 6 months and >1.04 for 12 months, respectively (Table 3).

Western blotting results obtained from both centers were analyzed in the validation cohort and their sensitivity and specificity were determined to predict exosomes with increased lung SAgs in LTx population studied. The values obtained from the validation cohorts, although significant, were not similar to those obtained for the discovery cohort, likely because of a smaller number of LTxRs analyzed for validation and potential differences in the diagnostic criterion between the 2 centers.

Discussion

Using rigorous study design (discovery and validation cohorts), we have demonstrated that circulating exosomes containing increased levels of lung SAgs (Kα1T and Col-V) are present, not only at the time of BOS diagnosis but also preceding the diagnosis, and might, therefore, be useful for predicting the development of BOS in LTxRs. Our results demonstrate that plasma collected at 6 and 12 months before the diagnosis of BOS has significantly increased levels of circulating exosomes with lung SAgs. This may allow the development of strategies for prevention and/or early treatment of LTxRs who are at risk for developing BOS.

Our group and others have demonstrated that development of Abs to mismatched donor HLAs and immune responses to lung SAgs (Col-V)[20] are associated with the development of primary graft dysfunction[21] (PGD) and BOS.[22–24] PGD and respiratory viral infections (RVIs) are widely recognized risk factors for BOS.[25–28] Based on these, we propose that stress to the transplanted organs either by PGD, RVI, or rejection can release circulating

Table 3 Statistical Analysis of Lung Self-Antigens for Discovery and Validation Cohorts

Lung SAgs	Time points (months)	Discovery cohort				Validation cohort			
		AUC	Cutoff	Sensitivity (%)	Specificity (%)	AUC	Cutoff	Sensitivity (%)	Specificity (%)
Col-V	6	0.99	>1.09	85.71	100	0.87	>0.99	70	80
	12	0.98	>1.18	90.48	100	0.82	>0.99	60	75
Kα1T	6	0.81	>1.06	61.9	90	0.85	>1.07	60	80
	12	0.74	>1.09	57.14	80	0.82	>1.04	65	80

Abbreviations: AUC, area under curve; Col-V, collagen type V; Kα1T, K-alpha 1 tubulin; SAg, self-antigen.

exosomes with lung SAgs. Persistence of stress may continue to induce and release the exosomes with lung SAgs into the circulation, which can lead to immune activation, increasing the risk of BOS. Therefore, circulating exosomes with lung SAgs could serve as a non-invasive biomarker for identifying LTxRs at risk for developing BOS. We recently demonstrated the presence of lung SAgs in circulating exosomes isolated from LTxRs diagnosed with PGD, RVI, and acute rejection and following de novo development of Abs specific to donor-mismatched HLA.[29] These exosomes also contain immunoregulatory proteins including transcription factors, adhesion and costimulatory molecules, and 20S proteasome. Immunization of mice with exosomes from LTxRs with BOS induced cellular and humoral immune responses to lung SAgs, strongly suggesting that these exosomes are immunogenic.[30] Thus, the detection of increased lung SAgs−containing exosomes not only might serve as a non-invasive biomarker for injury, but also their persistence may increase immune responses that result in chronic rejection. Studies from our laboratory have shown that lung injury owing to PGD, acute rejection, and RVIs, known risk factors for the development of CLAD, also induces circulatory exosomes with lung SAgs, and preliminary studies have shown that persistence of these exosomes with lung SAgs leads to development of Abs to mismatched donor HLA antigens (DSA), a known risk factor for the development of CLAD.[29,30] Therefore, removal of the circulating exosomes with lung SAgs by plasmapheresis may reduce the development of DSAs, which may result in increases in freedom from the development of CLAD. We also propose that early institution of extracorporeal photopheresis treatment, which is currently provided as a treatment option for LTxRs diagnosed with BOS, needs to be carried out when circulating exosomes with increased lung SAgs are detected early, that is, 6 to 12 months before clinical diagnosis of CLAD. Such an approach for instituting extracorporeal photopheresis therapy early based on detection of circulating exosomes with lung SAgs should assist in preventing and/or treating LTxRs diagnosed with CLAD before irreversible damage to the transplanted lungs.

During this study, the discovery cohort analysis of Col-V had a sensitivity of 85.7% and a specificity of 100% at 6 months and 90.48% sensitivity and 100% specificity at 12 months before clinical diagnosis of BOS. For the lung SAg Kα1T, there was 61.9% sensitivity and 90% specificity at 6 months and a sensitivity of 57% and a specificity of 80% at 12 months. For the validation cohorts, levels of exosomes containing Col-V demonstrated a sensitivity of 70% and a specificity of 80% at 6 months and a sensitivity of 60% with specificity of 75% at 12 months before clinical diagnosis of BOS. For the lung SAg Kα1T, sensitivity was 60% and specificity 80% at 6 months and had 65% sensitivity and 80% specificity at 12 months. These results demonstrate that determination of Col-V or Kα1T in circulating exosomes by western blot followed by semiquantitation possesses higher positive predictive value with excellent sensitivity and specificity. Further, circulating exosomes with lung SAgs can serve as a non-invasive biomarker in predicting risk for BOS at least 12 months before clinical diagnosis.

Previous reports have demonstrated that plasma from heart transplant recipients with cardiac allograft vasculopathy contain Abs to cardiac SAgs (myosin and vimentin).[31,32] In addition, kidney transplant recipients diagnosed with transplant glomerulopathy, a risk factor for developing chronic rejection, also develop Abs to kidney SAgs (fibronectin, Col-IV, and LG3).[33−36] Recently, we showed that exosomes isolated from heart and kidney transplant recipients contain tissue-associated SAgs[19] and demonstrated the exosomes' importance in graft failure using a syngeneic murine heart transplant model. A recent report demonstrated that circulating $C4d^+$ plasma endothelial macrovesicle levels were increased in human kidney transplant recipients with acute antibody-mediated rejection.[37] This signifies that de novo development of Abs can stimulate exosomes and may contribute to rejection. These results suggest that circulating exosomes containing tissue-associated SAgs can occur in other solid organ transplants, and exosomes with tissue-associated SAgs can serve as a non-invasive biomarker for kidney and heart transplant recipients at risk for developing chronic rejection.

There are some limitations for our study, including the following:

1. All of our analyses for exosomes containing lung SAgs are from samples collected retrospectively from LTxRs.
2. Because of the retrospective nature of our analysis, exact pairings of BOS and stable control patients were not carried out.
3. All of our analyses included only patients with BOS, and further analyses of restrictive allograft syndrome are needed.
4. Because we have performed quantitation of western blot using specific Abs to lung SAgs, this approach may not

be easily translatable into a clinical situation. However, availability of image screen analysis using labeled Abs specific to lung SAgs are possible and therefore should be translatable into a clinical laboratory.

In conclusion, our findings demonstrate the importance of circulating exosomes in the development of immune responses leading to chronic rejection. Our data, using different sets of plasma samples collected from 2 different LTx centers, demonstrated that circulating exosomes with lung SAgs can be detected 12 months before the diagnosis of BOS, indicating that circulating exosomes with lung SAgs can be a viable non-invasive biomarker for identifying patients at risk for developing CLAD. Early detection of patients at risk for developing chronic rejection provides an opportunity to develop strategies to prevent or intervene before the onset of irreversible damage to the transplanted organ. Further, based on the reports that Abs to tissue-associated SAgs can be detected before chronic rejection following human renal and cardiac transplantations, we propose that circulating exosomes with tissue-associated SAgs have the potential to be a non-invasive biomarker for identifying not only LTxRs at risk for developing CLAD but also other solid organ transplant recipients at risk for developing chronic rejection, a major problem in clinical transplantation.

Disclosure statement

The authors have no conflict of interest to disclose.

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Supplementary materials

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