ORIGINAL ARTICLE

Distinct molecular and immunological properties of circulating exosomes isolated from pediatric lung transplant recipients with bronchiolitis obliterans syndrome - a retrospective study

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ABSTRACT

Long-term success following human lung transplantation is poor due to chronic rejection. We demonstrated circulating exosomes of lung origin during acute and chronic lung allograft rejection. We analyzed plasma from pediatric lung transplant recipients (LTxRs) enrolled in the CTOT-C-03 to determine whether circulating exosomes are released into circulation during bronchiolitis obliterans syndrome (BOS). Plasma exosomes were isolated, and human leukocyte antigens (HLA) were detected. Exosomes were analyzed for lung self-antigens (SAgs), co-stimulatory molecules transcription factors, major histocompatibility complex class II (MHC-II), adhesion molecules, and 20S proteasome. Mice were immunized with exosomes from BOS or stable to determine their immunogenicity. Circulating exosomes from BOS LTxRs contained increased levels of SAgs, donor HLA class I, MHC-II, transcription factors, co-stimulatory molecules, and 20S proteasome compared with stable. Serial analysis of exosomes containing SAgs demonstrated that exosomes are detectable in the circulation before BOS. Mice immunized with exosomes from BOS, or stable, demonstrated that exosomes from BOS are distinct in inducing both humoral and cellular immune responses to SAgs. Circulating exosomes from BOS LTxRs elicit distinct humoral and cellular response. In addition, detection of SAgs on circulatory exosomes 12 months before diagnosis of BOS suggest that exosomes could serve as biomarker.

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Key words
bronchiolitis obliterans syndrome, exosomes, lung transplant

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Introduction

Lung transplantation (LTx) is a viable treatment option for pediatric patients with end-stage lung diseases such as cystic fibrosis, interstitial lung disease associated with pulmonary fibrosis, and congenital vascular diseases [1–3]. Short-term survival of LTx has improved, but acute rejection (AR) and chronic rejection in the form of chronic lung allograft dysfunction remain obstacles to long-term allograft function. Chronic lung allograft dysfunction currently includes both restrictive airway disease and an obstructive pathology known as

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bronchiolitis obliterans syndrome (BOS). Roughly 40% of pediatric LTx recipients (LTxRs) develop BOS within 5 years of LTx [4,5], however, the mechanisms underlying BOS development remain unclear.

Circulating exosomes (measuring < 200 nm in diameter) are membrane vesicles produced by the endocytic pathway and secreted into body fluids. They contain membrane and cytosolic proteins and molecules essential for exosome biogenesis. In addition, circulating exosomes have cell-specific components, including proteins, messenger RNA, and microRNA (miRNA [6]. Exosome-specific proteins include CD9, CD63, CD81, and CD82; and proteins involved in exosome biogenesis include actin, annexin, tumor susceptibility gene 101, fibronectin-1, and vesicle-associated membrane protein 8 [7]. Circulating exosomes derived from cancer cells harbor Dicer-AGO2 and TRBP proteins, which can convert pre-miRNA into mature miRNA, silencing messenger RNA of target cells and initiating tumor growth. Hence, it has been proposed that differentially expressed protein and miRNA in cancer-derived circulating exosomes may actually serve as potential biomarkers for cancer [8].

In murine transplant models, donor-derived circulating exosomes were shown to be a source of donor major histocompatibility complex (MHC) molecules. These circulating exosomes remain intact post-transplant thanks to the recipient's antigen-presenting cells, which prime T cells responding through the direct pathway [9,10]. In a previous study, we demonstrated that adult LTxRs with acute rejection (AR) and BOS contain circulating exosomes composed of donor human leukocyte antigen (HLA), lung-associated self-antigens (SAgs), and immunoregulatory miRNAs [11]. To determine whether pediatric LTxRs diagnosed with BOS also induce circulating exosomes, and to define whether circulating exosomes from pediatric LTxRs have immunological and molecular features distinct from those of adult LTxRs, we characterized the kinetics, and the immunological and molecular characteristics of circulating exosomes isolated from plasma samples of pediatric LTxRs diagnosed with BOS and from pediatric LTxRs without BOS from the Clinical Trials in Organ Transplantation in Children (CTOT-C)-03 observational cohort. Additionally, we determined the kinetics and persistence of circulating exosomes in the circulation. Finally, mice were immunized with circulating exosomes isolated from LTxRs with and without BOS and their immune capability was assessed, including distinct cytokines induced specific to lung SAgs.

Materials and methods

Patient selection

The Clinical Trials in Organ Transplantation in Children (CTOT-C-03, NCT00891865) is an observational cohort of 61 pediatric LTxRs enrolled between 2009 and 2013 and followed for 2 years. The primary goal of CTOT-C-03 was to assess relationships among clinical outcomes (AR, BOS, death), post-transplant infections, and various biomarkers. AR was defined pathologically [12] and occurred at an incidence of 30%. BOS was defined based on published criteria [13]. Informed consents from all LTxRs, and family, were obtained by each of the centers participating in CTOT-C-03 study. We studied serially obtained plasma samples from a subset of enrollees for whom samples were available: 6 pediatric LTxRs diagnosed with BOS, and 13 classified as stable, with lung function test results meeting criteria for BOS 0 (FEV_1 >90% of baseline and $FEF_{25–75}$ >75% of baseline) [13]. For this subset, the mean time to clinically detectable BOS was 11 months after LTx. Stable LTxRs had pulmonary function test results within the reference range at 1 year after LTx. Clinical features and demographic characteristics of the pediatric LTxRs enrolled in this study are presented in Table 1.

All patients received triple immunosuppression with tacrolimus, prednisone, and mycophenolate mofetil based on a mutually agreed protocol for dosing and

Table 1. Clinical features and demographic characteristics of 19 pediatric LTxRs

Variable	Stable (*n* = 13)	BOS (*n* = 6)
Sex, *n* (%)		
Male	8 (62)	5 (83)
Female	5 (48)	1 (16)
Mean age, years ± SD	13 ± 6	12 ± 6
Reason for transplant, *n* (%)		
Cystic fibrosis	9 (69)	6 (86)
Interstitial pneumonitis	1 (8)	0 (0)
Pulmonary hypertension	2 (15)	1 (14)
Other	1 (8)	0 (0)
Mean time to BOS development, months ± SD	N/A	11 ± 5.7
Mean time of sample collection, months ± SD		
Month 1	1.0 ± 0.4	0.9 ± 0.3
Month 6	6.3 ± 1.2	5.7 ± 1.3
Month 12	11.8 ± 1.8	12.7 ± 4.1

BOS, bronchiolitis obliterans syndrome; N/A, not applicable.

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trough targets. The majority of patients received anti-interleukin-2 Ab for induction. Roughly 25% received anti-leukocyte Ab induction [14]. Macrolides were included in the study protocol for treatment of BOS. LTxRs were treated with azithromycin three times weekly (250 mg for patients < 40kg, 500 mg for patients> 50 kg). Photopheresis was also included as an option for treatment in cases where BOS progressed in spite of initial therapy. We were unable to obtain the data from the clinical database.

Circulating exosome isolation

The ultracentrifugation method [Beckman Coulter; Swinging-Bucket (Ultracentrifugation); SW32Ti k-Factor (204)] was used to isolate circulating exosomes from the plasma of LTxRs, and the purity of these circulating exosomes was assessed by sucrose cushion method, as described in our earlier publications [11,15]. In brief, 1ml of plasma samples was centrifuged at 2000 and 10 000 *g*, and plasma samples were diluted with phosphate-buffered saline (PBS) and centrifuged at 100 000 *g*. The exosome pellet was washed and lysed with radio-immunoprecipitation assay buffer. The concentration of exosome proteins was determined by bicinchoninic acid assay. Quantification and size of exosomes [16] were analyzed using Nanosight and data are shown in Figure S1.

Enzyme-linked immunosorbent assay for mismatched donor HLA

To detect donor HLA in circulating exosomes, we used the enzyme-linked immunosorbent assay (ELISA) method as described by Logozzi et al., [17] with minor modifications. We set up an ELISA protocol for measuring HLA-A2 in the circulating exosomes since HLA-A2 is one of the high-frequency HLA antigen in our population. In brief, antibodies (Abs) to CD9 (BioLegend, San Diego, CA, USA) were coated in 96-well plates, followed by blocking with 0.5% bovine serum albumin in PBS) Circulating exosomes (1 mg/ml) isolated from sera of LTxRs were then added and incubated at 37°C for 12 h, followed by addition of a rabbit Ab specific to HLA-A2 (Abcam, Cambridge, UK) and secondary Ab, horseradish peroxidase (HRP) conjugated goat anti-rabbit (Abcam). Reaction was developed with chemiluminescent reagent (Millipore, Burlington, MA, USA) for 15 min and stopped with 0.1N HCl. Optical density was measured at 450 nm. Data were expressed as mean ± standard deviation, and *P*-values were deemed significant at less than 0.05.

Serial sample analysis

Serial plasma samples were available only from 2 pediatric LTxRs who developed BOS 20 and 28 months post-transplant. We also obtained time-matched plasma samples from stable LTxRs for use as controls. Serial plasma samples collected at 1, 6, and 12 months after LTx were used to determine the kinetics of exosome release and exosome persistence. Circulating exosomes were isolated as described above and subjected for Western blotting to analyze for the presence of lung SAgs.

Western blotting analysis

Isolated circulating exosomes were used to analyze samples for the presence of lung SAgs (Collagen V [Col-V] and Kα1 Tubulin [Kα1T]), co-stimulatory molecules (CA40, CD80, and CD86), transcription factors (nuclear factor-κB [NF-κB], hypoxia-inducible factor 1-alpha [HIF-1α], and CIITA), MHC-II, adhesion molecules (intercellular adhesion molecule [ICAM] and vascular cell adhesion protein [VCAM]), and 20S proteasome using Western blotting with specific Abs. TSG1, CD9, or Alix were used for loading control. Protein (3 μg) was resolved and transferred to a polyvinylidene difluoride membrane. The membrane was blocked and incubated with specific primary Abs and corresponding HRP labeled secondary Abs goat anti-rabbit or goat anti-mouse. Enhanced chemiluminescent substrate (Millipore) was used to develop blots, and imaging was done using the Odyssey CLx imaging system (LICOR Bioscience). Band intensity was quantified by Image J software. Rabbit anti-Col-V (Abcam) and mouse anti-Kα1T (Santa Cruz Biotechnology, Dallas, TX, USA) were used to detect lung-associated SAgs; rabbit anti-MHC-II anti-CD40, CD80, and anti-HIF-1α and CIITA (Abcam) anti-NF-κB (Cell signaling, Danvers, MA, USA) were used to detect MHC-II, co-stimulatory molecules, and transcription factors. Mouse Abs to ICAM and VCAM (Biolegend) and anti-α3 subunit 20S proteasomes (Santa Cruz Biotechnology) were used to detect adhesion molecules and immunoproteasome. Exosome-specific markers (Alix, CD9, TSG1) were detected by mouse anti-Alix, CD9 (Biolegend), and TSG-1(Santa Cruz Biotechnology).

Immunization of mice

Six-week-old male C57BL/6 mice (Jackson Laboratories, Sacramento, CA, USA) were immunized with either pooled circulating exosomes from six pediatric LTxRs

with BOS or from six stable pediatric LTxRs. Because we have found that lung lesions due to Abs to lung SAgs occur only if there is a primary insult [18], circulating exosomes were administered following intrabronchial administration of HCl (dose: 0.1N HCl) on day 0 is used as an acute lung injury model (<24 h) and has been established as a first-hit insult [19,20]. Circulating exosomes were then administered subcutaneously (100 μg in100 μl) in mice (n = 6/group) on days 1, 7, and 15 without adjuvants, as circulating exosomes contained 20S immunoproteasome. Plasma samples were collected on days 10, 20, and 30 and used for detection of Ab development to SAgs (Col-V, Kα1T), using ELISA. Animals were sacrificed on day 30, and spleens and lungs were harvested. Lungs were used for histopathological analysis by hematoxylin and eosin staining, as well as trichrome staining. Lymphocytes were isolated from the spleens by Ficoll-Hypaque density gradient centrifugation and were used in enzyme-linked immunospot (ELISpot) assay against lung-associated SAgs. The frequency of cells secreting SAg-specific cytokines was enumerated. All protocols and methods involving animals were approved in accordance with guidelines by the Institutional Animal Care and Use Committee at St. Joseph's Hospital and Medical Center.

Detection of humoral immune responses to lung SAgs

Development of Abs to lung-associated SAgs (Col-V, Kα1T) was determined by ELISA using sera collected on days 10, 20, and 30, as described by Fukami et al [21]. Briefly, 1μg/ml Col-V (Sigma) and Kα1T (prepared in the laboratory) were coated on 96-well ELISA plates (Corning) and stored at 4°C overnight. Plasma samples from mice immunized with circulating exosomes isolated from pediatric LTxRs with BOS and stable pediatric LTxRs were tested (1:100) for binding to Col-V and Kα1T. Detection was done with goat anti-mouse-conjugated HRP. Reactions were developed using TMB substrate solution (Millipore), and optical density was read at 450 nm. Plasma Ab concentrations were measured using standard curve of known concentrations of Abs to Col-V and Kα1T.

Detection of cellular immune response to lung-associated SAgs and analysis of cytokine-producing cells (IFN-γ, TNF-α, IL-10, IL-4, and IL-17)

Enzyme-linked immunospot assay was performed to detect cytokines that produced T cells specific to SAgs, using splenocytes harvested from mice immunized with circulating exosomes as described by Fukami et al. [21] (protocol detailed in Appendix S1). ImmunoSpot analyzer (Cellular Technology) was used to measure spots. Appropriate negative and positive control (Concanavalin-A) were used in the experiment. Spots per million were calculated after normalization with cytokine spots in the control.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6 (GraphPad software Inc.). Statistical data are expressed as mean ± standard deviation. We used two-tailed nonparametric *t-test* (Mann–Whitney) and two-way ANOVA test. Bonferroni correction was utilized to adjust for multiple comparisons and to detect statistical significance. *P*-values less than 0.05 were considered statistically significant. Image J software was used to quantify the optical density of blots.

Results

Circulating exosomes from LTxRs with BOS contain mismatched donor HLA, MHC-II molecule, and increased levels of lung SAgs (Col-V, Kα1T)

We characterized the composition of circulating exosomes obtained at the time of BOS diagnosis from patients enrolled in the aforementioned CTOT-C-03 study. Of the 6 LTxRs with BOS, 2 were mismatched for HLA-A2 (MHC class I). Mismatched donor HLA (HLA-A2) was detected in isolated circulating exosomes using ELISA compared with exosome from patients without HLA-A2 (optical density: 216.42 ± 56.7 vs. 112.8 ± 18.7; $P < 0.01$; Fig. 1a). Circulating exosomes isolated from LTxRs diagnosed with BOS also demonstrated significantly higher levels of MHC-II molecules by Western blotting analysis (37-fold increase; $P = 0.0002$) compared with stable LTxRs (Fig. 1b,c). Circulating exosomes from LTxRs with BOS showed significantly higher levels of lung SAgs, Col-V (ninefold increase; $P = 0.0006$), and Kα1T (3.1-fold increase; $P = 0.0006$), compared with circulating exosomes from stable LTxRs (Fig. 1d-e-b). These results agree with our previously reported findings in adult LTxRs [11,22] demonstrating the presence of donor HLA, increased levels of lung-associated SAgs, and increased levels of MHC class II molecules.

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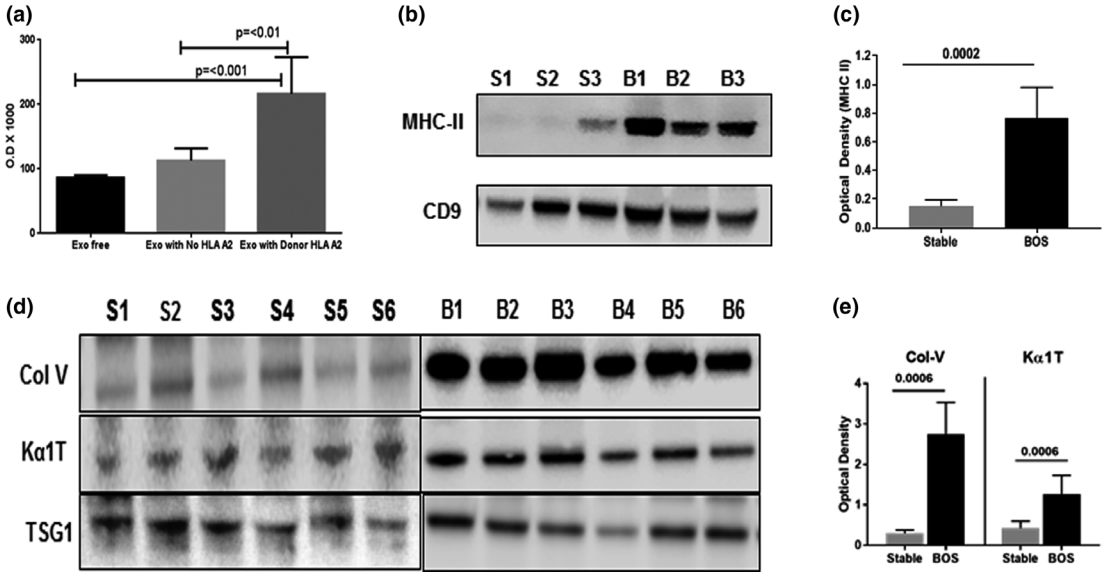

Figure 1 Donor HLA-A2 and MHC-II and SAgs expression within circulating exosomes of LTxRs with and without BOS: (a) Circulating exosomes were isolated from LTxRs (n = 2) transplanted with HLA-A2 donors and 50 μg/ml circulating exosomes/well was used to detect donor HLA-2 by enzyme-linked immunosorbent assay. Circulating exosomes isolated from sera of HLA-2 negative recipients showed presence of donor HLA-2 in comparison with exosomes from patients without HLA-A2. Experiment is repeated three times. (b) Circulating exosomes were isolated from LTxRs who were stable and from those with BOS (n = 6) and subjected to western blot assay to determine presence of MHC-II molecules. CD9 was used as a loading control. (c) Densitometry analysis. S: Stable group; B: BOS group. (d) Increased exosome-expressed lung-associated SAgs in LTxRs with BOS: Circulating exosomes were isolated from plasma samples from pediatric LTxRs with BOS (n = 6) and from stable pediatric LTxRs (n = 13). Western blot analysis was performed using lung-associated SAg-specific Abs (Col-V, Kα1T). TSG1 exosome-specific marker was used as loading control. Comparison of 6 stable LTxRs (top) and 6 LTxRs with BOS. (e) Densitometry revealed higher fold change in BOS vs. stable.

Circulating exosomes from LTxRs with BOS contain increased levels of co-stimulatory molecules, adhesion molecules, pro-inflammatory transcription factors, and 20S proteasome

We determined co-stimulatory molecules CD40, CD80, and CD86 in circulating exosomes isolated from pediatric LTxRs with BOS and stable pediatric LTxRs using specific Abs in a Western blotting analysis. We demonstrated that circulating exosomes isolated from pediatric LTxRs with BOS had significantly higher levels of co-stimulatory molecules CD40, CD80, and CD86 (20-fold increase; $P <$ or =0.0001) compared with circulating exosomes isolated from stable pediatric LTxRs (Fig. 2a,d).

We also noted higher expression of adhesion molecules ICAM (2-fold; P = 0.0007) and VCAM (2.1-fold; P = 0.0003) in circulating exosomes isolated from pediatric LTxRs with BOS compared with stable pediatric LTxRs (Fig. 2c,f). These circulating exosomes also contained significantly increased levels of pro-inflammatory transcription factors, NF-kB (16.2-fold increase; P = 0.0001), and HIF-1α (9.8-fold increase; P = 0.001; Fig. 2b,e). In addition, MHC class II activating transcription factor CIITA was also significantly increased in circulating exosomes isolated from pediatric LTxRs with BOS compared with circulating exosomes isolated from stable pediatric LTxRs (9.3-fold increase; P = 0.003 Fig. 2b,e). Furthermore, circulating exosomes from pediatric LTxRs with BOS also had higher levels of 20S proteasome (threefold increase; P = 0.0022) compared with stable pediatric LTxRs (Fig. 3a,b).

Densitometry analysis of co-stimulatory molecules, adhesion molecules, and transcription factors is shown in Fig. 2d–f. Taken together, these results suggest that circulating exosomes from pediatric LTxRs with BOS, like circulating exosomes from adult LTxRs with BOS [11], have the potential to augment immune responses and ultimately to increase the risk of BOS after LTx in pediatric patients.

Circulating exosomes contain lung SAgs before clinical diagnosis of BOS

To determine whether circulating exosomes with lung SAgs are detectable before clinical diagnosis of BOS, we

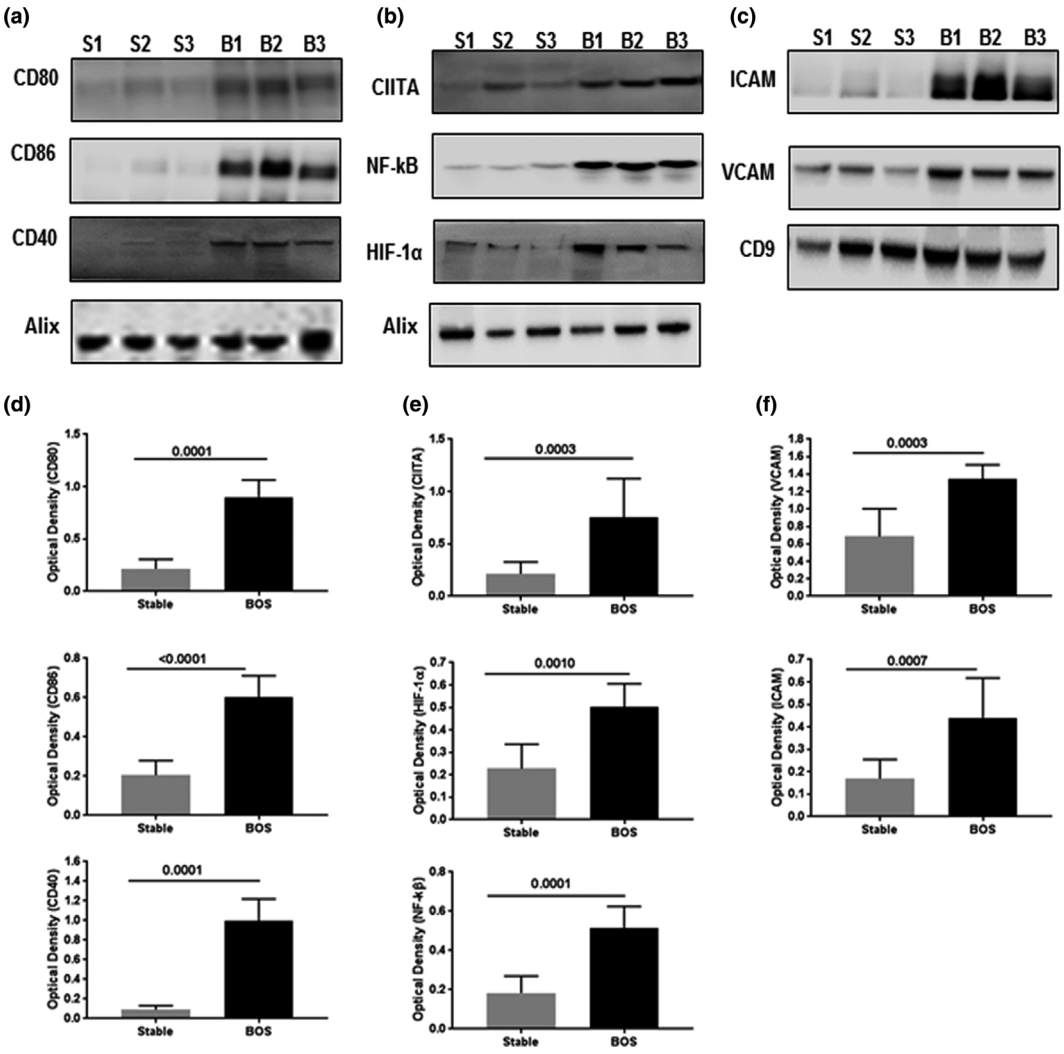

Figure 2 Increased expression of immunoregulatory proteins within circulating exosomes of LTxRs with BOS: Circulatory circulating exosomes were isolated from pediatric LTxRs with BOS (n = 6) and from stable pediatric LTxRs (n = 13). (a) Circulating exosomes from pediatric LTxRs with BOS contained co-stimulatory molecules (CD80, CD86), (b) transcription factor CIITA, NF-κB, and HIF-1α, and (c) adhesion molecules (ICAM, VCAM). CD9 and Alix (exosome-specific markers) were used as loading controls. S: Stable group; B: BOS group. Densitometry analysis of Western blot data presented in (d) co-stimulatory molecules CD40, CD80 and CD86; (e) transcription factors CIITA, NFκβ, HIFα; and MHC-II; (f) adhesion molecules ICAM and VCAM; of circulating exosomes isolated from pediatric lung transplant recipients with bronchiolitis obliterans syndrome and from stable pediatric lung transplant recipients. Image J is used to analyze intensity of bands.

analyzed serial plasma samples collected from two patients at 1, 6, and 12 months after LTx and developed BOS later and time-matched controls. Circulating exosomes isolated from time-matched stable LTxRs were used as controls. Circulating exosomes with lung-associated SAg (Col-V) were detectable in significantly higher levels in LTxRs with BOS than in stable LTxRs (Fig. 4a). The increased levels of circulating exosomes with lung SAgs were detected in the plasma samples collected at 6 and 12 months; BOS was not clinically diagnosed in these patients until 20 and 28 months, respectively (Fig. 4b). In the first LTxR with BOS (B1), BOS diagnosis was made 28 months post-transplant. The Col-V levels in exosome were significantly elevated compared with stable (S1) at 6-month (P value = 0.0080) and at 12-month (P value = 0.0001) prior to clinical diagnosis of BOS. The same was observed in the second LTxR with BOS (B2) when compared with stable (S2), in which BOS was diagnosed 20 months post-transplant, had significantly higher levels at 6-month (P value = 0.0190) and 12-month (P value = 0.0001), respectively, prior to BOS diagnosis.

These data indicate that circulating exosomes with lung SAgs are detectable early in patients prior to BOS

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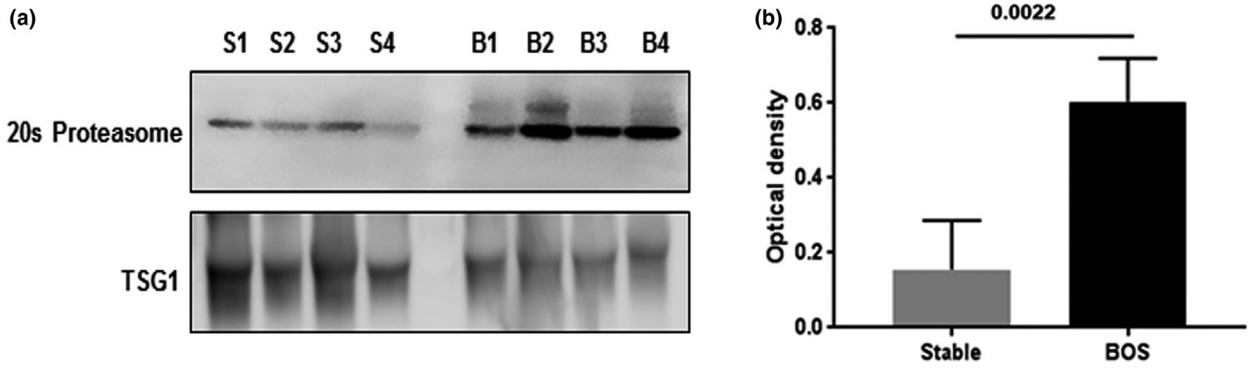

Figure 3 Increased expression of 20S proteasome components in circulating exosomes of LTxRs with BOS: (a) 20S proteasomes in circulating exosomes were analyzed in plasma samples from LTxRs with BOS and from stable LTxRs. 10μg protein was separated on 10% SDS-Page and transferred onto a polyvinylidene difluoride membrane. 20S proteasome-specific antibody was used to detect specific band of proteasome. Alix exosome-specific marker was used as loading control. S: Stable group; B: BOS group. (b) Densitometry analysis; $P = 0.0022$.

diagnosis. Even more significant is the evidence that the circulating exosomes persisted in the circulation for a long time. Therefore, induction of circulating exosomes and their persistence can induce immune responses that may lead to development of chronic rejection.

Circulating exosomes isolated from LTxRs with BOS can induce immune responses to SAgs in mice

To test our hypothesis that circulating exosomes isolated from six pediatric LTxRs with BOS would be distinct from circulating exosomes isolated from stable LTxRs in terms of immunogenicity, we injected groups of mice (6 per group) with circulating exosomes isolated from patients with and without BOS. We immunized (approximately $4.00e^{+08}$ +/− $2.82e^{+07}$ particles/ml; size 181.4 ± 4.2 nm, quantified by Nanosight) of stable exosomes and BOS exosome (approximately 4.54e + 08+/− $1.51e^{+08}$ particles/ml, size 189.9 ± 9.1 nm). There was no significant difference in number and size of exosome from both the patients with and without BOS. Plasma samples collected from

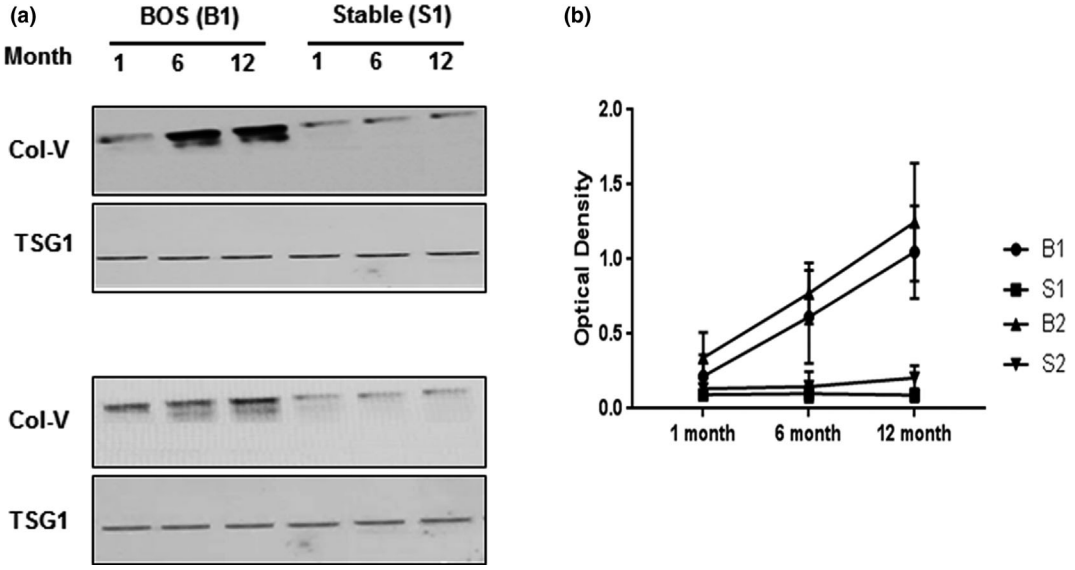

Figure 4 Kinetics of Col-V containing circulating exosomes in serially collected plasma samples from pediatric LTxRs with and without BOS: Col-V in circulating exosomes isolated from serial plasma samples collected at 6 and 12 months post-transplant (2 with BOS, 2 time-matched stable LTxRs). 10 μg of protein was separated on 10% SDS-Page and transferred onto a polyvinylidene difluoride membrane. Exosome-specific marker TSG1 was used as loading control; densitometry analysis was carried out using ImageJ software. (a) Western blotting analysis. (b) Densitometry analysis. In the first LTxR with BOS (B1), BOS diagnosis was made 28 months post-transplant; in the second LTxR with BOS (B2), BOS was diagnosed 20 months post-transplant. B1 vs. S1 (6-month P value = 0.0080, 12-month P value = 0.0001) and B2 vs. S2 (6-month P value = 0.0190, 12-month P value = 0.0001) is considered as significant. B = BOS; S: Stable.

immunized animals on days 3, 10, 20, and 30 were analyzed for Abs to lung SAgs. Meanwhile, spleen cells collected after sacrifice on day 30 were analyzed for lung-associated SAg-specific T cells and the frequency of cells secreting cytokines (IFNγ, TNFα, IL17, IL4, and IL10) was ascertained. Histopathology analysis of the lungs collected at day 30 after sacrifice was done using hematoxylin and eosin staining and trichrome staining. Significantly increased levels of Abs to lung SAgs were noted in mice immunized with circulating exosomes from pediatric LTxRs with BOS compared with mice immunized with circulating exosomes from stable pediatric LTxRs (Fig. 5a,b). Abs against lung-associated SAgs were detected in mice immunized with circulating exosomes isolated from pediatric LTxRs with BOS beginning on day 10, and we observed significant increases on days 20 and 30 for both lung-associated SAgs (Col-V, Kα1T).

Spleen cells isolated on day 30 after immunization with circulating exosomes were analyzed for the frequency of lung-associated SAg-specific cells secreting IFN-γ, TNF-α, IL-10, and IL-4 using ELISpot. As shown in Fig. 6, immunization with circulating exosomes from pediatric LTxRs with BOS resulted in a significant increase in cells secreting IFN-γ, TNFα, and IL-4 compared with circulating exosomes isolated from stable pediatric LTxRs (Fig. 5c–e). Interestingly, our results also demonstrated a significant reduction in the lung-SAgs-specific IL-10-secreting cells in mice immunized with circulating exosomes from pediatric LTxRs with BOS compared with circulating exosomes from stable pediatric LTxRs (Col-V: 71.5 ± 85.6 vs. 434.5 ± 38.9; Kα1T: 115.5 ± 23.3 vs. 374 ± 216.7, $P < 0.05$; Fig. 5f). These results demonstrate that circulating exosomes from pediatric LTxRs with BOS can not only induce humoral immune responses, but can also develop cellular responses with distinct types of cytokines. Immunization of nonrelevant antigen (ovalbumin: 100 μg) did not induce lung- SAg-specific cells secreting IFN-γ, TNF-α, IL-10, and IL-4 (Figure S2).

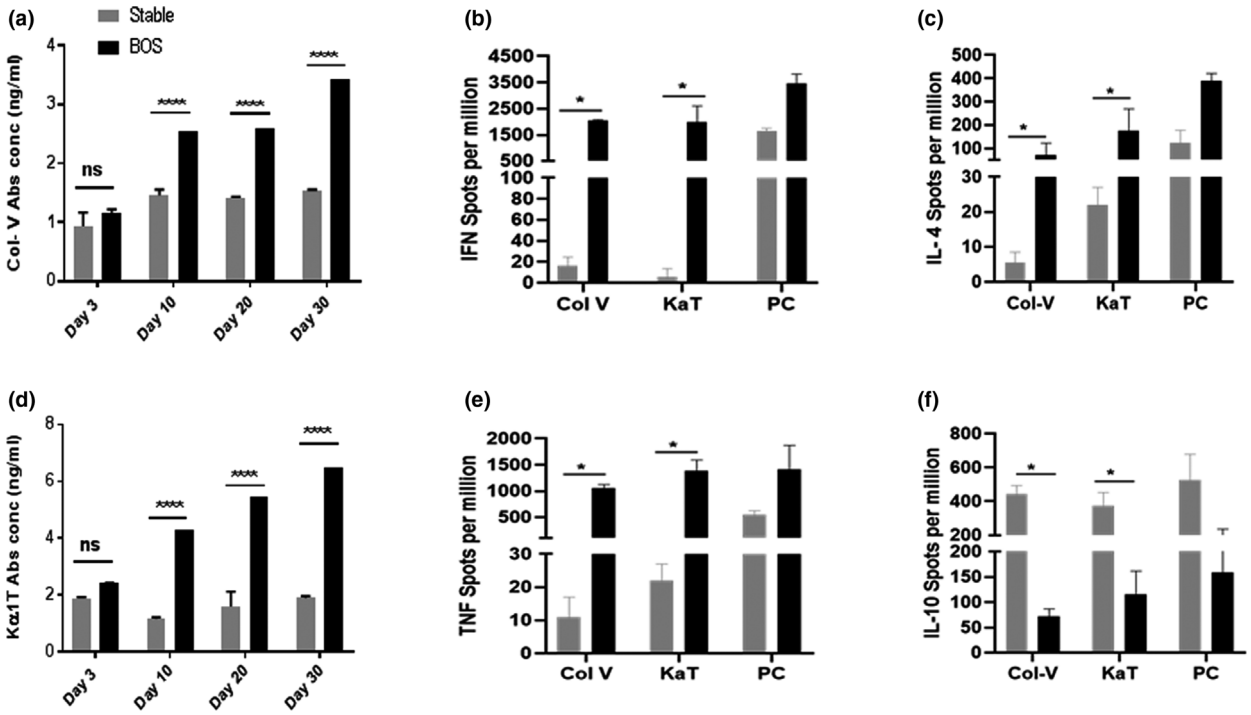

Figure 5 Induction of humoral and cellular response in mice after immunization with circulating exosomes. Sera from mice immunized with circulating exosomes isolated from six LTxRs diagnosed with BOS (pooled) and six stables (pooled) collected on day 3, 10, 20 30, and used to detect development of Abs against SAgs (Col-V, Kα1T) by ELISA. Sera of day 10 mice immunized with circulating exosomes from BOS developed Abs to Col-V: 2.55 ± 0.880 ng/ml (a) and Kα1T: 4.3 ± 1.102 ng/ml (b). *P* value, ns: Not significant; ****: <0.0001. Spleen was collected on day 30 from C57BL/6 mice immunized with circulating exosomes isolated from BOS and stable LTxRs were measured for frequency of T cells producing cytokines to lung SAgs by ELISPOT. Splenic cells collected on day 30 from mice immunized with circulating exosomes of BOS shown significant increase in T cells producing IFNγ (c), TNFα (d), and IL-4 (e) to SAgs. Stable LTxRs exosome immunized mice showed increased frequency of IL 10 producing T cells (f). *P* value, *: <0.05 considered as significant.

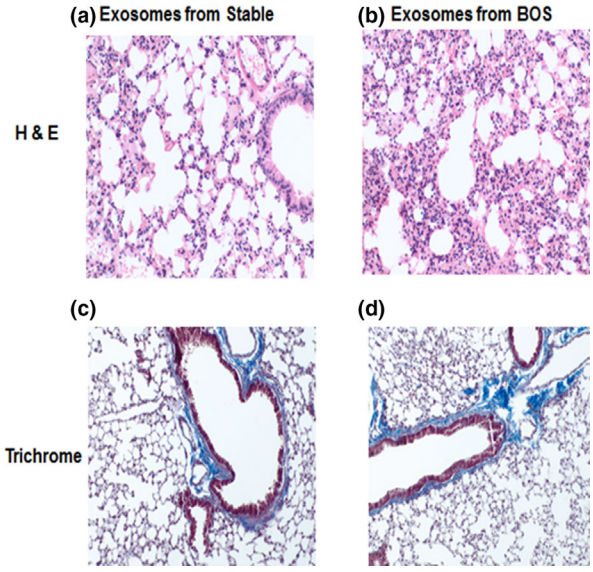

Figure 6 Histology of lung samples from mice immunized with circulating exosomes: Lungs were collected on day 30 to analyze lesions for cellular infiltration, alveolar damage, and fibrosis by hematoxylin and eosin and trichrome staining. (b and d) Alveolar lesions and fibrosis were observed in mice (6 mice/group) immunized with circulating exosomes from LTxRs with BOS. Alveolar lesions and fibrosis were more severe in these mice than in (a and c) mice immunized with circulating exosomes isolated from stable LTxRs. Images were obtained on a Leica microscope at 40×.

Histopathological analysis of animal lungs harvested on day 30 after immunization with circulating exosomes from pediatric LTxRs with BOS had moderate to severe inflammation involving approximately 70% of tissue. On the other hand, mice immunized with circulating exosomes isolated from stable pediatric LTxRs induced only mild inflammation involving approximately 15% of tissue (Fig. 6). To assess lung fibrosis trichrome staining was performed. Inflammatory cells in the bronchioles and vessels were observed in mice immunized with circulating exosomes from pediatric LTxRs with BOS, and we also observed cellular infiltration, lesions on the bronchioles, and increased fibrosis in these samples (Fig. 6d). Mice immunized with circulating exosomes from stable pediatric LTxRs showed no significant cellular infiltration and lesions.

Discussion

We recently demonstrated that circulating exosomes isolated from plasma samples and bronchoalveolar lavage fluid (BAL) of adult human LTxRs diagnosed with AR or BOS contain not only mismatched donor HLA, but also lung-associated SAgs (Col-V, Kα1T), indicating that the circulating exosomes are induced and released from the transplanted organ [23]. Few have characterized circulating exosomes in BAL and sera post-LTx. We showed that LTxRs with acute and chronic rejection induced circulating exosomes containing donor HLA and lung SAgs, along with immune-regulatory miRNAs [11,24]. Circulating exosomes interact with cells via ligand–receptor interactions [25]. Circulating exosomes released by dendritic cells (DCs) bearing donor H2 had high levels of heat shock proteins and mir-155, which are powerful inducers of DC activation [25]. Cross-dressed recipient DCs presenting donor MHC acquired via donor-derived circulating exosomes effectively activate the allo-reactive T cells through the semi-direct pathway [10,26]. Circulating exosomes have also been shown to induce autoantibodies involved in allograft rejection [27,28].

In order to determine whether pediatric LTxRs also undergo this process of induction and release of circulating exosomes, and to define the phenotypic and immunological characteristics of these circulating exosomes, we analyzed pediatric LTxRs enrolled in the CTOT-C-03 study irrespective of their gender difference. The results presented here in demonstrate increased levels of circulating exosomes containing lung-associated SAgs (Col-V and Kα1T) as well as mismatched donor HLA in pediatric LTxRs with BOS, compared to circulating exosomes isolated from stable pediatric LTxRs (Fig. 2). In addition, the circulating exosomes isolated from pediatric LTxRs with BOS contained transcription factors (CIITA, NF-κB HIFα), adhesion molecules (ICAM, VCAM), co-stimulatory molecules (CD40, CD80, CD86; Fig. 3), MHC-II (Fig. 1b), and 20S proteasome (Fig. 4). These results suggest that circulating exosomes induced during chronic rejection are immunogenic and are similar in their phenotypic and immunological profiles as those observed in rejection in adult LTx [29]. We have analyzed both sera and BAL exosomes in adult LTxRs and found they had similar pattern as in our previous studies [11].

To determine whether circulating exosomes isolated from pediatric LTxRs with BOS can be immunogenic, we immunized wildtype C57BL/6 animals with circulating exosomes isolated from pediatric LTxRs with BOS or from time-matched, stable pediatric LTxRs. Based on a published report that demonstrated that circulating exosomes containing 20S proteasome are highly immunogenic and do not require adjuvants [27], we immunized mice with circulating exosomes isolated from pediatric LTxRs with BOS or from stable pediatric LTxRs without adjuvants. Mice immunized with circulating exosomes isolated from pediatric LTxRs with BOS induced both

humoral and cellular immune responses to the lung SAgs (Col-V, Kα1T). Mice immunized with circulating exosomes isolated from stable pediatric LTxRs, on the other hand, did not induce these responses (Fig. 6).

Enumeration of cytokine-secreting cells from splenic lymphocytes of animals immunized with circulating exosomes from pediatric LTxRs with BOS clearly demonstrated a distinct pattern—that is, higher levels of SAg-specific IFN-γ- (Fig. 6c), TNFα- (Fig. 6d), and IL-4 (Fig. 6f) secreting cells and a marked reduction in IL-10-secreting cells. In contrast, splenic lymphocytes from mice immunized with circulating exosomes from stable pediatric LTxRs demonstrated a significantly higher number of SAg-specific IL-10-secreting cells and reduced pro-inflammatory cytokine-producing cells (Fig. 6f). It was surprising that circulating exosomes isolated from humans would have such a profound effect in mice. We propose that this effect could be either due to the contents (various miRNAs regulating cytokine responses) or, more likely, that the administered circulating exosomes are taken up by murine antigen-presenting cells along with miRNAs, which regulate both cytokine responses and Ab development. These results suggest that circulating exosomes can play an important role in regulating immune responses, including possibly regulating peripheral tolerance induced by IL-10-secreting cells. This is supported by our finding of immune responses against lung SAgs when immunized with circulating exosomes from pediatric LTxRs with BOS, but not after immunization with lung SAgs from stable pediatric LTxRs.

Serial analysis of circulating exosomes with lung-associated SAgs in LTxRs with BOS, though preliminary, suggests that circulating exosomes with lung-associated SAgs (Col-V and Kα1T) are present in the plasma before clinical diagnosis of BOS is possible. Furthermore, these circulating exosomes seem to persist (from 6 to 12 months), suggesting that they can induce immune responses that lead to BOS (Fig. 4). This needs further validation in a larger cohort of pediatric LTxRs with and without BOS.

Our study is subject to some limitations. The sample size is relatively small; however, one should take into consideration that this study is limited to pediatric LTxRs. Furthermore, serial analysis is done only on a limited number of patients who developed BOS due to limited availability of samples. The practical difficulty in performing cell analysis by flow cytometry is the study involves pediatric population and the volume of blood obtained is very low and hard to perform those studies. Therefore, additional analysis in a larger cohort is needed to validate the findings presented in this manuscript.

In conclusion, our results demonstrate that circulating exosomes isolated from pediatric LTxRs with BOS are distinct in their properties compared with circulating exosomes isolated from stable pediatric LTxRs with respect to their contents and biomolecules, including mismatched donor HLA, lung-associated SAgs (Col-V, Kα1T), co-stimulatory molecules, MHC-II, adhesion molecules, pro-inflammatory transcription factors, and 20S proteasome. This finding is similar to the results obtained in adult human LTxRs (9). We propose that these differences play an important role in induction of immune responses, and increase the risk of BOS development. This is supported by our results suggesting that circulating exosomes with lung SAgs can be detected in the plasma of LTxRs before BOS can be clinically diagnosed and that circulating exosomes with lung-associated SAgs persist longer than 12 months in the circulation. We propose that LTxRs with increased levels of exosomes with lung-associated SAgs can be considered for performing bronchoscopy to look for and treat subclinical causes of lung injury (i.e., low-grade acute rejection, subclinical antibody-mediated rejection, or infection) and also consider empiric prophylactic therapies such as extracorporeal photopheresis or changing baseline by addition of mTOR inhibitors.

Finally, we demonstrated that circulating exosomes are highly immunogenic in a murine model of immunization. This murine model confirmed distinct humoral and cellular immune responses when mice were immunized with circulating exosomes isolated from pediatric LTxRs with BOS or from stable pediatric LTxRs.

Authorship

MS and TM involved in research design. LDI and SCS: involved in sample collection. RR performed ELISA for HLA and assisted in writing of manuscript. MS performed research. MS, SP; TM: Data analysis: MS, TM, LDI, PSH, SCS: Writing of manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest. All authors have approved the final version of this manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Characterization of exosomes.

Figure S2. Immunization of ovalbumin and frequency of T cells producing cytokines to lung SAgs by ELISPOT

Appendix S1. ELISPOT.

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