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# Exosomes as Modulators and Biomarkers of Transplant Immunity

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## Abstract

**Purpose of Review—**Exosomes have garnered increasing interest due to their involvement in a wide array of biological processes, including immunity and regeneration. In this review, we outline our current understanding of the role of exosomes in modulating transplant immune responses and as biomarkers of allograft function or rejection.

**Recent Findings—**The exosomal effect on post-transplant immunity is heterogeneous and context dependent. They are critical for priming anti-donor T cell immunity via semi-direct presentation but have also been shown to promote tolerance to graft-expressed non-inherited maternal antigens. Post-transplant, proteomic and gene expression profiling of exosomes collected from blood, urine, or bronchoalveolar lavage can discriminate between cellular and antibody-mediated rejection and as a potential early prognostication tool.

**Summary—**Secreted by both the donor and recipient cells after solid organ transplantation, exosomes are mechanistic mediators of the allogeneic immunity and have shown promise as non-invasive biomarkers of graft function.

### Keywords

Extracellular vesicles; Exosome; Transplant biomarker; Rejection; Tolerance

## Introduction

Extracellular vesicles (EVs) are small, lipid bi-layered, non-replicative subcellular particles released from cells. The three main EVs, exosomes, microvesicles, and apoptotic bodies [1], are stratified based on features including size, originating cell compartment, surface and internal cargo, and function [2]. Exosomes are the smallest EVs (50–150 nm) and are formed from multivesicular bodies (MVBs) in a ceramide and endosomal sorting complex

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(ESCRT)-dependent manner [3, 4]. They are released via exocytosis [5] from nearly all cell types and can be found in myriad fluid compartments (e.g., blood [6], urine [7], breast milk [8], amniotic fluid [9], semen [10], tears [11], bile [12], lymph [13], saliva [14], synovial fluid [15], cerebral spinal fluid [16, 17], bronchial fluid [18, 19]). Initially dismissed alongside other EVs as cellular debris [20], exosomes have since been identified as important biologic mediators of diverse biologic functions including angiogenesis, tumor progression, injury response, and immunity [21, 22, 23]. Due to the complexity and overlap of subcellular particles, specific methodologies have been outlined to ensure proper characterization in scientific research [4, 24].

While exosomes are heterogeneous, they all express a combination of tetraspanins that include CD63, CD9, CD81, CD37, CD53, and CD82. The varied internal cargo can include proteins such as heat shock (HSP70, HSP90), transport/binding (annexins, galectin), cytoskeleton (actin, tubulin, cofilin, moesin), cytokines (TNFa, IL6), and MHC complexes, as well as lipids (flotillin, ceramide, sphingo-myelin, phosphatidylserine, cholesterol) and nucleic acids (mRNA, miRNA, long non-coding RNAs) [5, 19, 25, 26] from the parent cell.

Exosomes are thus of particular interest in the setting of transplantation where graft- and recipient-derived particles can provide granular information about the state of the transplanted organ and/or anti-donor immune response, respectively. Further, there is compelling evidence that exosomes are not just potential biomarkers but also critical mediators of allogeneic immunity making them novel therapeutic targets. Herein, we will review the current literature on exosome function after solid organ transplantation.

## Antigen Presentation and Rejection

Naïve T cell priming requires T cell receptor (TCR) recognition of peptide antigen bound to the major histocompatibility complex (MHC) of an antigen presenting cell (APC). After solid organ transplantation, donor peptide from the allograft primes the allogeneic response via three different presentation pathways: the indirect, direct, and semi-direct [27, 28]. The indirect pathway requires donor peptide to be captured, internalized, and processed by a professional APC then presented on an MHC class II molecule to naïve T cells. This priming pathway is slower, but critical for the emergence of later alloimmunity and chronic graft rejection [29, 30]. In the direct pathway, donor APCs within the allograft, activated by transplant-associated inflammation, traffic to secondary lymph organs and present allo-peptide-MHC to recipient T cells. This “passenger leukocyte model” was historically considered the dominant form of early allo-T cell priming leading to rejection [31].

However, preclinical murine heart, skin, and islet transplant models all showed a paucity of detectable donor passenger APCs in the recipient draining lymph nodes/spleens early after transplantation and no donor cells by day 7 post-transplant [32, 33••, 34••]. How so few donor APCs could induce such a rapid and robust anti-donor response remained an open question until the paradigm shifting descriptions of semi-direct presentation. Semi-direct presentation occurs when intact donor MHC and peptide are transferred from donor cells and presented on self-APCs. This process was also termed “cross decoration” [35, 36] and

mechanistic work by Liu et al. [33••] and Marino et al. [34••] showed that cross presentation is mediated by donor-derived exosomes expressing donor class I and II MHC that bind to recipient DCs in the secondary lymph node. Whether this binding is non-specific, receptor driven, or both remains unknown. Blocking exosome release by the donor allograft at the time of transplant decreased donor antigen cross presentation on recipient DC [33••, 34••] and significantly increased graft survival [20]. This antigen/MHC transfer by exosomes appears to have broad biologic relevance, including in cancer immunity where tumor-derived exosomes can prime cytotoxic CD8 T cells by transporting tumor antigen to host DCs [37].

One reason that exosomes may be particularly effective at priming anti-donor T cell responses is that in addition to donor antigen, exosomes have been shown to carry costimulatory molecules and pro-inflammatory microRNA. After lung transplantation, only exosomes from rejecting patients expressed CD40, CD80, and CD86 [38], while exosomes from patients with acute or chronic lung rejection contained miR-182 and 155 (inflammation), miR-92a (endothelial activation), and miR-142-5p (associated with antibody-mediated rejection (AMR)) [38, 39]. In skin transplant models, exosomes derived from mature DCs expressed high levels of MHC class I/II, ICAM-1, and the costimulatory molecules CD80 and CD86 and accelerated rejection [40]. Moreover, exosomes from bronchoalveolar lavage (BAL) fluid in patients with acute lung rejection showed increased expression of genes involved in pathways for both innate and adaptive immune systems as well as olfactory receptor genes and downregulation of genes associated with allograft quiescence (CXCL16, IL-33, EEA-1) [39]. Based on this growing body of literature, exosome-mediated semi-direct presentation is now considered the dominant mode of early anti-donor T cell priming [41] with endocytosed donor-derived exosomes presumed to be an additive source of antigen for indirect presentation [42].

## Tolerance

As noted, the cellular context of released exosomes is a major determinant of subsequent function. In murine transplant studies, Ma et al. [43] built on data showing that exosomes from immature DCs prolonged intestinal and cardiac allograft survival [44, 45, 46] and found that immature-DC derived exosomes promoted long-term liver allograft survival in rats when co-administered with regulatory T cells by enhancing donor-specific Treg expansion and stability in a DC-dependent manner [43].

Studying kidney transplant outcomes between non-haploidentical siblings, Burlingham et al. [47] observed a paradoxical finding wherein kidney allografts expressing the maternal, but not paternal, non-inherited HLA allele had higher rates of early acute rejection yet greater long-term graft survival. Tolerance to maternal cells and associated non-inherited maternal antigens (NIMA) beyond fetal life has previously been ascribed to multiple mechanisms including induction of regulatory T cells (Treg) [48] and induction of B cell anergy during development [49]. Expanding on these findings, Bracamonte et al. [50•] newly showed that persistent maternal microchimerism (NIMA tolerance) was also mechanistically linked to circulating exosomes expressing the non-inherited MHC molecules. As predicted, these exosomes increased the anti-maternal CD8 T cell response via semi-direct presentation, but unexpectedly arrested anti-maternal CD4 T cell responses in a PD-L1-dependent manner.

Since exosomes can carry immunoregulatory molecules/microRNA [51], the authors posited that the NIMA-expressing exosomes delivered these molecules to host dendritic cells to enhance PD-L1 expression. Functionally, this “split tolerance” or “Janus effect” with enhanced CD8- and inhibited CD4-T cell responses helps explain the observation by Burlingham et al. [47] showing increased early acute rejection yet prolonged graft survival of NIMA-expressing kidney transplants.

## Biomarkers

The standard of care for organ transplant function surveillance includes non-specific serologic testing and invasive biopsy procedures. For example, in kidney transplantation, a rise in serum creatinine is a late manifestation of injury, cannot differentiate etiology of disease, and is often due to transient non-pathologic hemodynamic shifts [52]. Detection of donor-specific antibodies (DSAs) alone is also nei-ther specific nor sensitive enough to diagnose AMR [52]. Thus, there is a need for a non-invasive, specific, and early indicator of post-transplant allograft rejection.

The plasma level of donor-derived cell free DNA (dd-cfDNA) as a ratio of total cfDNA is a newer screening modality for solid organ transplant rejection that has shown promise in kidney [53, 54, 55] and heart [56, 57] transplant patients. The testing is non-invasive and samples are shelf-stable at room temperature for up to a week, making analysis at specialized central labs possible. Both antibody-mediated rejection and cellular rejection associate with increased dd-cfDNA levels, and though AMR consistently induces higher dd-cfDNA levels than acute cellular rejection (ACR), no threshold value has been shown to reliably differentiate one type of rejection from the other. A newer caveat to cfDNA testing was discovered during the COVID pandemic where infection with the virus led to marked rises in total cfDNA, thus depressing the dd-cfDNA ratio and leading to potential false negative (missed rejection) results [58, 59] .

Graft-derived exosomes and extracellular vesicles are also readily obtainable from accessible fluids, including blood and urine, and carry a broad array of intracellular and membrane components, including nucleic acids, from their parent cell [60]. Exosomes are not as easily isolated or stored as cfDNA samples, but the diverse protein, lipid, and RNA components make exosomes a more complete “snapshot” of their cells of origin and make them ideal biomarker candidates. Indeed, early studies have shown that exosomes hold promise as markers of rejection and allograft function after heart, kidney, lung, and islet transplantation [60].

### Kidney

Kidney transplant presents a unique opportunity to use both serum and urinary exosomes to monitor allograft function. Since urinary exosomes can be derived from any cell in contact with the urinary space, they provide a broad picture of the allograft environment. In fact, in disease states such as kidney transplantation or chronic kidney diseases, the number of urinary EVs, including exosomes, increases, supporting EVs as a mechanistically relevant biomarker [61]. Suthanthiran et al. [62] previously found that sequencing the urine sediment provided useful prognostic and diagnostic information. They identified a urinary

mRNA signature enriched for activated T cells that associated with acute cellular graft rejection (ACR), which was supported by a subsequent proteomic study by Park et al. that found elevated CD3 expression in urinary exosomes from patients with acute rejection [63]. Beyond T cell-associated proteins, urinary exosomes from patients with ACR also have increased expression of proteins associated with kidney injury (APOM), inflammation and innate immune responses (CLCA1, homopexin, tetraspanin-1), and coagulation (PROS1) [64•, 65, 66] identifying additional relevant pathogenic pathways.

In AMR, exosomes may prove particularly useful since the cell mediators of disease (B cells or plasma cells) may not be present in significant numbers in the graft itself. Jung et al. [67] found six EV proteins in urine that were significantly upregulated in chronic AMR patients compared to long-term graft survival patients: PIGR, APOA1, HHPX, AZGP1, CP, and TTR. Specifically, AZGP1, a zinc binding glycoprotein implicated in several diverse processes including antigen processing, lipolysis, and cachexia, was found to be an AMR-specific proteomic biomarker [67]. Additionally, serum exosomes from patients with AMR have increased RNA levels of *gp130*, *CCL4*, *TNF*, *SH2D1B*, *CAV1*, and *atypical chemokine receptor 1*, consistent with increased systemic inflammation [68].

Moreover, much as early allograft gene transcripts can predict long-term transplant outcomes [69], exosomal protein content also correlates with late graft outcomes. A recent study described 66 urinary extracellular vesicle proteins associated with stable post-transplant kidney function [62, 70] and identified the stress-response metabolism protein PCK2/PEPCK found as an independent predictor of long-term graft function [66]. PCK2 levels in urinary EVs (size 100–400 nm, which broadly included exosomes and microvesicles) on 1-day post-transplant positively correlated with both 6- and 12-month eGFR.

Exosomes can also identify non-immune-mediated kidney allograft pathology. Increased urine volume and decreased urinary osmolality immediately post-transplant correlated with decreased AQP-2 in urinary EVs. Subsequent recovery in exosome AQP-2 levels by day 6 post-transplant coincided with normalization of urine parameters, supporting the hypothesis that urinary exosomes reflect the intragraft cellular environment [71]. Additionally, in separate studies of post-transplant hypertension, ENaC subunits were elevated in urinary exosomes of albuminuric kidney patients [72] while the tubular $Na^+$-$K^+$ $-2Cl^-$ cotransporter was increased in cyclosporine-A-associated hypertension [73, 74], and the thiazide-sensitive sodium chloride transporter (NCC) was increased with tacrolimus use [75]. Serum exosomes also have utility as biomarkers for graft injury with increased expression of the kidney-specific antigens collagen type IV and fibronectin associating with transplant glomerulopathy and graft loss [76••, 77•].

## Heart

Non-invasive imaging modalities such as echocardiography can provide information on allograft function but not pathogenesis, leaving endomyocardial biopsy as the gold standard to assess disease in heart transplant patients. After cardiac transplantation, circulating plasma-derived EVs show promise as a non-invasive alternative to monitor graft rejection and function [60]. Heart transplant alters the exosomal proteome independent of rejection

[78] and Castellani et al. [79•] found that the total number of EVs was significantly greater in ACR and AMR patients compared to non-rejecting controls. Specifically, there was an increase in the number of smaller EVs (30–150 nm) expressing CD9, CD63, and CD81, specifically suggesting a mechanistic role for exosomes [79•]. The EVs were also found to express proteins that could discriminate between ACR (CD2, CD3, HLA-1, CD41b, ROR1, SSEA-4) and AMR (CD19, CD20, HLA-II, CD25, and CD326) and which encouragingly also correlated with previously described mediators of disease [79•]. Furthermore, in patients diagnosed with coronary artery vasculopathy (CAV), there were significantly higher levels of the cardiac-specific antigens vimentin and cardiac myosin found in the exosomes compared with stable heart transplant patients, suggesting that exosomes can diagnose both acute and chronic forms of graft injury [77•].

## Lung

EVs can be collected from both the sera and BAL fluid of lung transplant patients, which are significantly lower risk procedures in comparison to lung biopsy [18]. Lung transplant recipients with both acute rejection (AR) and chronic lung rejection manifesting as bronchiolitis obliterans (BOS) have been found to have EVs containing donor HLA and lung antigen that correlate with early detection of disease. Gunasekaran et al. detected higher donor, but not recipient, HLA expression, as well as increases in pulmonary antigens collagen-V (Col-V) and K-alpha 1 tubulin (K1T) in both serum and BAL-derived exosomes from both BOS and AR patients compared to stable transplant controls. In fact, increased serum exosomal Col-V preceded the clinical diagnosis of rejection by as much as 2 months [38, 80]. Sharma et al. confirmed elevated levels of Col-V and K1T in serum exosomes from lung transplant patients diagnosed with BOS and also found that serum exosomes in patients with detectable DSA had greater pulmonary antigen levels compared to DSA-negative recipients, together suggesting that exosomes may be early markers of subclinical graft injury [77•].

## Islets

Recent translational work using clinical samples and a humanized mouse model found that allo- and xenogeneic human islets release donor HLA-expressing exosomes that can be tracked in the recipient plasma to monitor graft health [81••]. In the preclinical mouse model, when acute islet rejection was induced by adoptive transfer of islet-sensitized T cells, a significant drop in xenoexosomes occurred by day 1 but hyperglycemia was not detected until day 5. Further, islet-derived exosomes from rejecting animals showed altered proteomic cargo including complement C3 and homopexin along with a range of almost 40 microRNAs. By assaying for the known donor-recipient HLA mismatch, transplanted islet-derived exosomes from the plasma of type 1 diabetic human recipients could similarly be detected in recipient plasma up to 5 years after transplantation. Intriguingly, in the one patient that had a recurrence of autoimmune diabetes, a significant drop in the number of donor exosomes preceded the clinical parameters of rejection (hyperglycemia, C-peptide levels) by over 6 months.

## Conclusion

Exosomes and other EVs are now widely recognized as mediators and biomarkers of immune activation and graft function after solid organ transplantation. The exosome compartment is dynamic and heterogeneous with the protein and nucleic acid content of released exosomes (and thus their downstream function) dependent upon the state of the parent cell. Activated DCs release exosomes with greater MHC class I/II and costimulatory molecule expression than naïve DCs, and in murine models, donor exosomes expressing donor MHC I and II have been shown as critical modulators of graft rejection by priming the allogeneic T cell response via semi-direct presentation [28]. Conversely, exosomes from naïve DCs adoptively transferred along with regulatory T cells prolong liver allograft survival by stabilizing and expanding the Treg population [48]. Additionally, exosomes derived from maternal microchimerism induce “split tolerance” to non-inherited maternal antigens post-transplant, with an increased risk of early cellular rejection likely via semi-direct priming, but paradoxically better over-all graft survival associated with induced PD-L1 expression by donor antigen-expressing recipient APCs [50•].

As biomarkers, exosomes are well positioned to provide information about both the allograft and the recipient’s allogeneic immune response. Expression of organ-specific antigens by exosomes is associated with graft dysfunction across solid organ transplantation, while differentially expressed exosomal protein or RNA content has been shown to correlate with ACR or AMR in kidney, heart, and lung transplant recipients. Early post-transplant PCK2/PEPCK expression in urinary exosomes has also been shown to have long-term kidney graft survival prognostic value [66, 76••], and a reduction in circulating donor-derived exosomes appears to precede clinically detectable islet loss by 6 months [39]. Released by essentially all allograft cells as well as infiltrating immune cells, exosomes show great promise as a potential “liquid biopsy” tool. In fact, exosomes are now being used clinically to screen patients at risk for high-grade prostate cancer [82] (ExoDx, Exosome Diagnostics, Waltham, MA).

Given their small size and inherent heterogeneity, exosome isolation and analysis platforms will need continued refinement for widespread clinical use, but their mechanistic role in solid organ alloimmunity and potential use as biomarkers is now undeniable. The field of extracellular vesicle research is primed for exciting breakthroughs in the near future that will add significantly to our understanding of post-transplant biology and patient care.

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