





Impact of donor extracellular vesicle release on recipient cell “cross-dressing” following clinical liver and kidney transplantation

Sotiris Mastoridis¹  | María-Carlota Londoño^{1,2} | Ada Kurt¹ | Elisavet Kodela¹ | Elena Crespo³ | John Mason⁴ | Oriol Bestard³  | Marc Martínez-Llordella¹  | Alberto Sánchez-Fueyo¹ 

¹Medical Research Council (MRC) Centre for Transplantation, Institute of Liver Studies, King's College London, London, United Kingdom

²Liver Unit, Hospital Clinic Barcelona, IDIBAPS, CIBEREHD, Barcelona, Spain

³Kidney Transplant Unit, Bellvitge University Hospital, Barcelona, Spain

⁴Department of Physiology, Anatomy & Genetics, University of Oxford, United Kingdom

Correspondence

Alberto Sánchez-Fueyo
Email: sanchez_fueyo@kcl.ac.uk

Funding information

King's College Hospital Medical Research Trust; National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' National Health Service Foundation Trust and King's College London; Medical Research Council Centre for Transplantation, Medical Research Council Centre for Transplantation, Grant/Award Number: J006742/1

Abstract

In several murine models of transplantation, the “cross-dressing” of recipient antigen presenting cells (APCs) with intact donor major histocompatibility complex (MHC) derived from allograft-released small extracellular vesicles (sEVs) has been recently described as a key mechanism in eliciting and sustaining alloimmune responses. Investigation of these processes in clinical organ transplantation has, however, been hampered by the lack of sensitivity of conventional instruments and assays. We have employed advanced imaging flow cytometry (iFCM) to explore the kinetics of allograft sEV release and the extent to which donor sEVs might induce cross-dressing following liver and kidney transplantation. We report for the first time that recipient APC cross-dressing can be transiently detected in the circulation shortly after liver, but not kidney, transplantation in association with the release of HLA-bearing allograft-derived sEVs. In liver transplant recipients the majority of circulating cells exhibiting donor HLA are indeed cross-dressed cells and not passenger leukocytes. In keeping with experimental animal data, the downstream functional consequences of the transfer of circulating sEVs harvested from human transplant recipients varies depending on the type of transplant and time posttransplant. sEVs released shortly after liver, but not kidney, transplantation exhibit immunoinhibitory effects that could influence liver allograft immunogenicity.

KEYWORDS

antigen presentation/recognition, basic (laboratory) research/science, immune regulation, immunobiology, kidney transplantation/nephrology, liver allograft function/dysfunction, liver transplantation/hepatology, lymphocyte biology: activation, translational research/science

Abbreviations: APC, antigen presenting cell; BF, brightfield; CD, cross-dressed; CFDA-SE, 5-(and-6-)carboxyfluorescein diacetate succinimidyl ester; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; ESRD, end-stage liver disease; EV, extracellular vesicle; fPBS, filtered phosphate-buffered saline; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HHT, hereditary hemorrhagic telangiectasia; HLA, human leukocyte antigen; iFCM, advanced imaging flow cytometry; ISx, Amins ImageStream[®] MKII; KTx, kidney transplant; LTx, liver transplant; MHC, major histocompatibility complex; NAFLD, nonalcoholic fatty liver disease; PBMC, peripheral blood mononuclear cell; PD-L1, programmed death ligand 1; PL, passenger leukocyte; PPP, platelet-poor plasma; PSC, primary sclerosing cholangitis; SBDS, similarity bright detail score; SEC, size-exclusion chromatography; sEV, small extracellular vesicle; SSC, side-scatter.

Marc Martínez-Llordella and Alberto Sánchez-Fueyo Shared senior authorship

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2020 The Authors. American Journal of Transplantation published by Wiley Periodicals LLC on behalf of The American Society of Transplantation and the American Society of Transplant Surgeons

1 | INTRODUCTION

Induction of immune responses to major histocompatibility complex (MHC) mismatched allografts has been traditionally considered to result from the migration of graft-derived antigen presenting cells (APCs), or “passenger leukocytes,” to secondary lymphoid organs where they directly present intact donor MHC molecules to recipient alloreactive T cells. This model has recently been called into question, with mounting data from both vascularized and non-vascularized animal transplant models showing that in the early post-transplant period few if any such passenger donor leukocytes are found in secondary lymphoid organs.¹⁻³ In contrast, within hours of transplantation, a much larger number of recipient APCs in the graft-draining lymph nodes or spleen carry donor-type MHC molecules on their surface.^{1,3,4} The transfer of intact donor MHC to recipient APCs is known as “cross-dressing” and is partly mediated by allograft-derived extracellular vesicles (EVs) released into the circulation.^{1,3} EVs are nanosized membranous particles released by most cell subsets, including graft parenchymal cells, endothelium, and passenger leukocytes. Owing to their small size and their capacity to transport a variety of biomolecules, they function as key mediators of intercellular communication, relayed across a spectrum of biofluids and tissue types. Among their surface protein cargo, EVs carry intact MHC and peptide/MHC complexes, which confers to them the capacity to activate T cells.⁵⁻⁷ Although their direct allostimulatory capacity is weak and requires high EV concentrations,^{5,6,8} the potential for such peptide/MHC complexes to elicit alloresponses can be markedly enhanced if transferred to APCs.⁸⁻¹² Among the range of EV subtypes, those most widely reported to play a key part in the cross-dressing of APCs are “exosomes,” small EVs (50-200 nm) of endosomal origin, exhibiting characteristic morphological features, and thought to bear a characteristic complement of tetraspanin protein surface markers. In recent years, it has become evident that commonly employed exosome isolation techniques in fact isolate heterogeneous populations of small EVs with the size characteristics of exosomes, not necessarily of endosomal origin. In the interests of definitional accuracy, such isolates are increasingly referred to as small EVs (sEVs). Though no single marker is known to uniquely identify the exosomal fraction of such isolates, proteomic analyses of sEV subpopulations have identified CD63 as a candidate – thus sEVs bearing CD63 are putatively designated exosomes.¹³⁻¹⁵

In most experimental transplant models, allo-MHC cross-dressing has been shown to be a highly immunogenic phenomenon. The capacity of cross-dressed dendritic cells (DCs) to elicit alloreactive T cell responses *in vitro* was first described by Herrera and colleagues¹⁶ and subsequently confirmed *in vivo* by the same group in a mouse skin transplant model.⁴ More recently, Benichou and colleagues showed that injection into naïve mice of allogeneic EVs or of recipient cells cross-dressed *in vitro* with donor EVs was sufficient to elicit a donor-specific inflammatory alloresponse *in vivo*.³ Furthermore, Morelli and colleagues demonstrated that upon acquiring donor EVs, recipient DCs became activated, stimulated alloreactive T cells, and promoted allograft rejection. The immunogenicity of allo-MHC

cross-dressed DCs is not restricted to secondary lymphoid organs and is also apparent in the allograft itself. Thus, in rodent models of islet and kidney transplantation, rejection was preceded by the engagement of effector T cells with cross-dressed graft-infiltrating host DCs.¹⁷ However, in contrast to these observations, in a model of spontaneous tolerance following MHC-mismatched murine liver transplantation, recipient intrahepatic DCs cross-dressed with donor sEVs markedly suppressed alloreactive host T cell responses,¹⁸ suggesting that the outcome of recipient APC cross-dressing may vary depending on the type of allograft.

The characterization of the kinetics of allograft sEV release and elucidation of the extent to which donor sEVs induce recipient APC cross-dressing following clinical organ transplantation have been hampered by the lack of sensitivity of conventional flow cytometric instruments. We recently developed a method by which circulating small EVs, including exosomes, can be characterized and quantified using advanced imaging flow cytometry (iFCM).¹³ We report here the results of applying this technique to investigate the kinetics of donor-derived, CD63 bearing, sEV release in the clinical contexts of liver and kidney transplantation and describe for the first time the development of recipient APC cross-dressing in clinical transplantation. Our findings confirm that following liver, but not kidney, transplantation, the majority of circulating cells exhibiting donor MHC are cross-dressed cells and not passenger leukocytes. This is, however, a transient phenomenon, which is no longer detectable beyond the first weeks posttransplant regardless of the liver allograft status.

2 | MATERIALS AND METHODS

2.1 | Study population and design

The study was approved by the North of Scotland Research Ethics Committee (REC Ref: 15/NS/0062) and all participants provided written informed consent. Peripheral blood samples were collected from liver (LTx) and kidney (KTx) transplant recipients immediately before transplantation and at posttransplant days 1, 4, 10, and 30. Additional sequential peripheral blood specimens were collected from stable LTx >3 years posttransplant who experienced an episode of histologically confirmed rejection following attempted complete immunosuppression withdrawal (clinicaltrials.gov, NCT02498977). All participants were human leukocyte antigen (HLA) genotyped by polymerase chain reaction sequence-specific oligonucleotide probes (PCR-SSOP, Luminex, Austin, TX) at the Clinical Transplantation Laboratory at Guy's Hospital London.

2.2 | Circulating sEV and peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood was collected following standard procedures that minimize contamination by platelets and platelet-derived vesicles.¹⁹ Following cubital vein venepuncture, 3 mL of blood was discarded

before collection of 9 mL into BD Vacutainer® K3-EDTA-coated collection tubes (Becton, Dickinson, Franklin Lakes, NJ). Tubes were inverted gently 5 times and blood was allowed to sit at room temperature for 30 minutes. Whole blood was centrifuged at 400 g (Heraeus Megafuge 40R with 195 mm 7500-3180 rotor, ThermoFisher Scientific, Waltham, MA) for 10 minutes at 20°C to remove cells. The plasma layer was collected and centrifuged again at 5000 g for 10 minutes at 20°C. The resulting platelet-poor plasma (PPP) was aliquoted and stored at -80°C. small EVs (sEVs) were isolated from PPP by size-exclusion chromatography (SEC) using CellGS Exo-Spin™ Mini Columns according to manufacturer's instructions, and as previously validated.^{13,20,21} Specifically, PPP was thawed and centrifuged at 16 000 g for 30 minutes (Sorval Legend Micro 21R equipped with 7500-3424 rotor, ThermoFisher Scientific) to remove large particles and cell fragments, and supernatant transferred to a new microcentrifuge tube and set on ice. CellGS columns were equilibrated 15 minutes prior to use. Using a micropipette, the preservative buffer on top of the column was discarded, the outlet plug removed, and 200 µL of 0.22 µm-filtered phosphate-buffered saline (fPBS) was added to the top of the column. The column was then centrifuged for 10 seconds at 50 g (Centrifuge 5430 R, equipped with FA-45-24-11-HS rotor, Eppendorf, Hamburg, Germany). 0.1 mL PPP was applied to the column and centrifuged at 50 g for 60 seconds. The column was then transferred to a new 1.5 mL collection tube, 200 µL fPBS applied to the top, and elution of sEVs performed by a final centrifugation step at 50 g for 60 seconds. Confirmation of sEV isolation was performed as previously described (Figure S1).¹³ Eluate volume was topped up to 200 µL if necessary with fPBS, aliquoted, and stored at -80°C. Peripheral blood mononuclear cell (PBMC) isolation was performed by Ficoll-Paque (GE Healthcare, Danderyd, Sweden) density-gradient sedimentation and stored in liquid nitrogen.

2.3 | Circulating sEV analyses by ImageStream^x

Multispectral imaging flow cytometric acquisition of sEVs was performed using Amnis ImageStream^x MKII (ISx, EMD Millipore, Seattle, WA) as previously described.¹³ ISx fluidics were set at low speed, sensitivity set to high, magnification at 60x, core size 7 µm, and the "Hide Beads" option unchecked before every acquisition in order to visualize speed beads in analyses. The ISx was equipped with the following lasers run at maximal power to ensure maximal sensitivity: 405 nm (120 mW), 488 nm (200 mW), 561 nm (200 mW), and 642 nm (150 mW). Upon each start-up, the instrument calibration tool ASSIST® was performed to optimize performance and consistency. Two channels (Ch01 and Ch09) were set to brightfield (BF), permitting spatial coordination between cameras. Channel 12 was set to side-scatter (SSC), and further fluorescence channels were used for antibody detection as required. To avoid the risk of coincident particle detection, sEV samples were not run at concentrations greater than 10¹⁰ objects/mL.²² sEV labeling was performed as previously described.^{13,22-24} Briefly, fPBS diluted sEV sample was incubated with Fc receptor blocker (Human TruStain FcX™, BioLegend, San Diego, CA) for 10 minutes at room temperature. The pan-EV label 5-(and-6-)-carboxyfluorescein diacetate succinimidyl

ester (CFDA-SE, ThermoFisher) was added at a concentration of 10 µM and incubated in the dark for 10 minutes at 4°C. This was followed by further staining in the dark at room temperature for 15 minutes with master-mix preparations of the following monoclonal antibodies (mAbs) as appropriate: anti-human CD63-PE (BioLegend, H5C6); PD-L1-BV605 (BioLegend, 29E.2A3); IgG1 (BioLegend, MOPC-21); IgG2b (BioLegend, MPC-11); HLA-B7/27-APC (MACS); HLA-A3-APC (eBioscience, San Diego, CA); HLA-A2-APC (BioLegend); HLA-A2-PECy7 (BioLegend); HLA-A2-PE (BD Biosciences, San Jose, CA); HLA-B8-APC (MACS); HLA-A24-FITC (MBL); HLA-A1-Biotin (OneLambda, Canoga Park, CA); HLA-A11-Biotin (OneLambda); HLA-A26-Biotin (OneLambda). All mAbs were centrifuged at 5000 g for 5 minutes prior to use, as clumps could be mistaken for EVs.^{25,26} To avoid false positive events, all antibodies used were run on ISx in buffer (fPBS) alone to ensure antibody clumps were not present. Technical controls and isotype controls, in conjunction with fluorescence-minus-one (FMO) controls, were employed where appropriate for EV gating. All samples were acquired using INSPIRE® software. Data analyses were performed, and spectral compensation matrices produced, using ISx Data Exploration and Analysis Software (IDEAS®).

2.4 | PBMC analyses by ImageStream^x

PBMCs were incubated with viability dye-v450 (eBioscience); according to manufacturer's instructions, and Fc receptor block was performed (Human TruStain FcX™, BioLegend). For surface staining, cells were incubated for 20 minutes at 4°C with different combinations of the following antibodies: CD63-PE7 (BioLegend), PD-L1-BV605 (BioLegend), and appropriate HLA antibodies among those described. Following staining cells were fixed in fixation buffer (BioLegend). Whole-cell image acquisition was performed with fluidics set at low speed, sensitivity set to high, magnification at 60x, and SSC and BF channels set as above. Live cells were identified after sequential gating for (1) single cells, using BF aspect ratio vs Area Feature gating; (2) in focus cells, using BF gradient root mean square (RMS) Feature; and (3) LIVE/DEAD™-stain negative cells (Ch02). Colocalization analyses were performed using the Similarity Bright Detail Score (SBDS) feature in IDEAS® as previously described.^{13,27-29} ISx enables quantitative analysis of the degree of colocalization between fluorophores on a pixel-by-pixel basis by comparing digital images captured in each of its image detection channels. The Similarity Bright Detail R3 algorithm within IDEAS® produces a score (SBDS) serving as a measure of the degree of colocalization between these.

2.5 | PBMC analyses by conventional flow cytometry

PBMCs were incubated with LIVE/DEAD™-APC (ThermoFisher Scientific) according to manufacturer's instructions, and treated with Fc receptor blocker (Human TruStain FcX™, BioLegend). For surface staining, cells were incubated for 20 minutes at 4°C with

different combinations of the following fluorochrome conjugated antibodies: CD19-BV711, CD3-BV605, HLA-DR-BV785, CD123-PE-Dazzle, CD11c-PE, CD16-PerCP-Cy5.5 (BioLegend); Viability-V450, CD14-AF780 (eBioscience); and appropriate HLA antibodies. Following staining cells were fixed in fixation buffer (BioLegend). Conventional flow cytometry was performed using LSR Fortessa flow cytometer (BD) and analyzed using FlowJo software v7.6 (Tree Star, Ashland, OR).

2.6 | Generation of monocyte-derived dendritic cells (DCs)

Monocytes from HLA-A2 negative healthy volunteers were isolated from PBMCs using the EasySep™ Human CD14 positive selection kit (Stemcell Technologies, Vancouver, BC). Monocyte-derived DCs were generated by culturing freshly isolated monocytes for 5 days in EV-free complete media with 50 ng/mL of granulocyte-macrophage colony-stimulation factor (GM-CSF) and 800 U/mL IL-4 (R&D Systems, Minneapolis, MN), at 37°C in 5% CO₂. Following 5 days of culture, cells were harvested, washed, and cultured for a further 48 hours with a cytokine cocktail of GM-CSF (50 ng/mL), interleukin-4 (IL-4; 800 U/mL), tumor necrosis factor alpha (TNFα; 10 ng/mL), IL-6 (10 ng/mL), IL-1β (10 ng/mL), and prostaglandin E2 (1 μg/mL, all from R&D Systems) to induce DC maturation.³⁰ DCs generation was confirmed by flow cytometric analysis of the expression of surface markers assessed at days 0, 5, and 7 using the following conjugated antibodies: CD14-V450, CD40-APCCy7, CD1a-BV605, CCR7-BV711, HLA-DR-PerCPCy5, CD80-FITC, CD86-PE (Figure S2).

2.7 | Cross-dressing of monocyte-derived DC and CD8 T cell proliferation assays

Monocyte-derived DCs were co-cultured for 24 hours in serum-free media (X-VIVO™ 15, Lonza, Basel, Switzerland) with sEVs freshly isolated as described previously from HLA-A2 positive and HLA-A2 negative healthy volunteers; 5 × 10⁵ DCs were co-cultured with 50 μl sEV isolate, and successful cross-dressing of DCs confirmed by flow cytometric analysis of HLA-A2 (Figure 4A-B). This approach achieves consistent cellular cross-dressing, as we have previously described.¹³ Harvested DCs were immediately cultured for a further 5 days with 10⁵ CD8 T cells at a 1:1 or 1:2 ratio. CD8 T cells were isolated from PBMCs from HLA-A2 negative and HLA-A2 positive healthy controls using the EasySep™ Human CD8 + T Cell Isolation Kit (Stemcell Technologies). To detect T cell proliferation, isolated CD8 T cells were incubated with eFluor™ 670 proliferation dye (eBioscience) for 20 minutes in the dark at room temperature and then washed and counted. At the end of co-culture, CD8 proliferation was quantified by flow cytometry. In order to determine the role of human sEVs in allostimulation, equivalent experiments were conducted employing circulating sEVs isolated from kidney and liver transplant recipients pretransplant and at days 1 and 10 following

transplantation. To investigate whether cross-dressing with sEVs isolated from transplant recipients modifies the phenotype and cytokine production of DCs, we cultured for 24 hours DCs derived from healthy individuals' monocytes with sEVs isolated from either healthy individuals, liver transplant recipients, or kidney transplant recipients (at day 1 and day 10 posttransplant), and (1) used flow cytometry to analyze the changes in the expression of costimulatory molecules (CD40, CD86) and PD-L1; and (2) quantified IL-6 and IL-10 levels in culture supernatants using Human IL-6 and Human IL-10 ELISA MAX™ kits (Biolegend, London, UK) according to manufacturer's instructions. For ELISA experiments, supernatant of DCs cultured for 24 hours with or without sEVs were thawed on ice prior to use and diluted 1:10 for IL-6 and 1:2 for IL-10; analysis of standards and samples was carried out in duplicate; and plates were read at 450 nm and values extrapolated from a 5-parameter curve.

2.8 | Statistical analysis

Statistical analyses were performed by GraphPad Prism v7.0 Software. Student's *t* and Mann-Whitney tests were used for comparisons between two groups as appropriate, and analysis of variance (ANOVA; with Tukey's posttest) to compare more than two groups. Paired samples were compared by nonparametric Wilcoxon tests (**P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001).

3 | RESULTS

3.1 | Host cells cross-dressed with donor HLA molecules are highly prevalent following liver transplantation

In order to evaluate the presence of cross-dressed recipient cells following liver transplantation, we enrolled 8 liver and 3 kidney transplant recipients who exhibited HLA class I mismatches amenable to discrimination using available fluorescent anti-HLA antibodies (Table 1). Conventional flow cytometry was used to assess the presence of circulating passenger leukocytes (displaying donor- but not recipient HLA) and cross-dressed recipient cells (displaying both donor- and recipient HLA) following kidney and liver transplantation. Monocytic, dendritic, T cell, and B cell subsets were specifically analyzed (Figure 1A). Although circulating cross-dressed cells were noted to be present in all 8 liver transplant recipients analyzed, only 5 exhibited detectable levels of passenger leukocytes. Both cross-dressed and passenger leukocyte numbers peaked in the first 24 hours following liver transplantation, with their number waning to negligible levels by postoperative day 30 (Figure 1B). By day 30 postoperatively, only 2 liver transplant recipients were found to have detectable levels of cross-dressed cells, and none had detectable levels of passenger leukocytes in circulation. Neither cross-dressed nor passenger leukocytes were detectable in the circulation of subjects having undergone kidney transplantation throughout the duration of follow-up

TABLE 1 Clinical characteristics and HLA-type of transplant recipients

Pt#	Recipient age	Organ transplanted (indication)	Posttransplant complications	Recipient HLA I type	Donor HLA I type
1	23	Liver (Biliary atresia)	Nil	A01/ A02 B35/ B40	A02/ A03 B07/ B12
2	59	Liver (HCC/HCV)	Nil	A03/ A11 B5/ B17	A02/ A03 B07/ B07
3	65	Liver (HHT)	Nil	A02/ A09 B40/ B17	A01/ A01 B08/ B08
4	47	Liver (Cryptogenic)	Death (Pulmonary embolus)	A03/ A11 B18/ B22	A2/ A28 B14/ B15
5	60	Liver(NASH)	Nil	A01/ A01 B08/ B08	A02/ A03 B27/ B12
6	19	Liver (PSC)	Acute cellular rejection – POD7	A2/ A11 B40/ B52	A2/ A3 B15/ B44
7	22	Liver (Biliary atresia)	Nil	A24/ A33 B40/ B44	A1/ A23 B8/ B44
8	65	Liver (PSC)	Nil	A1/ A2 B41/B44	A1/ A3 B7/ B44
9	61	Kidney (ESRD)	Nil	A2/ A29 B18/ B44	A3/ A29 B7/ B44
10	35	Kidney (ESRD)	Nil	A24/ A24 B15/ B18	A11/ A24 B7/ B18
11	60	Kidney (ESRD)	Nil	A11/ A30 B15/ B18	A3/ A30 B7/ B18

Abbreviations: ESRD, end-stage renal disease; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HHT, hereditary hemorrhagic telangiectasia; NASH, nonalcoholic fatty liver disease; POD, postoperative day; PSC, primary sclerosing cholangitis.

(Figure 1B). On subset analysis, CD14 + monocytes formed the bulk of cross-dressed cells (Figure 1B, left panels), and passenger leukocytes were made up of varying proportions of cells across the whole spectrum of immune cell subsets analyzed (Figure 1B, right panels), but their overall numbers were significantly lower than those of cross-dressed cells (Figure 1C). Advanced imaging flow cytometry was performed by ImageStream^x in order to achieve visual corroboration of the cross-dressing of recipient APCs with donor HLA molecules. As compared to recipient HLA, which is diffusely present, donor HLA was seen as discrete spots on the recipient cell, a pattern in keeping with our previously published *in vitro* analyses of sEV uptake kinetics.¹³

3.2 | Circulating sEVs bearing donor HLA peak early following liver transplantation and are no longer detectable at late time points after transplantation

Next, we investigated whether circulating EVs of donor origin could be detected following liver or kidney transplantation. To do this, we used advanced imaging flow cytometry – an approach that allows sensitive multiparameter characterization of nanosized particles.^{13,22,23} Small EVs were isolated from plasma by size-exclusion chromatography as previously described (Figure S2). SEC offers significant advantages over alternative methods of sEV isolation. These include a reduced risk of sEV damage during isolation, relatively

low co-precipitation of nonvesicular contaminants, the capacity to extract sEVs from low-volume clinical samples, and the reduction of user-variability through the use of commercial SEC columns. Although no single marker can serve to uniquely identify exosomes, comprehensive proteomic analyses of sEV subtypes identified CD63 as among the most suitable.¹⁴ Thus, isolated sEVs bearing exosomal marker CD63 (putative exosomes) were assessed for their expression of donor HLA (Figure 2A,B). Circulating CD63 + sEVs bearing donor HLA were identified in all 8 liver transplant recipients in the early posttransplant period, with a peak at postoperative day 1, though falling to undetectable levels by day 4 posttransplant (Figure 2C). The peak in donor sEV number corresponded with the peak in cross-dressed circulating leukocytes in liver transplant recipients. No circulating donor sEVs were detectable in any kidney transplant recipients investigated, which also corresponded to the absence of cross-dressed cells detectable in these patients.

3.3 | Circulating sEVs bearing donor HLA are not detected in recipients undergoing allograft dysfunction due to rejection at late time points posttransplant

To determine whether donor sEVs release can be detected in circumstances of liver allograft dysfunction taking place beyond the

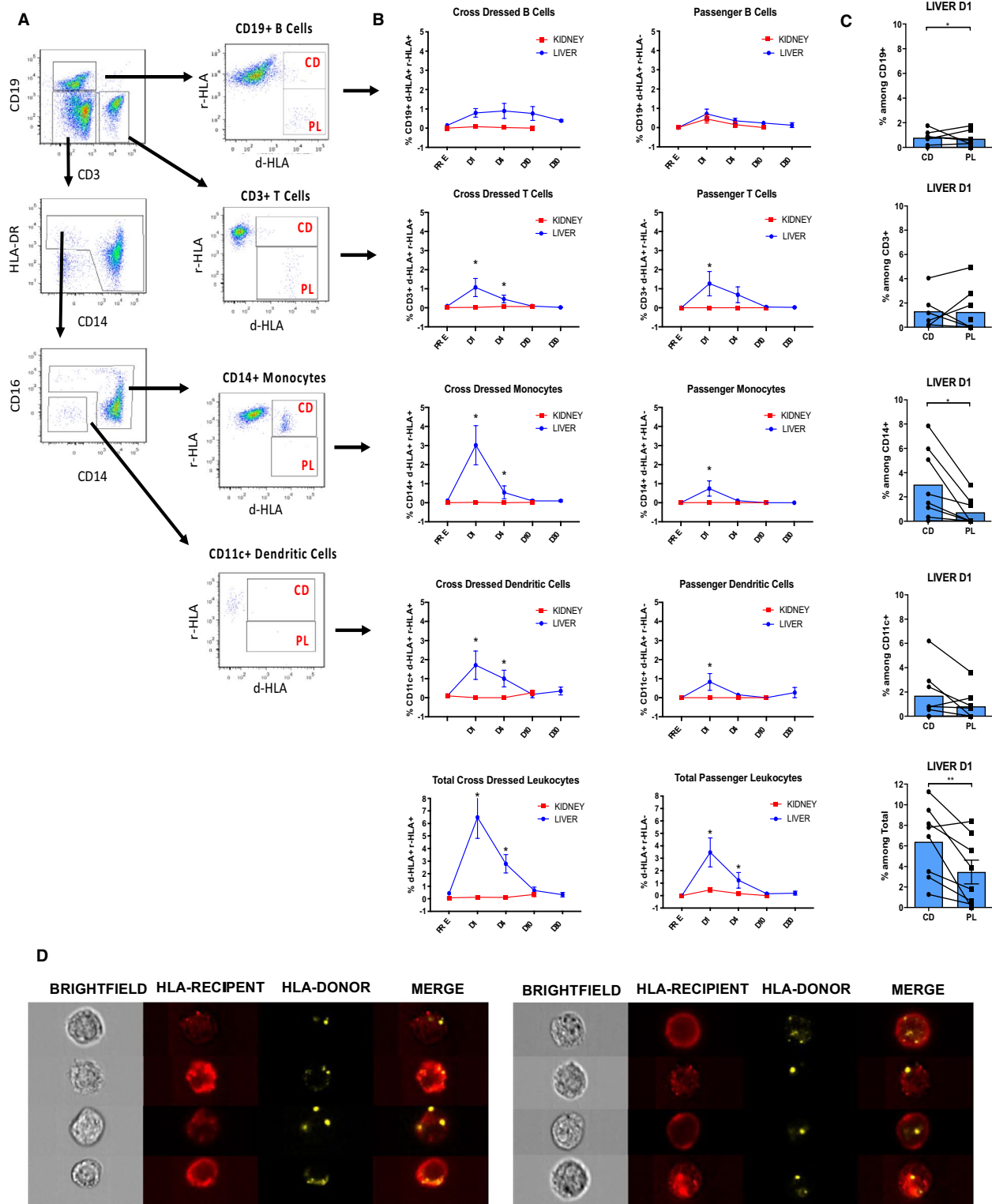


FIGURE 1 Circulating cross-dressed leukocyte subsets peak early following liver transplantation. (A) Gating strategy for the identification of CD19 + B cells, CD14 + monocytes, CD11c + dendritic cells, and CD3 + T cells (CD: cross-dressed; PL: passenger leukocytes). (B) Distribution of cross-dressed (left) and passenger (right) leukocyte subsets pre- and 1, 4, 10, and 30 days post- liver (n = 8) or kidney (n = 3) transplantation. Analysis of variance with Tukey's posttest highlighted significance relative to PRE time point. (C) Differential percentages of circulating cross-dressed and passenger leukocytes in liver recipients 1 day after transplantation. Wilcoxon paired test. (D) Analysis by advanced imaging flow cytometry (ImageStream^X) of recipient (recipient HLA⁺, red) circulating cells cross-dressed with donor HLA (yellow) observed as characteristically discrete spots. Each cell image is representative of 1 of 8 liver transplant recipients analyzed. (*P < .05, **P < .01)

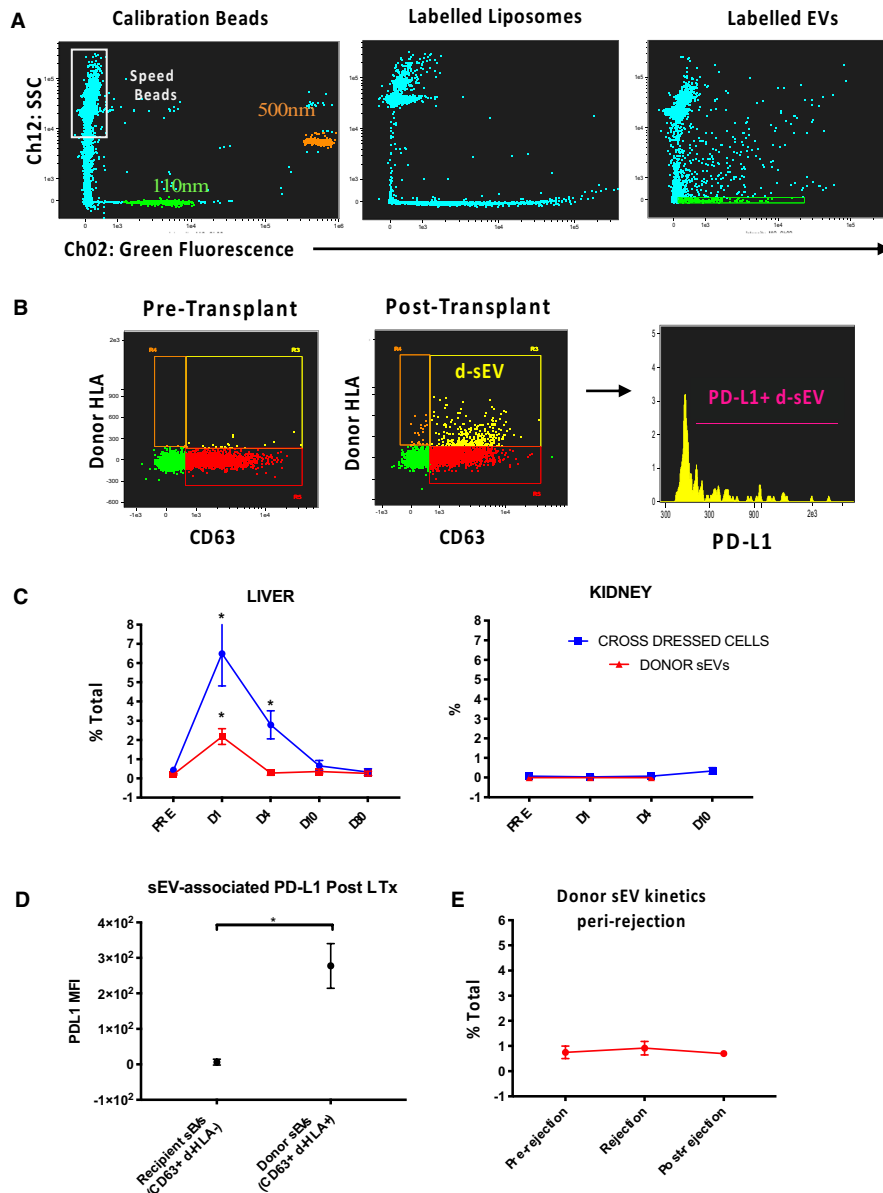


FIGURE 2 Circulating donor-derived CD63 + small extracellular vesicles (sEVs) bearing PD-L1 peak early following liver transplantation (LTx). Advanced imaging flow cytometry was used for the multiparametric analysis of sEVs using Amnis ImageStream^x (ISx). (A) Polystyrene calibration beads of known size demonstrate the capacity for small particle acquisition by ISx. Speed beads and their aggregates serve as internal calibrators for the cytometer and the fluidics system (left panel). Gating of CFDA-SE-labeled small EVs including exosomes (right panel) was performed with reference to fluorescently labelled liposomes of known size and displaying scatter characteristics resembling exosomes (middle panel). Representative plots modified with permission from Mastoridis et al.¹³ (B) Gated small EVs were further analysed for the expression of exosomal marker CD63 and donor HLA, and double-positive events then interrogated for the presence of programmed death ligand 1 (PD-L1). Representative plots modified with permission from Mastoridis et al.¹³ (C) The kinetics of donor sEV (d-sEVs: CD63 + donor HLA+) release into the circulation in the peritransplant period in both liver and kidney allograft recipients were compared to the presence of cross-dressed cells in circulation (ascertained by conventional flow cytometry, see Figure 1; ANOVA with Tukey's posttest, significance indicated relative to PRE timepoint). (D) The expression of programmed death ligand 1 (PD-L1) on sEVs of donor and recipient origin were compared among liver transplant recipients. (E) Liver allograft rejection following immunosuppression withdrawal is not associated with a significant increase in circulating sEVs bearing donor HLA. (n = 3; mean ± SD; Wilcoxon test; *P < .05)

immediate posttransplant period, we conducted circulating sEV analyses on sequential plasma samples collected from liver transplant recipients experiencing biopsy-proven T cell-mediated rejection following an attempt at complete immunosuppression withdrawal. These rejection episodes were, on average, seven and a half years

following transplantation (range: 4.9-10.5 years). In contrast to the very consistent release of donor sEVs observed shortly after liver transplantation, no significant rise in sEVs bearing donor HLA was noted before or at the time of rejection in this context (Figure 2E, and Table S1).

3.4 | sEVs of donor origin are enriched in PD-L1 that is transferred in tandem with donor HLA to cross-dressed recipient cells

The intrahepatic expression of PD-L1 is upregulated following liver transplantation in both mice and humans and has been implicated in the regulation of alloimmune responses and the establishment of liver allograft tolerance.^{18,31,32} We observed that a higher proportion of circulating donor-derived sEVs carried membrane-bound PD-L1 than sEVs of recipient origin (Figure 2B,D). Likewise, recipient cross-dressed cells (ie, displaying donor HLA molecules in their membrane) expressed higher PD-L1 than recipient cells not bearing donor HLA (Figure 3A,B), in keeping with what has been previously described in a murine liver transplant model.¹⁸ To determine whether PD-L1 on recipient cross-dressed cells is transferred in tandem with donor HLA, we conducted colocalization analyses by ISx using SBDS scoring. PD-L1 colocalized with donor HLA, suggesting that the elevated PD-L1 expression observed on cross-dressed cells may be due to tandem transfer on donor-derived sEVs (Figure 3A,C). In contrast, donor HLA did not colocalize with the exosomal marker CD63 (data not shown), likely reflecting the fact that CD63 is borne by both donor and recipient sEVs alike but also the possibility that donor HLA may also be derived by non-sEV-dependent pathways such as cell-cell contact and cell nibbling.

3.5 | sEVs derived from liver but not kidney recipients transiently inhibit allogenic T cell responses

We sought to investigate the extent to which cross-dressing influences human APC immunogenicity, both in healthy individuals and in the setting of clinical transplantation. First, we employed HLA-A2 negative

monocyte-derived dendritic cells (DCs) from healthy individuals that had been cross-dressed in vitro with plasma-derived HLA-A2 positive sEVs harvested from an allogeneic healthy control (Figure 4A,B). Following culture with allogeneic sEVs, DCs displayed HLA-A2 expression on their surface and acquired the capacity to elicit proliferation of syngeneic CD8 + T cells (Figure 4C). Next, we conducted experiments in which monocyte-derived DCs from healthy controls were cultured for 24 hours with plasma-derived sEVs harvested from allogeneic liver (n = 7) and kidney (n = 3) recipients on days 1 and 10 posttransplant. DCs were then washed and replated for 5 days with allogeneic third-party CD8 T cells. sEVs isolated on day 2 post-livertransplantation (Figure 5A), but not those harvested from day 1 kidney recipients (Figure 5B), significantly inhibited T cell proliferation ($7.4\% \pm 3.1\%$ vs $22.4\% \pm 2.4\%$ as compared to non-cross-dressed DCs; $P < .001$; Figure 5A). The inhibition of CD8 T cell proliferation observed when employing day 1 liver transplant recipient sEVs was reduced when DCs were cultured with day 10 sEVs, although the effect was still significant in comparison with non-cross-dressed DCs (Figure 5A). In contrast, circulating sEVs from kidney transplant recipients did not induce significant changes in third-party CD8 T cell responses (Figure 5B). To investigate the effect of sEVs isolated from liver transplant patients on DCs, we analyzed the changes in the expression of costimulatory molecules (CD40 and CD86) in DCs cultured with sEVs for 24 hours. DCs cultured with sEVs isolated from liver transplant recipients at day 1 following transplantation expressed significantly less CD40 than DCs cultured with sEVs at day 10 ($P = .005$), or DCs without sEVs. This was not observed in kidney transplant recipients (Figure S3A). In addition, DCs cultured with sEVs from liver transplant recipients produced less IL-6 and more IL-10 when compared to the use of sEVs from kidney transplant recipients (Figure S3B). Altogether, these data suggest that sEVs from liver transplant patients have a heightened capacity to induce an inhibitory phenotype in DCs.

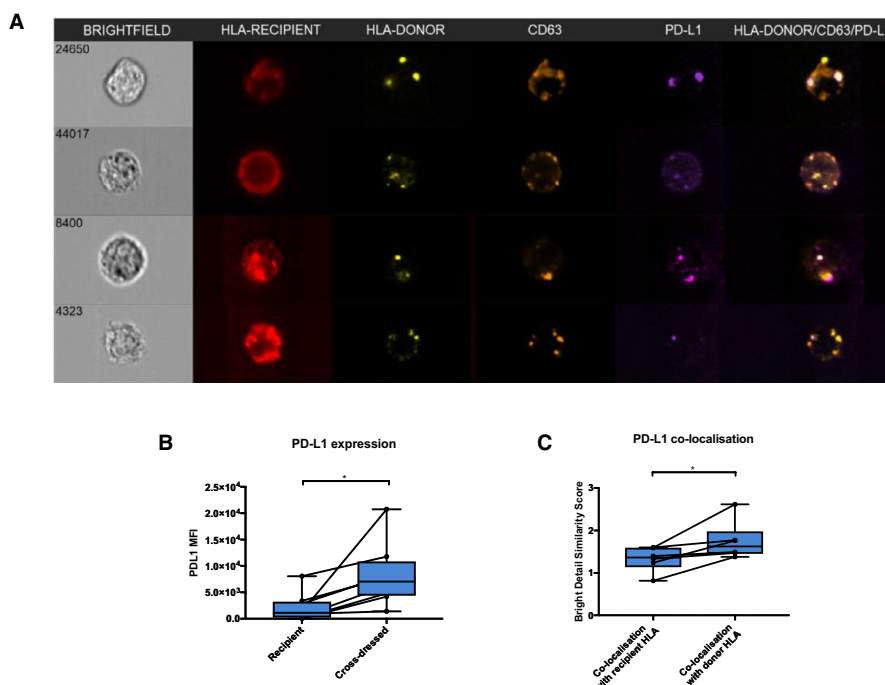
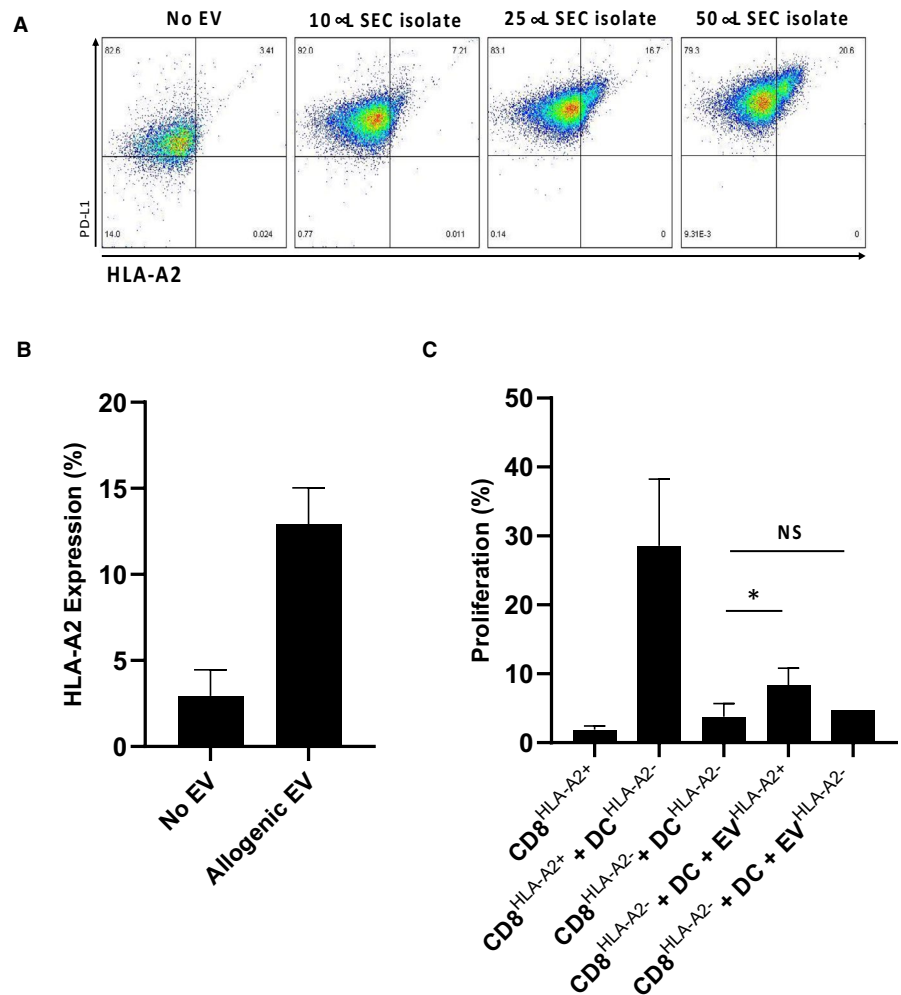


FIGURE 3 Cross-dressed recipient cells are enriched in programmed death ligand 1 (PD-L1), and this colocalizes with donor HLA molecules. Analysis of circulating peripheral blood mononuclear cells (PBMCs) by imaging flow cytometry of liver transplant recipients 1-day posttransplant. (A) Representative profiles of CD14 + recipient cells cross-dressed with donor HLA. The antibodies used are indicated at the top of each column. (B) PD-L1 expression in recipient and cross-dressed cells (n = 8). (C) Colocalization analysis of PD-L1 with recipient or donor HLA molecules by Bright Detail Similarity Score (n = 6). (Wilcoxon test; * $P < .05$)

FIGURE 4 Dendritic cells cross-dressed with allogenic small extracellular vesicles (sEVs) induce T cell proliferation in vitro. (A) Representative profiles of change in HLA-A2 expression in HLA-A2 negative cells following 24 hours co-culture with increasing quantities of HLA-A2 positive (allogenic) sEVs. (B) Summary of 3 independent experiments showing increase in HLA-A2 expression in monocyte-derived dendritic cells following co-culture with HLA-A2 positive sEVs. (C) HLA-A2 negative monocyte-derived dendritic cells were cross-dressed with either HLA-A2 positive or HLA-A2 negative sEVs, and their capacity to affect CD8 T cells proliferation compared using flow cytometry (4 independent experiments; Student's *t* test, mean \pm SD, where **P* < .05). DC, dendritic cell; SEC, size-exclusion chromatography



4 | DISCUSSION

In the context of transplantation, T cells can be activated through the direct, indirect, and semidirect allorecognition pathways. The semidirect pathway, which involves the activation of directly alloreactive T cells upon recognition of intact donor MHC molecules presented by recipient APCs, was originally proposed as a conceptual solution to the “three cell model” conundrum, that is, the need for recipient CD4⁺ T cells to recognize donor allopeptides on the same APCs directly presenting intact donor MHC molecules to alloreactive CD8⁺ cells, in order for CD4⁺ subsets to be capable of regulating alloreactive CD8⁺ T cells.¹⁶ Recent studies in experimental models indicate that the semidirect allopresentation elicited by the release of donor allograft-derived small EVs is indeed key in initiating and sustaining alloimmune responses. Thus, in skin, heart, kidney, and islet murine transplant models the cross-dressing of recipient APCs by EV-mediated delivery of intact donor MHC in secondary lymphoid tissues and/or the graft, and not direct allopresentation by allograft-derived APCs, is the main pathway through which directly alloreactive T cells are activated.^{1,3,17,33}

In the current study we sought to investigate whether, and in which circumstances, cross-dressing of recipient APCs occurs in

clinical transplantation and the extent to which it might contribute to allopresentation. We provide for the first time evidence indicating that cross-dressed recipient leukocytes can be found in the circulation following liver transplantation, albeit transiently, and that their numbers significantly exceed those of circulating passenger leukocytes. This is in keeping with the observations made in the animal transplant models referenced previously, wherein cross-dressed recipient-type cells make up the majority of DCs presenting donor MHC molecule both in the graft and the secondary lymphoid tissue. Thus, in mice, shortly after liver and kidney transplantation 60% and >70% of intra-graft DCs respectively were found to be cross-dressed,^{17,18} whereas following heart transplantation, the number of splenic APCs carrying donor MHC molecules was 100-fold greater than the number of donor DCs that had migrated to the spleen.¹ Of note, the number of cross-dressed APCs described in these murine models is substantially greater than what we observed in the circulation of human liver recipients. Furthermore, in contrast to liver, following kidney transplantation we detected no cross-dressed cells or passenger leukocytes, and even after liver transplantation the number of circulating cross-dressed cells rapidly waned and became undetectable by day 30 posttransplant. This likely reflects the limitations of being restricted to the analysis of blood specimens and we speculate that, as shown in murine models, following clinical organ transplantation recipient APC cross-dressing

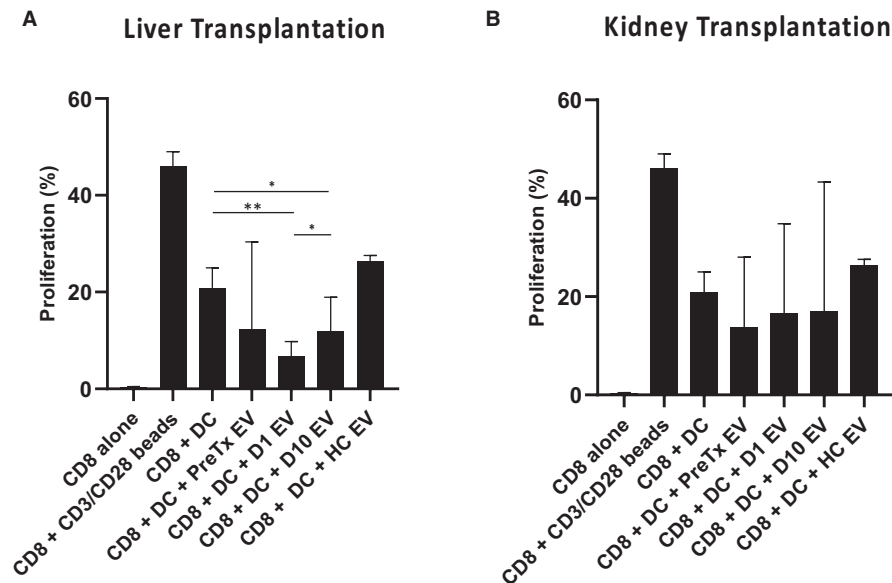


FIGURE 5 Small extracellular vesicles (sEVs) derived from liver but not kidney transplant recipients transiently inhibit allogenic T cell responses. Dendritic cells (DCs) were cross-dressed with sEVs isolated from liver ($n = 7$) and kidney ($n = 3$) transplant recipients isolated pretransplant (Pre-Tx), and at postoperative days 1 (D1) and 10 (D10), as well as from healthy control subjects (HC). (A) DCs cross-dressed with sEVs harvested from liver transplant recipients on D1 and D10 posttransplant significantly inhibited third-party CD8 T cell proliferation as compared to non-cross-dressed DCs or DCs cross-dressed with syngeneic sEVs. (B) DCs cross-dressed with sEVs harvested from kidney recipients on D1 and D10 posttransplant did not significantly influence third-party CD8 T cell proliferation (paired and unpaired Student's t test, mean \pm SD, where $*P < .05$, $**P < .01$). DC, dendritic cell; SEC, size-exclusion chromatography; HC, healthy control

continues to occur in the allograft and/or secondary lymphoid tissues for prolonged periods of time. This is supported by the demonstration that in longstanding kidney transplant recipients with poor graft function not only indirectly but also directly primed alloreactive T cells persist in the circulation.³⁴

The mechanistic basis for APC cross-dressing is an area of ongoing research. Analyses by immunoelectron microscopy following murine cardiac transplantation have shown recipient DCs to acquire intact donor MHC molecules in graft-draining lymphoid organs by capturing clusters of donor-derived sEVs.¹ Our data suggest that this is likely to be the case in humans as well. First, because the presence of circulating cross-dressed cells in the early posttransplant period coincides with a rise in circulating allograft-derived sEVs bearing donor HLA. Second, because in circulating cross-dressed cells donor HLA colocalizes with PD-L1, a molecule that is enriched in liver allograft-derived sEVs. Nevertheless, the contribution of other mechanisms to the transfer of donor HLA molecules (eg, trogocytosis, or cell nibbling, either following graft infiltration by recipient cells or in association with passenger leukocytes³⁵) cannot be excluded. The fact that we observe that not all donor HLA colocalizes with CD63 might offer limited support to the notion that such alternative mechanisms of transfer are also at play. However, this absence of observed colocalization, in addition to the finding that not all PD-L1 colocalizes with donor HLA, should also be understood with the following considerations in mind. First, it is unlikely that all sEVs bearing the particular donor HLA subtype analyzed will also carry PD-L1 or CD63 and vice versa. Second, there are nongraft sources of PD-L1, as well as nongraft sEVs bearing CD63. Third, target-cell intrinsic

PD-L1 expression will not necessarily bear any spatial relation to, for instance, donor HLA or CD63. Finally, we have little understanding of the potential for variability in the cycling of different sEV constituent proteins by target cells and the impact of this on the probability of concurrent detection.

sEVs are prone to rapid systemic dilution and clearance, predominantly by the reticuloendothelial system. Such clearance might account for the rapid diminution of donor-derived sEVs we observed following liver transplantation. There have been no studies assessing whether different transplanted organs release differing quantities of HLA-bearing sEVs, but our observations indicating that in humans much fewer sEVs find their way into the systemic circulation after kidney than after liver transplantation suggest that the size of the organ and/or its particular cytoarchitecture and vascular anatomy influence the quantity of sEVs being released.

Beyond quantitative differences among transplanted organs, qualitative variation in sEVs – that is, in the repertoire of surface molecules and cargo that they contain – may also affect downstream functional outcomes. This has been clearly demonstrated in animal models. Thus, in a murine liver transplant model, graft-infiltrating DCs cross-dressed by donor sEVs expressed high levels of PD-L1 and markedly suppressed donor-reactive CD8 + T cell proliferative responses by inducing an exhausted phenotype.¹⁸ In contrast, cross-dressed DCs isolated from the lymphoid tissues of heart recipients failed to increase PD-L1 expression and promoted proliferation of directly alloreactive CD8 + T cells.¹ Likewise, in a kidney transplant model,

cross-dressed DCs sustained the effector function of alloreactive CD8 + T cells and induced acute and chronic rejection.¹⁷ Our findings employing control DCs cross-dressed in vitro with sEVs sequentially collected from both kidney and liver human recipients confirm the unique inhibitory properties of the sEVs released after liver transplantation. This is an important observation that could mechanistically underpin the reduced immunogenicity of liver allografts described both in animal models and in the clinic. Of note, the capacity of circulating sEVs harvested from liver recipients to inhibit CD8 + T cell proliferation decreased with time, in keeping with the temporal changes in circulating donor-derived sEVs release observed after liver transplantation. Whether the inhibitory effect persists in the liver graft itself for longer periods of time remains to be established.

In summary, we have described for the first time the kinetics of allograft-derived sEV release and circulating cell cross-dressing in human kidney and liver transplantation. Despite the limitation of being restricted to blood specimens, our data confirm several observations made in experimental animal models, in particular the clear preeminence of recipient cross-dressed cells over donor passenger leukocytes as a potential source for allostimulation and the unique downstream functional effects mediated by liver-derived sEVs.

ACKNOWLEDGMENTS

This work was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust and King's College London, and by the King's Medical Research Trust, King's College London.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Sotiris Mastoridis  <https://orcid.org/0000-0001-7689-2330>

Oriol Bestard  <https://orcid.org/0000-0001-9468-7920>

Marc Martínez-Llordella  <https://orcid.org/0000-0003-1939-4102>

Alberto Sánchez-Fueyo  <https://orcid.org/0000-0002-8316-3504>

REFERENCES

- Liu Q, Rojas-Canales DM, Divito SJ, et al. Donor dendritic cell-derived exosomes promote allograft-targeting immune response. *J Clin Invest*. 2016;126(8):2805-2820.
- Celli S, Albert ML, Bousso P. Visualizing the innate and adaptive immune responses underlying allograft rejection by two-photon microscopy. *Nat Med*. 2011;17(6):744-749.
- Marino J, Babiker-Mohamed MH, Crosby-Bertorini P, et al. Donor exosomes rather than passenger leukocytes initiate alloreactive T cell responses after transplantation. *Sci Immunol*. 2016;1(1):aaf8759.
- Smyth LA, Lechler RI, Lombardi G. Continuous Acquisition of MHC: Peptide Complexes by Recipient Cells Contributes to the Generation of Anti-Graft CD8+ T Cell Immunity. *Am J Transplant*. 2016;17(1):60-68.
- Raposo G, Nijman HW, Stoorvogel W, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med*. 1996;183(3):1161-1172.
- Muntasell A, Berger AC, Roche PA. T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes. *EMBO J*. 2007;26(19):4263-4272.
- Admyre C, Bohle B, Johansson SM, et al. B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce TH2-like cytokines. *J Allergy Clin Immunol*. 2007;120(6):1418-1424.
- Vincent-Schneider H, Stumptner-Cuvelette P, Lankar D, et al. Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells. *Int Immunol*. 2002;14(7):713-722.
- Segura E. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood*. 2005;106(1):216-223.
- Montecalvo A, Shufesky WJ, Beer Stolz D, et al. Exosomes as a Short-Range Mechanism to Spread Alloantigen between Dendritic Cells during T Cell Allorecognition. *J Immunol*. 2008;180(5):3081-3090.
- Mallegol J, Van Niel G, Lebreton C, et al. T84-Intestinal Epithelial Exosomes Bear MHC Class II/Peptide Complexes Potentiating Antigen Presentation by Dendritic Cells. *Gastroenterology*. 2007;132(5):1866-1876.
- Wakim LM, Bevan MJ. Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. *Nature*. 2011;471(7340):629-632.
- Mastoridis S, Bertolino GM, Whitehouse G, Dazzi F, Sanchez-Fueyo A, Martínez-Llordella M. Multiparametric Analysis of Circulating Exosomes and Other Small Extracellular Vesicles by Advanced Imaging Flow Cytometry. *Front Immun*. 2018;9:1-13.
- Kowal J, Arras G, Colombo M, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *PNAS*. 2016;113(8):E968-E977.
- Tkach M, Kowal J, Théry C. Why the need and how to approach the functional diversity of extracellular vesicles. *Phil Trans R Soc B*. 2017;373(1737):20160479.
- Herrera OB, Golshayan D, Tibbott R, et al. A Novel Pathway of Alloantigen Presentation by Dendritic Cells. *J Immunol*. 2004;173(8):4828-4837.
- Hughes AD, Zhao D, Dai H, et al. Cross-dressed dendritic cells sustain effector T cell responses in islet and kidney allografts. *J Clin Invest*. 2020;130(1):287-294.
- Ono Y, Perez-Gutierrez A, Nakao T, et al. Graft-infiltrating PD-L1(hi) cross-dressed dendritic cells regulate antidonor T cell responses in mouse liver transplant tolerance. *Hepatology*. 2018;67(4):1499-1515.
- Witwer KW, Buzás EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles*. 2013;2: 20360.
- Welton JL, Webber JP, Botos L-A, Jones M, Clayton A. Ready-made chromatography columns for extracellular vesicle isolation from plasma. *J Extracell Vesicles*. 2015;4:23430-23439.
- Lane RE, Korbie D, Anderson W, Vaidyanathan R, Trau M. Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing. *Sci Rep*. 2015;5:7639.
- Lannigan J, Erdbruegger U. Imaging flow cytometry for the characterization of extracellular vesicles. *Methods*. 2017;112:55-67.
- Headland SE, Jones HR, D'Sa ASV, Perretti M, Norling LV. Cutting-Edge Analysis of Extracellular Microparticles using ImageStreamX Imaging Flow Cytometry. *Sci Rep*. 2014;4:1-10.

24. Morales-Kastresana A, Telford B, Musich TA, et al. Labeling Extracellular Vesicles for Nanoscale Flow Cytometry. *Sci Rep*. 2017;7(1):1-10.
25. Pospichalova V, Svoboda J, Dave Z, et al. Simplified protocol for flow cytometry analysis of fluorescently labeled exosomes and microvesicles using dedicated flow cytometer. *J Extracell Vesicles*. 2015;4:25530.
26. Flow NJP. Cytometry of Extracellular Vesicles: Potential, Pitfalls, and Prospects. *Curr Protoc Cytom*. 2015;73(13):14.1-14.16.
27. Zuba-Surma EK, Kucia M, Abdel-Latif A, Lillard JW, Ratajczak MZ. The ImageStream System: a key step to a new era in imaging. *Folia Histochem Cytobiol*. 2007;45(4):279-290.
28. Hennies CM, Lehn MA, Janssen EM. Quantitating MHC class II trafficking in primary dendritic cells using imaging flow cytometry. *J Immunol Methods*. 2015;423(C):18-28.
29. Beum PV, Lindorfer MA, Hall BE, et al. Quantitative analysis of protein co-localization on B cells opsonized with rituximab and complement using the ImageStream multispectral imaging flow cytometer. *J Immunol Methods*. 2006;317(1-2):90-99.
30. Trabanelli S, La Manna F, Romano M, et al. The Human Mesenchymal Stromal Cell-Derived Osteocyte Capacity to Modulate Dendritic Cell Functions Is Strictly Dependent on the Culture System. *J Immunol Res*. 2015;2015(6):1-10.
31. Morita M, Joyce D, Miller C, Fung JJ, Lu L, Qian S. Rejection triggers liver transplant tolerance: Involvement of mesenchyme-mediated immune control mechanisms in mice. *Hepatology*. 2015;62(3):915-931.
32. Shi X-L, Mancham S, Hansen BE, et al. Counter-regulation of rejection activity against human liver grafts by donor PD-L1 and recipient PD-1 interaction. *J Hepatol*. 2016;64(6):1274-1282.
33. Zeng F, Morelli AE. Extracellular vesicle-mediated MHC cross-dressing in immune homeostasis, transplantation, infectious diseases, and cancer. *Semin Immunopathol*. 2018;40:1-14.
34. Bestard O, Nickel P, Cruzado JM, et al. Circulating alloreactive T cells correlate with graft function in longstanding renal transplant recipients. *J Am Soc Nephrol*. 2008;19(7):1419-1429.
35. Zhang Q-J, Li X-L, Wang D, et al. Trogocytosis of MHC-I/peptide complexes derived from tumors and infected cells enhances dendritic cell cross-priming and promotes adaptive T cell responses. Gregson A, ed. *PLoS ONE*. 2008;3(8):e3097.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Mastoridis S, Londoño M-C, Kurt A, et al. Impact of donor extracellular vesicle release on recipient cell "cross-dressing" following clinical liver and kidney transplantation. *Am J Transplant*. 2020;00:1-12. <https://doi.org/10.1111/ajt.16123>