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ORIGINAL ARTICLE

Cross-decoration of dendritic cells by non-inherited maternal antigen-containing extracellular vesicles: Potential mechanism for PD-L1-based tolerance in cord blood and organ transplantation

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Abstract

Exposure to non-inherited maternal antigens (NIMA) during the fetal period induces lifelong split tolerance to grafts expressing these allo-antigens. In adult mice, the production of extracellular vesicles (EVs) from maternal microchimeric cells causes cross-decoration (XD) of offspring dendritic cells (DC) with NIMA and upregulation of PD-L1, contributing to NIMA tolerance. To see how this may apply to humans, we tested NIMA acquisition by fetal DCS in human cord blood. The average percentage of NIMA-XD among total DCs was 2.6% for myeloid and 4.5% for Plasmacytoid DC. These cells showed higher PD-L1 expression than their non-XD counterparts (mDC: $p = .0016$; pDC: $p = .024$). We detected $CD9^+$ EVs bearing NIMA and PD-L1 in cord blood. To determine if this immune regulatory mechanism persists beyond the pregnancy, we analyzed NIMA-expressing kidney and liver transplant recipients. We found donor antigen XD DCs in peripheral blood and graft-infiltrating DCs. As in cord blood, the pattern of donor antigen expression was punctate, and PD-L1 expression was upregulated, likely due to both protein and miRNA acquired from EV. Our findings support a mechanism for split tolerance to NIMAs that develops during pregnancy and is recapitulated in adult transplant recipients.

KEYWORDS
basic (laboratory) research / science, translational research / science, immunobiology, kidney transplantation / nephrology, liver transplantation / hepatology, alloantigen, antigen presentation / recognition, tolerance, chimerism, tolerance, mechanisms, dendritic cell

Abbreviations: APC, antigen-presenting cell; DC, dendritic cell (mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell); EV, extracellular vesicles; HLA, human leukocyte antigen; mAb, monoclonal antibody; MHC, major histocompatibility complex; MMc, maternal microchimerism; NIMA, non-inherited maternal antigen; XD, cross-decorated.

1 | INTRODUCTION

Ample evidence exists of NIMA-specific "split" tolerance, a phenomenon first reported by Ray Owen in 1945, and expanded upon by Medawar, Billingham, and Brent to describe the active development of lifelong immuno-tolerance to allo-antigens introduced during the fetal period.[1,2] Acquired tolerance to non-inherited maternal antigens (NIMA) during the fetal (and perinatal) period has persistent, lifelong consequences. Clinical studies have demonstrated that organ transplant recipients when mismatched HLAs are NIMAs, show decreased rates of chronic rejection, but paradoxically with increased acute rejection rates.[3,4] This "split tolerance," wherein an individual may mount certain types of immune responses to a particular antigenic challenge, but is tolerant of the same antigen in other immunological settings can be seen as beneficial because, clinically, acute rejection is generally treatable with immuno-suppressive drugs, while chronic rejection is more intractable. Pediatric liver transplant recipients where the donor is the mother have better outcomes, possibly due to immunological benefits afforded by maternal microchimerism (MMc).[5]

While the presence of MMc has long been established,[6-8] these studies and the pioneering experiments on split tolerance in animals have yet to describe a mechanism by which split tolerance is generated and maintained in humans. Our group has previously reported that MMc that persists in mice during adulthood secrete NIMA-bearing EVs that are acquired by host DCs, which thereby become "cross-decorated" (XD) with intact MHC class I and II of maternal origin.[9] Additionally, these XD cells upregulate PD-L1, in surface areas distinct from the acquired (intact) NIMAs, but coincident with areas where NIMA-MHC breakdown products (allopeptides) are being presented. This results in tolerogenic allopeptide pathway alloreactive presentation to host indirect T cells and thus in abortive activation of NIMA-specific T cell clones.[9]

We now know of the myriad immunological consequences of MMc.[10] Because MMc levels are associated with tolerance to NIMAs,[11] it was hypothesized that these microchimeric cells orchestrate the acquisition of tolerance to these targets. The mechanisms of how such a small population could have a global impact on the immune system remained obscure until it was demonstrated it was largely due to extracellular vesicle (EV)-mediated modifications of host antigen-presenting cells (APCs).[9] In this phenomenon, MMc cells secrete EVs that transport NIMAs, especially intact class I and II MHC molecules. The offspring's APCs, including both myeloid (mDC) and plasmacytoid dendritic cells (pDC), acquire intact maternal HLAs, and upregulate PD-L1. This results in tolerogenic antigen presentation together with PD-L1 signaling.[9] Although not demonstrated, the authors speculated that the cellular pattern of PD-L1 expression suggested endogenous upregulation due to EV-transported microRNA (miRNA).[9] If true, this is in contrast to the punctate cell surface pattern of expression of maternal MHC class I / II antigens and CD86. Consequently, semi-direct presentation occurs with CD86 co-signaling, while indirect antigen presentation occurs elsewhere in the cell with the endogenously upregulated PD-L1 and is thus inhibited. Because it is thought that (semi)direct presentation is more important for acute rejection, while the indirect presentation is for chronic rejection, the consequent tolerance is "split," with increased rates of the former and reduced rates of the latter.

Another type of antigenic "chimerism," the presence of non-self-antigens of maternal or donor origin in host cells transferred via EVs, has been much less characterized. We describe here, for the first time, acquired expression of NIMA along with PD-L1 in fetal dendritic cells, associated with a tolerogenic immuno-phenotype. Furthermore, we observed a parallel phenomenon in NIMA$^+$ transplant recipients. Our findings expand on the known mechanisms of fetal immune regulation to NIMAs during pregnancy, showing that EVs released by NIMA$^+$ cells could extend into adulthood a PD-L1-dependent mechanism that similarly regulates immune responses against NIMAs expressed by grafts.

2 | MATERIALS AND METHODS

2.1 | Subjects

Cord blood was collected from healthy women with uncomplicated pregnancies from Seattle, Washington (Fred Hutchinson) and Madison, Wisconsin (University of Wisconsin Hospital, Meriter Hospital) area hospitals in accordance with local Internal Review Board guidelines.[12,13] PBMC and/or explanted grafts were obtained from healthy individuals, kidney, or liver transplant recipients in accordance with local Institutional Internal Review Board guidelines.

2.2 | Polymorphism-specific qPCR for microchimerism detection

DNA was extracted using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) and resuspended in Tris-HCl (pH 8.5). Each sample was assayed for MMc by selecting a qPCR assay specific to the mother of the cord blood from a panel of HLA-specific qPCR or non-HLA polymorphism-specific qPCR assays. The assays were all previously validated for specificity for the intended polymorphism, and to be able to amplify up to a single DNA copy in a background of four orders of magnitude of non-specific copies per test aliquot.[14] Real-time qPCR reactions were performed on ABI® Prism 7700 (Applied Biosystems, Waltham, MA, USA), as described previously.

2.3 | Multiparameter and imaging flow cytometry

Fluorochrome-labeled monoclonal anti-human antibodies were used at optimized concentrations according to vendor technical documents and titration experiments in our lab as previously described.[15] Following exclusion of debris using a SSC-A vs FSC-A gate, live singlets were stained and visualized for multi-parameter flow cytometry using a BD LSR-II, or Amnis ImageStream

instrument acquired at 60X magnification as previously described.[15] DCs were gated as $CD45^+$, Lineage negative ($CD3^-$, $CD14^-$, $CD16^-$, $CD19^-$, $CD20^-$, $CD56^-$) $HLA\text{-}DR^+$ cells, which were further classified into mDCs ($CD11c^+$, $CD123^-$) and pDCs ($CD11c^-$, $CD123^+$).

2.4 | HLA antibodies

Antibodies specific for HLA alleles were chosen and characterized to confirm specificity. The antibody clones used were: anti-HLA-A1 (0289HA), -A2 (BB7.2), -A2,24,28 (CR11-351; REA142), -A2, B57 (MA2.1), -A3 (GAP-A3), -A9 ([A23,24], REA127), -A24 (17A10), -B7,27,w22 (ME-1), -B7,40 (MB40.2), -B8 (REA145), -B12 ([B44,45] REA138), Pan-HLA-I (W6/32), and Pan-HLA-DR (L243). Antibodies were obtained from ATCC cell line cultures and purified in-house, or purchased from Biolegend, Miltenyi Biotech, MLB Sciences or OneLambda.

For flow cytometry experiments samples, were stained with HLA antibodies exclusive to baby and mother, or donor and recipient and, as a control, in addition to a fluorescence minus one control and antibodies against HLA not present in the pair (irrelevant antibody control) to assess background fluorescence and control for cross-reactivity.

2.5 | EV isolation and analysis

EVs were isolated from cord blood or adult plasma as previously described. Briefly, samples were subjected to serial centrifugation at 4°C, 300 × g for 10 min (removing cells), 2000 × g for 10 minutes (removing dead cells), and 10,000 × g for 30 min (removing cell debris). Supernatants were filtered (0.45 μm) and run through a 30 nm size exclusion chromatography column (iZON qEV30). The EV-containing fractions were pooled and subjected to 100,000 × g ultracentrifugation for 2 h at 4°C. Supernatants were collected as an EV-free fraction, and the pellets were re-suspended in PBS.

2.6 | Particle size and concentration analysis

The size distribution and particle concentration of the isolated EVs were measured using a Nanosight NS300 equipped with Nanosight NTA 3.3 software and 532 nm laser (Malvern Instruments, UK) as previously described.[15]

2.7 | EV miRNA analysis

Total RNA was isolated from EV pellets collected from donor plasma using miRNeasy kit (Qiagen). Seventy-five nanograms of RNA were reverse transcribed using miScript RT II kit (Qiagen) using HiFlex buffer to amplify miRNA and mRNA concurrently. Expression of miR-24, miR-30b-5p, and miR-142-3p (primers purchased from Qiagen), PD-L1 and β-actin (primers purchased from Sigma) was assessed by real-time quantitative PCR, and average Ct values were plotted.

2.8 | Transmission electron microscopy—Negative Staining and Immuno-gold labeling

Isolated EVs were fixed and prepared for TEM or immune-gold labeling as previously described. Grids were stained with either anti-HLA-B8 (1:10; REA145, Miltenyi), anti-PD-L1 (1:50; R&D, AF156), anti-CD9 (1:30; Santa Cruz, C-4), overnight at 4°C. followed by incubation with the appropriate secondary antibody conjugated gold bead of either 10nm (HLA, PD-L1) or 6nm (CD9). Stained grids were viewed using a FEI Tecnai T-12 transmission electron microscope.

2.9 | ELISA

ELISAs for extracellular vesicles were done using a modification of the method of Logozzi et al[16] and previously described in Sullivan et al.[15] Briefly, half-area-well microtiter plates were coated with various capture HLA antibodies or anti-PD-L1 antibody (R&D Systems, AF156) at 10μg/ml each in 10 mM Tris (pH 9) and detected with biotinylated anti-tetraspanin monoclonal antibodies (anti-CD9 [Ancell, SN4/C3-3A2]), anti-CD63 [Ancell, AHN16.1/46-4–5] or anti-CD81 [Ancell, 1.3.3.22]) at 1 μg/ml concentration.

2.10 | Immunoprecipitation and Western blot

Equal amounts of isolated EV or non-EV solutions were boiled in loading buffer containing sample buffer and RIPA lysis buffer, and electrophoresed on a 12% Mini-Protein TGX Precast Gels (BioRad) as previously described.[15] Membranes were incubated with anti-CD9 (1:500; C-4 [Santa Cruz Biotechnology]), anti-PD-L1 (1:666; E1L3N [Cell Signaling Technology]), anti-GM130 (1:1000; D6B1 [Cell Signaling Technology]), and anti-gp96. Appropriate HRP-conjugated secondary antibodies were incubated for 1 h at room temperature, and target bands were visualized using a Las-4000 mini imager.[15]

For immunoprecipitation experiments, cyanogen-bromide-activated Sepharose 4B beads were coupled to either anti-HLA-A3 antibody (GAP-A3) or anti-A24 antibody (17A10) to achieve a concentration of 2 mg of antibody per ml of swollen beads. Antibody binding efficiency was corroborated by the determination of the protein concentration before and after overnight incubation with swollen beads. Beads were blocked by incubation with 1 M ethanolamine followed by two washes with PBS and three washes with alternating cycles of 0.1 M borate/0.5 M NaCl pH 8.0 and 0.1 M acetate/0.5 M NaCl pH 4.0. 12.5 μls of antibody-coupled bead slurry were incubated with EV samples overnight at 4°C with tilting and rotation. The supernatant was collected, and the beads washed 3x

with PBS-Tween 0.05% and resuspended in SDS-PAGE sample buffer (containing 2% SDS, EDTA, and β-mercaptoethanol) and heated to 100°C before proceeding to SDS-PAGE.

2.11 | Statistical analysis

Statistical analyses were performed using Graph Pad Prism (GraphPad Software v 5.01, San Diego, CA). *p*-values were calculated using paired student's *t* tests.

3 | RESULTS

3.1 | Fetal cord blood dendritic cells become cross-decorated with NIMA *in utero* and express high levels of PD-L1

While the presence of MMc in human, adult peripheral blood, and tissues has been reported,[8] the XD of host APC with NIMA has not yet been thoroughly investigated in humans. Using multi-parameter flow cytometry, we attempted to identify acquired NIMA expression in recirculating mDCs and pDCs, however, we found no such cells among PBMCs of healthy adults (data not shown).

To maximize the probability of detecting NIMA-XD host cells, we analyzed umbilical cord blood, as this would contain the highest concentration of maternal antigen exposure. We performed qPCR on isolated cord blood mononuclear cells from 13 full-term, non-complicated pregnancies to detect non-shared maternal HLA and non-HLA polymorphic alleles. We found MMc in 61.5% of these cord blood (Table 1). In addition, we performed multi-parameter flow cytometry experiments on the cord blood mononuclear cells with antibodies against IPA (present only in fetal cells) and NIMA (the maternal HLA haplotype not inherited by the baby). We found XD cells in 50% (6/12) of these samples (Table 1; one cord blood, 294C2, was excluded due to low cell viability).

Figure 1A shows representative FACS plots of myeloid (top left) and plasmacytoid DCs (bottom left) isolated from cord blood samples positive for fetal HLA-A1 and maternal HLA-A2. HLA-A2 specific staining was confirmed by flow cytometry in samples from both cord blood and healthy, adult individuals (Figures S1 and S2). Among the 6 cords that had XD, 2.6% (±0.5) of mDCs and 4.5% (±1.7) of pDCs showed dim surface NIMA expression (Figure 1A). NIMA-positive and IPA-negative cells (i.e., maternal cells) were not observed by flow cytometry, consistent with their infrequency in cord blood and only be reliably detected by qPCR.[17] While we did not find a significant difference between the frequency of XD-mDC vs XD-pDC (Figure 1B), analysis of PD-L1 expression among the NIMA-positive and NIMA-negative groups of mDC and pDC (Figure 1A) showed PD-L1 expression was significantly increased in XD-mDC and XD-pDC vs their non-XD counterparts (Figure 1C). The expression of PD-L1 was relatively low to absent in the vast majority (>95%) of both DC subsets that remained non-XD. Specifically, non-XD mDCs and pDCs both showed low levels of PD-L1 positivity, i.e., 15.07% (± 4.3) for mDC and 19% (± 8.3) for pDC. In contrast, PD-L1 expression was consistently high in the small number of XD cells: 53.7% (± 8.4) in mDCs and 54.7% (± 15) in pDC, a difference that was highly significant (Figure 1C; $p = .0016$ mDCs, 0.024 pDCs).

We further analyzed isolated cord blood cells using imaging flow cytometry (ImageStream) for NIMA, IPA, and PD-L1 expression patterns. Consistent with conventional multiparameter flow cytometry, nearly all cells showed a bright, uniform, global expression of the IPA. NIMA expression was limited to a minority of cells in those samples that were XD positive by conventional flow cytometry. By ImageStream analysis, the staining for acquired NIMA in the XD subset of mDCs ($CD11c^+$) was punctate and discontinuous, suggesting

TABLE 1 Cross decoration, microchimerism, and demography of utilized cord blood samples

ID	Maternal age	Race	Sex	NIMA	MMc in CB (qPCR)	XD
237C1	23	Caucasian	F	HLA-B8	Yes	Yes
250C1	33	Caucasian	F	HLA-A2	Yes	Yes
256C2	28	Caucasian	F	HLA-A2, HLA-B7	No	Yes
274C1	27	Caucasian	F	HLA-B27	No	No
294C2	36	Caucasian	F	HLA-A2	Yes	NA
324C2	30	Mixed	F	HLA-A24	Yes	No
329C1	35	Asian	F	HLA-B7	Yes	No
333C1	34	Mixed	F	HLA-B7	Yes	No
365C2	38	Caucasian	F	HLA-B7	No	No
366C2	39	Caucasian	F	HLA-A24	Yes	No
371C1	29	Caucasian	M	HLA-B8	No	Yes
374C2	33	Mixed	F	HLA-B7	No	Yes
379C1	30	Caucasian	M	HLA-B44	Yes	Yes

Note: The age and race of each mother, the antibodies used to detect NIMA XD on newborn DC, and the result(Y/N) of evaluation of maternal micro-chimerism (MMc) by PCR, are shown.

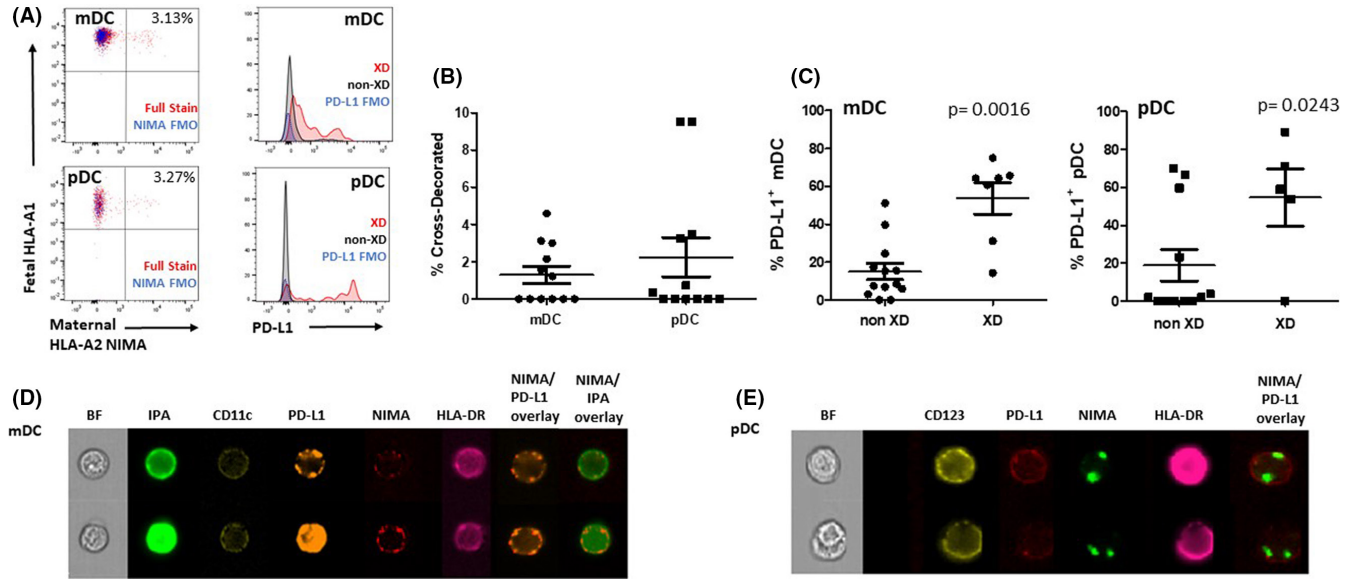

FIGURE 1 Fetal cord blood dendritic cells become cross-decorated with NIMA in utero and express high levels of PD-L1. (A) Representative flow cytometry plots of mDCs (top) and pDCs (bottom) isolated from human cord blood showing fetal (IPA-HLA-A1$^+$) mDCs and pDCs expressing NIMA (HLA-A2$^+$) (A, left). (1A, left) Flow cytometry histograms comparing expression of PD-L1 in non-XD (quadrant 1) vs XD (quadrant 2) mDCs and pDCs from panel A right. Red: cross-decorated, black: non-cross-decorated, blue: PD-L1 FMO control. (B) Graph of the percentage of XD mDC and pDC isolated from cord blood. (C) Plot of the percentage of PD-L1 expression on XD vs non-XD mDCs and pDCs. Data are from $n = 12$ samples. Significant differences between groups were determined by paired student's t tests. (D) Representative ImageStream examples of cross-decorated mDCs (CD11c$^+$) in a cord blood sample. While the IPA (fetal marker) expression is bright, uniform, and continuous, NIMA expression is punctate and discontinuous. PD-L1 expression is also punctate and mostly co-localized in the same areas of the cell surface that show NIMA as shown in the overlay. In the second example, the PDL-1 expression appears less constrained than the NIMA. (E) Representative ImageStream examples from a cord blood sample of cross-decorated pDCs (CD123$^+$) showing continuous expression of PD-L1 with a punctate NIMA appearance along the surface of the cell

exogenous acquisition through EVs (Figure 1D). PD-L1 expression had a somewhat similar pattern of expression to the NIMA staining profile. In pDCs (CD123$^+$), we also found a punctate pattern of NIMA expression, but PD-L1 appeared to be less linked to those spots of acquired antigen than the mDC (Figure 1E). Analysis of the bright detail similarity index (BDSI), a measurement of co-localization, for the NIMA and PD-L1 signal was 1.7 in mDCs and 1.4 in pDCs (Figure S3A, B), indicating a potential co-localization of these markers.

3.2 | Cord blood plasma contains EVs of maternal origin containing NIMA and PD-L1

To determine whether EVs could carry NIMA and PD-L1, we characterized EVs from isolated cord blood samples with direct and indirect assays of EV-PD-L1-NIMA association. Transmission electron microscopy (TEM) of isolated EV samples showed morphology consistent with other EV reports (Figure 2A). Nanoparticle tracking analysis (NTA) from isolated cord blood EVs showed a mean size of 116 nm ±5.3, a size distribution consistent with that of exosomes, a subset of EVs (Figure 2B,C).

Immunoblotting of isolated EV samples corroborated their EV identity as they expressed the tetraspanin CD9, an EV-associated protein, but lacked GM130, gp96, and calnexin (indicating no contamination with Golgi or endoplasmic reticulum membranes) (Figure 2D). To indicate the association of PD-L1 and NIMA on EVs, we used NIMA antibody-coupled beads to capture maternal EVs from cord blood plasma and separate them from fetal EVs. We detected PD-L1 and CD9 on maternal, NIMA-carrying EVs (Figure 2E). Some PD-L1 was detected in the fetal EVs, albeit at a lower signal despite representing the bulk of the cord EVs. Thus, maternal EVs circulating in cord blood plasma are enriched in PD-L1 protein, and DCs that acquire NIMA through maternal EVs will potentially co-acquire PD-L1 in the same area.

We next investigated the presence of PD-L1 on cord blood EVs using ELISA. Using a sequential dilution ELISA and coating ELISA plates with a PD-L1 antibody followed by tetraspanin (CD9, CD63, CD81) detection antibodies, we found CD9 to be the highest associated tetraspanin with PD-L1 (Figure 2F, top left). Both CD63 and CD81 exhibited low levels of detection with PD-L1. Most of the signal in this ELISA was in the initial dilutions and weakened after diluting beyond fourfold when it approached our level of detection, thus we confirmed this result using a more stepwise dilution strategy (Figure 2F, bottom left). This ELISA approach was also used to detect NIMA in tetraspanin-expressing EVs. Using a stepwise dilution of cord blood samples, we detected HLA-B7-NIMA$^+$ (middle top) and HLA-B40- NIMA$^+$ (bottom middle) on CD9$^+$ EVs. The signal for NIMA (HLA-B7) in cord blood EVs was much lower than for self-HLA EVs in cord blood (Figure 2F top right) or adult plasma (Figure S4), consistent with the notion that a minority of cord blood EVs are maternal in origin, and most originate from fetal-derived tissues.

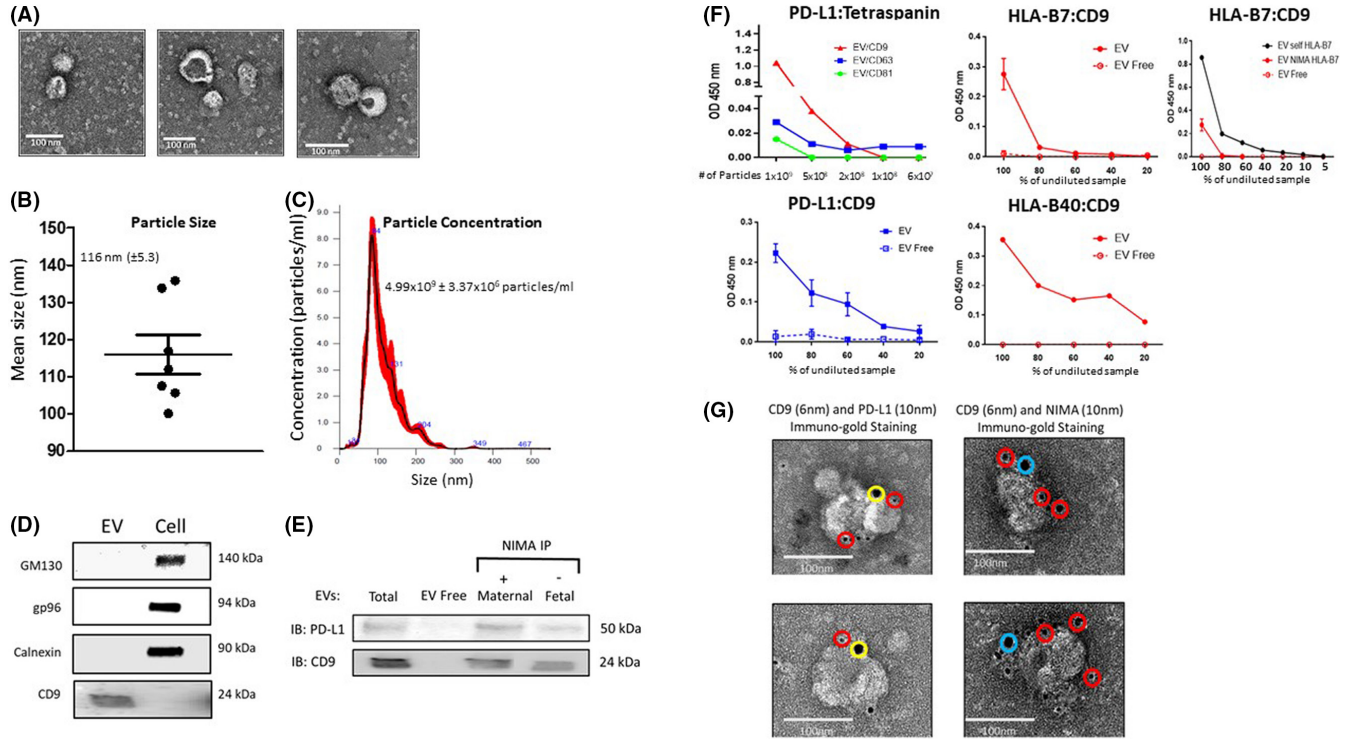

FIGURE 2 Cord blood plasma contains EVs of maternal origin containing NIMA and PD-L1. (A) Representative transmission electron microscopy images of cord blood EVs, showing the expected morphology of EVs. (B) Graph of the mean particle size of isolated EVs from cord blood samples as determined by NTA. The mean particle size of isolated EVs was 116nm +/ 5.3. (C) Representative NTA data showing the size and concentration of isolated EVs. The mean concentration of all EV samples measured was 4.99×10^9 +/− 3.37×10^6 particles/ml of plasma. (D) Western blot of isolated EVs or whole cell lysate illustrating the presence of EV associated proteins (CD9) and the absence of cell-associated contaminants (Golgi-GM130, endoplasmic reticulum-gp96, and calnexin) in isolated EV preparations. (E) Immuno-precipitation of isolated EVs illustrates the association of CD9, PD-L1, and NIMA-HLA on EVs. EVs from cord blood were immuno-precipitated using appropriate anti-NIMA beads. Bead-bound (NIMA, maternal) and non-bead-bound (fetal) fractions were separated in a polyacrylamide gel, before immunoblotting with PD-L1 and CD9. Non-immunoprecipitated extracellular vesicles and extracellular vesicle-depleted culture supernatants were included as controls. The experiment shows enrichment of PD-L1 in maternal (immunoprecipitated) EVs. (F) ELISAs for PD-L1, tetraspanin, and NIMA-HLA support the association of these three molecules on isolated EVs from cord blood. Tetraspanins (CD9, CD63, and CD81) were used in ELISA assays to determine the relative association of the number of nanoparticles containing tetraspanins and PD-L1 in EVs isolated from cord blood (top left). CD9 was the highest detected tetraspanin using a step-wise dilution of total particles, determined by NTA analysis, of cord blood EVs. ELISAs focused on CD9-PD-L1 association were performed in a minimal dilution strategy to show the concentration-dependent trend in the CD9-PD-L1 signal (bottom, left). ELISAs examining individual NIMA association with CD9 from cord blood EVs were also performed (middle and right). The middle top and bottom graphs show the association of HLA-B7 (a NIMA) and CD9 in isolated cord blood EVs from HLA-B7 NIMA samples. Panel F, right, shows the NIMA-B7 signal juxtaposed with a sample in which HLA-B7 was the self-HLA. **(G)** Immuno-gold labeling TEM illustrates the association of CD9, PD-L1, and HLA-NIMA on EVs isolated from cord blood. Representative TEM images after immuno-gold double-antibody labeling, with PD-L1 (10-nm gold beads, yellow circles) and CD9 (6-nm gold beads, red circles) (left panels) or NIMA (10-nm gold beads, blue circles) and CD9 (6-nm gold beads, red circles) (right panels) of purified EVs isolated from cord blood samples

To confirm that maternal $CD9^+$ EVs carry both NIMA and PD-L1 in cord blood plasma, we performed immuno-gold labeling TEM to detect specific HLA-NIMAs, CD9, and PD-L1 on cord blood EVs. Using secondary antibodies conjugated to varying sized gold beads, we were able to identify CD9 (6nm bead) and PD-L1 (10 nm) (Figure 2G left) and CD9 (6nm bead) and NIMA (10 nm bead) (Figure 2G, right) on isolated EVs from cord blood further confirming our observation that PD-L1 and NIMA are found on EVs from cord blood.

To determine whether EVs are causing an upregulation of PD-L1 outside of acquired PD-L1 protein, we analyzed the miRNA content of cord blood EVs and found the presence of miR-24, miR-30b, and miR-142-3p, three miRNAs that upregulate PD-L1 expression in APCs, together with PD-L1 mRNA (Figure S5A–D).[18,19] The potential of this nucleic acid cargo leading to PD-L1 upregulation in XD cells outside of the domains of acquired NIMA and complimenting acquisition of protein PD-L1 via EVs is outlined in Figure 5SF.

3.3 | Cross decoration of DCs with NIMA andPD-L1 in human kidney and liver grafts

To determine if NIMA/PD-L1 XD is a common characteristic of acquired tolerance, we looked for the presence of NIMA/PD-L1 XD in kidney or liver transplant patients. Using an explanted maternal

kidney graft, mismatched with the recipient (daughter) at donor HLA-A2, and removed due to high-grade vesico-ureteral reflux with no features of rejection, we found 1.96% of XD mDCs and 4.79% of XD pDCs in peripheral blood, and 14.7% of XD mDCs and 5.2% of XD pDCs within the graft (Figure 3A, left and right, Figure S6). XD- DCs with donor antigen exhibited the characteristic punctate expression pattern (Figure S7).

In peripheral blood, PD-L1 was virtually absent in the non-XD compartment, whereas 100% of XD-mDCs and 80.3% of XD-pDCs were PD-L1-positive (Figure 3B top). Within the graft, this association was even more pronounced, with 97% of XD-mDCs and 95.3% of XD-pDCs expressing PD-L1 (Figure 3B bottom).

To investigate if this phenomenon was also observed in liver transplants, we studied a living sibling donor liver transplant recipient (Figure 3C, D). As with the kidney, this liver transplant was mismatched with the recipient at donor HLA-A2, which was a NIMA to the recipient. The graft did not show signs of immunological rejection at this timepoint (Table 2). Using PBMCs, we found acquired HLA-A2 (NIMA) in 2.05% of mDCs and 1.71% of pDCs (Figure 3C). Among the DCs that remained non-XD, PD-L1 expression was found in 13.3% of mDCs and 5% of pDCs, but in those that became XD the expression of PD-L1 increased to 81.1% in mDCs and 59.5% in pDCs (Figure 3D).

4 | DISCUSSION

The immunological challenge posed during pregnancy wherein the fetal and maternal environment co-exist while exhibiting varying levels of allogenic mismatch led to the hypothesis of acquired immunological tolerance[1,2] and the idea that mechanisms designed to maintain maternal and fetal tolerance to allogenic mismatch develop in utero and persist lifelong.[20] Consistent with the data proposed in this manuscript, EVs of maternal origin cross the placental barrier to the cord blood circulation where they are acquired by fetal dendritic

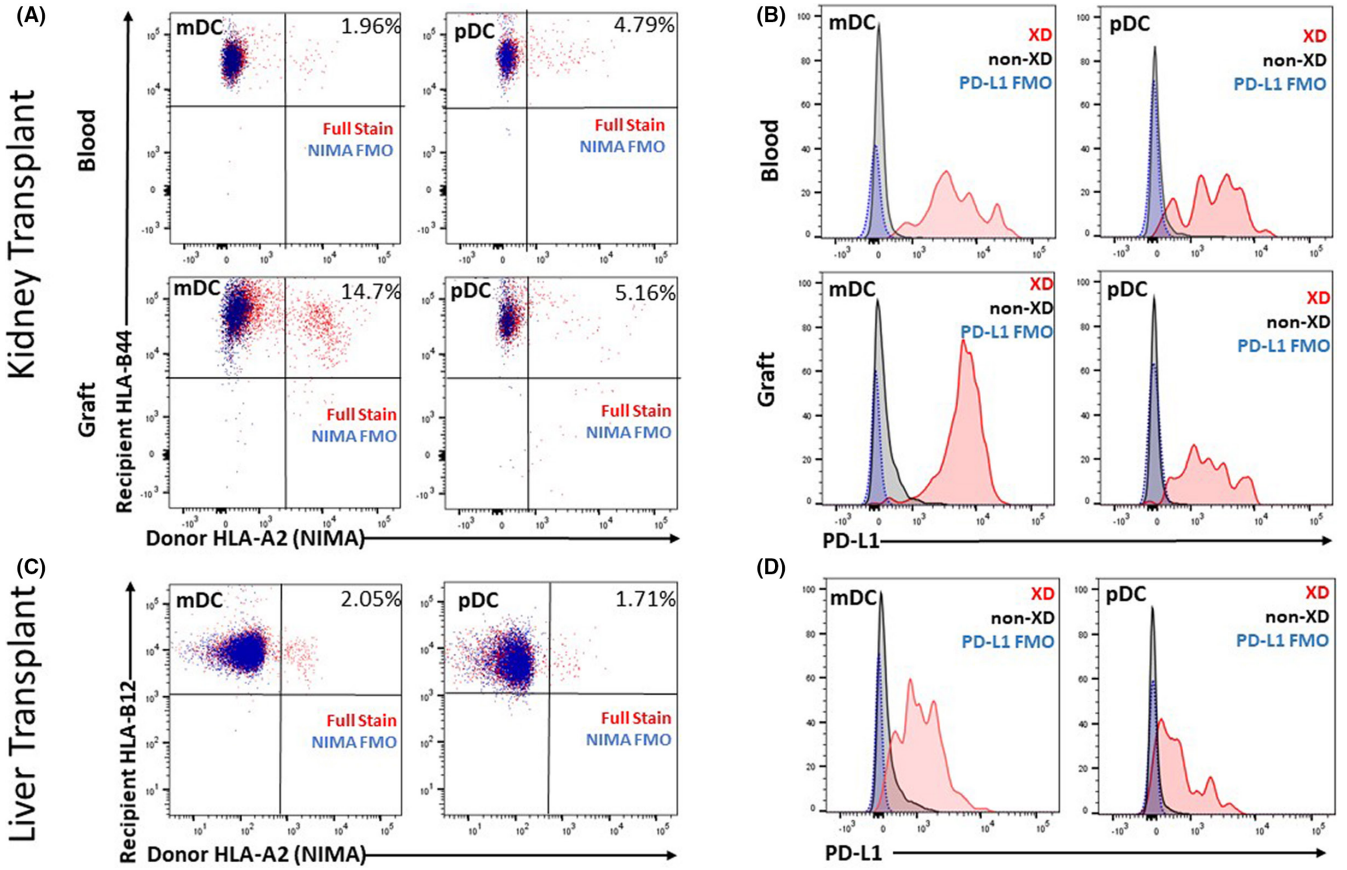

FIGURE 3 NIMA-mismatched kidney and liver grafts recapitulate the cord-blood cross-decorating phenomenon. (A) Representative flow cytometry plots of XD in mDC (left) and pDC (right) populations from the blood (top) or explanted graft (bottom) of a living donor mother to daughter transplant (where all the mismatches are NIMAs). While all the cells were positive for the recipient-exclusive HLA-B44, we detected surface expression of donor antigen (NIMA) HLA-A2 in a subset of circulating mDCs and pDCs (top), and a much larger fraction in graft infiltrating mDCs and pDCs (bottom). (B) Representative histograms of PD-L1 expression on XD and non-XD-mDCs (left) and pDCs (right). PD-L1 expression was higher in XD vs non-XD mDCs and pDCs, both in blood (top) and the graft (bottom). (C, D) A living donor brother-to-sister liver transplant, mismatched at HLA-A2, a NIMA, was analyzed for donor antigen acquisition and PD-L1 expression. (C) While virtually all the DCs were positive for the recipient-exclusive HLA-B12, we detected surface expression of donor antigen (NIMA) HLA-A2 in a subset of circulating mDCs and pDCs. (D) Determination of PD-L1 expression in populations of XD vs non-XD mDCs and pDCs was observed and XD of mDCs and pDCs, similar to the kidney transplant patient, was associated with a higher expression of PD-L1

cells, cross-decorating them with maternal HLA class I together with EV PD-L1 (Figure 4). The NIMA is presented with co-acquired PD-L1 to fetal T cells by the semi-direct pathway, leading to suppression of these alloreactive cells. Additionally, EV-transferred miRNAs and PD-L1 mRNA contribute to PD-L1 detection in other areas of the cell membrane, leading to suppression of the indirect pathway.

We detected NIMA (HLA class I) on the surface of both mDCs and pDCs in half the cords studied, which is remarkably similar to the frequency of adult mice that demonstrate NIMA XD in a previous report from our lab (45%).[9] The similarity between these two studies, one in mice and our current study in humans implies an evolutionarily conserved mechanism to afford the development of fetal-maternal tolerance in eutherian animals.

One of the most significant observations from this study is the fact that acquired-fetal NIMA tolerance from EVs may help determine solid organ transplantation outcomes throughout life. Assaying several different live-donor liver transplant recipients for alloantigen XD and PD-L1 expression on circulating and graft-infiltrating DC (data not shown), we found the highest expression of PD-L1 in XD-DCs was from a NIMA mismatch. Thus, the tolerogenicity of a well-accepted organ graft (such as liver) could be enhanced by the benefits of the NIMA effect. Indeed, 60% of mother-to-child liver transplants (where all mismatches are NIMA) could undergo immunosuppression withdrawal with operational tolerance.[5] Similarly, peripheral blood and the explanted kidney of a NIMA (mother-daughter) mismatch kidney transplant patient, a feature that is well

TABLE 2 Transplant patient summary table

Patient ID	Sex	Age (years)	Transplant type	Diagnosis at transplant	Assessment of XD post-transplant	Immunosuppressive regimen	HLA mismatches (bold = NIMA)
1	Female	39	Kidney: living mother-to-daughter	Crescentic glomerulonephritis	19 years	Prednisone +tacrolimus. Induction: basiliximab, mycophenolate	HLA-A2, HLA-B14, HLA-DR13, HLA-DQ6
2	Female	20	Liver: living sibling	Autoimmune hepatitis	3 years	Prednisone +mycophenolate + tacrolimus	HLA-A2, HLA-B7, HLA-DR3, HLA-DQ2

Note: Pertinent information including immunosuppressive regimen, NIMA mismatches, and time of XD assessment following transplantation is illustrated for both kidney and liver transplant patients.

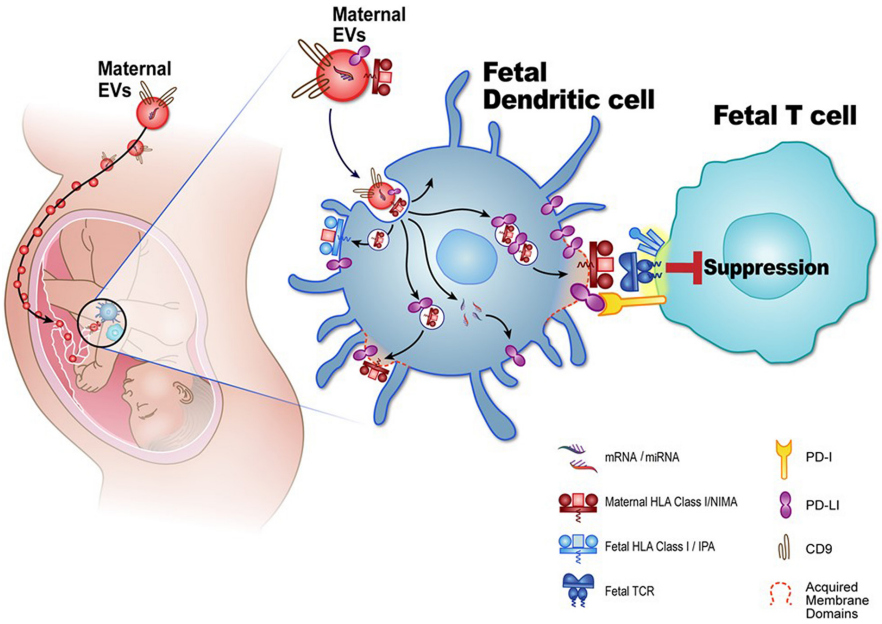

FIGURE 4 Model of fetal dendritic cell cross-decoration with maternal antigens and PD-L1 in utero. Maternally derived EVs carrying HLA-NIMA and PD-L1 enter the cord blood and are acquired by fetal dendritic cells, leading to the expression of NIMA and PD-L1 on the surface of the fetal DC. Tolerogenic semi-direct presentation of NIMA by PD-L1-expressing dendritic cells contributes to tolerance to NIMAs by helping to suppress fetal T cell activation through co-inhibitory receptor engagement. Additionally, maternal EVs may transfer PD-L1 mRNA/miRNAs that upregulate PD-L1 production outside of the domains of maternal HLA and PD-L1 acquisition leading to a further anti-activation/inhibitory phenotype of the fetal DC and inhibition of indirect presentation of NIMA epitopes

known to be tolerogenic in this transplant type,[3,4] exhibited NIMA/PD-L1 acquisition.

In our investigation of a mother-to-daughter kidney transplant, the punctate pattern of NIMA HLA-A2 expression (Figure S4) was consistent with the acquisition of HLA class I through EVs. Because both cords with and without MMc showed NIMA-XD, and these are the only maternal cells present in cord blood, we believe NIMA EVs are transferred through the placenta to impact fetal immune cells (Figure 4). Although there are reports of fetal-to-maternal EV transfer through the placenta, the literature reporting transfer in the opposite direction (mother to fetus) is lacking.[21,22] This in utero maternal-to-fetal transfer of physiologically active EVs opens the door to a multiplicity of immuno-therapeutic approaches to address pregnancy-related disorders, including preeclampsia, intra-uterine growth retardation, and pathologies specific to the fetus that can be ameliorated through natural or artificial EV-mediated interventions. This area of investigation is currently ongoing.

There was no association between the presence of MMc and XD in our cord blood samples. While the prevalence and level of detection of MMc in cord blood are an ongoing debate[14,23] there is, however, agreement in the literature that these cells are infrequent (<0.1%)[24] and below the level of detection by FACS.[17] A major implication of our work is that a very small number of cells can have an exponential effect by producing considerable amounts of EVs containing NIMA and PD-L1 that when acquired effect a large number of cells. The level of XD, therefore, is reflective of a NIMA$^+$ cell secreting these tolerogenic EVs promoting acquired tolerance. The tolerogenic effect of the EVs may not just be the result of simply transferring PD-L1 protein on an EV to an acquiring cell. We detected miRNAs and PD-L1 mRNA in cord blood EVs (Figure S5). This suggests a possible induction of endogenous PD-L1 that could complement PD-L1 protein transfer in maternal cross-decorated cells. This could also be one explanation for our observation of surface PD-L1 on acquiring cells, beyond the "pockets" of EV acquisition on the plasma membrane. These two distinct patterns of expression have previously been suggested as the basis for NIMA-associated "split" tolerance;[9] in kidney transplantation, this refers to the increase of direct/semi-direct alloreactivity leading to more early acute rejection episodes in NIMA$^+$ grafts, coupled with a decrease of indirect pathway alloreactivity resulting in less chronic/late rejection incidence.[3,4]

In transplantation, cross-decoration of recipient DCs is central to acute rejection, and to sensitize the recipient immune system to alloantigens, cross-decoration causes widespread semi-direct alloantigen presentation, thus amplifying the immune response to them.[25,26] The context of alloantigen engagement will skew this response to tolerance or rejection. For example, immunogenic models of skin, heart, pancreatic islet, lung, or kidney transplantation are spontaneously rejected in an EV-dependent fashion.[27-29] More tolerogenic grafts (e.g., liver, kidney grafts that express NIMA) might use EVs to instigate immune regulation. In a spontaneously tolerated murine liver transplant model, graft-infiltrating host DCs become cross-decorated with donor MHC-I. XD-DCs can represent up to 60% of all DCs at their peak but decline until reaching a steady level that persists indefinitely. Additionally, XD DC shows high PD-L1 expression.[30] All these findings are remarkably similar to our own in humans.

It has been reported that the appearance of circulating XD-leukocytes in peripheral blood after liver transplantation peaks in the first week following transplantation and then quickly abates.[31] In this report, they also found no XD cells in peripheral blood after kidney transplantation.[31] In contrast to this report, we found XD-DCs in both liver and kidney transplant recipients, both circulating and, in the graft, years after transplantation.

This manuscript puts forth a novel EV-dependent mechanism by which maternal-fetal NIMA-tolerance can be generated and maintained throughout life. With both NIMA and PD-L1 being transferred to acquiring cells, an anti-inflammatory milieu can be generated and acquired tolerance can persist. While important for maternal-fetal tolerance in utero, this form of NIMA tolerance persists throughout life and can be exploited in strategies to utilize naturally acquired split tolerance in solid organ transplantation. NIMA mismatch and manipulation of co-activation markers/inhibitory receptor levels by therapeutic application of EVs can be the difference between acute and chronic graft rejection or operational tolerance.

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DISCLOSURE

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

DATA AVAILABILITY STATEMENT

We cannot host the data as per the data-sharing policy.

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REFERENCES

1. Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science*. 1945;102:400-401.
2. Billingham RE, Brent L, Medawar PB. 'Actively acquired tolerance' of foreign cells. *Nature*. 1953;172:603-606.
3. Burlingham WJ, Grailer AP, Heisey DM, et al. The effect of tolerance to noninherited maternal HLA antigens on the survival of renal transplants from sibling donors. *N Engl J Med*. 1998;339:1657-1664.
4. Smits JM, Claas FH, van Houwelingen HC, et al. Do noninherited maternal antigens (NIMA) enhance renal graft survival? *Transpl Int*. 1998;11:82-88.
5. Kim MH, Akbari O, Genyk Y, et al. Immunologic benefit of maternal donors in pediatric living donor liver transplantation. *Pediatr Transplant*. 2019;23:e13560.
6. Hall JM, Lingenfelter P, Adams SL, et al. Detection of maternal cells in human umbilical cord blood using fluorescence in situ hybridization. *Blood*. 1995;86:2829-2832.
7. Socié G, Gluckman E, Carosella E, et al. Search for maternal cells in human umbilical cord blood by polymerase chain reaction amplification of two minisatellite sequences. *Blood*. 1994;83:340-344.
8. Maloney S, Smith A, Furst DE, et al. Microchimerism of maternal origin persists into adult life. *J Clin Investig*. 1999;104:41-47.
9. Bracamonte-Baran W, Florentin J, Zhou Y, et al. Modification of host dendritic cells by microchimerism-derived extracellular vesicles generates split tolerance. *Proc Natl Acad Sci*. 2017;114:1099-1104.
10. Kinder JM, Stelzer IA, Arck PC, et al. Immunological implications of pregnancy-induced microchimerism. *Nat Rev Immunol*. 2017;17:483-494.
11. Dutta P, Burlingham WJ. Correlation between post transplant maternal microchimerism and tolerance across MHC barriers in mice. *Chimerism*. 2011;2:78-83.
12. Lee TH, Paglieroni T, Ohto H, et al. Survival of donor leukocyte subpopulations in immunocompetent transfusion recipients: frequent long-term microchimerism in severe trauma patients. *Blood*. 1999;93:3127-3139.
13. de bellefon LM, Heiman P, Kanaan SB, et al. Cells from a vanished twin as a source of microchimerism 40 years later in a male with a scleroderma-like condition. *Chimerism*. 2010;1:56-60.
14. Kanaan SB, Gammill HS, Harrington WE, et al. Maternal microchimerism is prevalent in cord blood in memory T cells and other cell subsets, and persists post-transplant. *Oncoimmunology*. 2017;6:e1311436.
15. Sullivan JA, Tomita Y, Jankowska-Gan E, et al. Treg-cell-derived IL-35-coated extracellular vesicles promote infectious tolerance. *Cell Rep*. 2020;30:1039-1051.e5.
16. Logozzi M, De Milito A, Lugini L, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One*. 2009;4:e5219.
17. Opstelten R, Slot MC, Lardy NM, et al. Determining the extent of maternal-foetal chimerism in cord blood. *Sci Rep*. 2019;9:5247.
18. Naqvi AR, Fordham JB, Ganesh B, et al. miR-24, miR-30b and miR-142-3p interfere with antigen processing and presentation by primary macrophages and dendritic cells. *Sci Rep*. 2016;6:32925.
19. Naqvi AR, Fordham JB, Nares S. miR-24, miR-30b, and miR-142-3p Regulate Phagocytosis in Myeloid Inflammatory Cells. *J Immunol*. 2015;194:1916-1927.
20. Eikmans M, van Halteren AG, van Besien K, et al. Naturally acquired microchimerism: implications for transplantation outcome and novel methodologies for detection. *Chimerism*. 2014;5:24-39.
21. Sheller-Miller S, Lei J, Saade G, et al. Feto-maternal trafficking of exosomes in murine pregnancy models. *Front Pharmacol*. 2016;7:432.
22. Adam S, Elfeky O, Kinhal V, et al. Review: Fetal-maternal communication via extracellular vesicles - Implications for complications of pregnancies. *Placenta*. 2017;54:83-88.
23. Kanold AMJ, Westgren M, Götherström C. Cellular subsets of maternal microchimerism in umbilical cord blood. *Cell Transplant*. 2019;28:522-528.
24. Mold JE, Michaëlsson J, Burt TD, et al. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. *Science*. 2008;322:1562-1565.
25. Dolan BP, Gibbs KD, Ostrand-Rosenberg S. Dendritic cells cross-dressed with peptide MHC class I complexes prime $CD8^+$ T cells. *J Immunol*. 2006;177:6018-6024.
26. 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;130:287-294.
27. Nayak DK, Zhou F, Xu M, et al. Zbtb7a induction in alveolar macrophages is implicated in anti-HLA–mediated lung allograft rejection. *Sci Transl Med*. 2017;9:398.
28. 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:aaf8759.
29. Liu Q, Rojas-Canales DM, Divito SJ, et al. Donor dendritic cell–derived exosomes promote allograft-targeting immune response. *J Clin Investig*. 2016;126:2805-2820.
30. 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;67:1499-1515.
31. Mastoridis S, Londoño MC, Kurt A, et al. Impact of donor extracellular vesicle release on recipient cell "cross-dressing" following clinical liver and kidney transplantation. *Am J Transplant*. 2021;21(7):2387-2398.

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