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Donor-derived exosomes: The trick behind the semi-direct pathway of allorecognition

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Abstract

Purpose of review—The passenger leukocyte hypothesis predicts that after transplantation, donor antigen (Ag)-presenting cells (APCs) from the graft present donor MHC molecules to directly alloreactive T cells in lymphoid organs. However, in certain transplantation models, recent evidence contradicts this long-standing concept. New findings demonstrate that host, instead of donor, APCs play a prominent role in allo-sensitization against donor MHC molecules via the semi-direct pathway. A similar mechanism operates in development of T-cell split tolerance to non-inherited maternal Ags (NIMAs).

Recent findings—Following fully-mismatch skin or heart transplantation in mice, no or extremely few donor migrating APCs (i.e. conventional dendritic cells, DCs) are detected in the draining lymphoid organs. Instead, recipient DCs that have captured donor extracellular vesicles (EVs, i.e. exosomes) carrying donor MHC molecules and APC co-stimulatory signals, present donor MHC molecules to directly allo-reactive T cells. This semi-direct pathway can also give rise to a form of “split” tolerance during chronic alloantigen exposure, since indirectly alloreactive T helper cells and directly alloreactive T cell effectors are differentially impacted by host DCs “cross-dressed” with EVs/exosomes derived from maternal microchimerism.

Summary—Acquisition by recipient APCs of donor exosomes (and likely other EVs) released by passenger leukocytes or the graft, explains the potent T-cell allo-sensitization against donor MHC molecules, in absence or presence of few passenger leukocytes in lymphoid organs. It also provides the basic mechanism and in vivo relevance of the elusive semi-direct pathway. Its degree of coordination with the allopeptide –specific, indirect pathway of T cell help may determine whether semi-direct allopresentation results in a sustained, effective, acute rejection response, or rather, in abortive acute rejection and “split” tolerance.

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Introduction

The cellular adaptive immune response against allografts is mediated through recognition by donor-reactive T cells of donor intact alloMHC molecules presented by donor cells -via the direct pathway of allo-recognition-, and of self-MHC molecules loaded with peptides derived from polymorphic regions of the donor MHC molecules or of non-MHC proteins, the latter known as the indirect pathway. More recently, it became evident that directly allo-reactive T cells also recognize donor MHC molecules acquired intact by recipient Ag-presenting cells (APCs) through a third mechanism dubbed the semi-direct pathway.

It has been classically accepted that after organ/tissue transplantation, donor leukocytes transplanted with the graft -mainly conventional dendritic cells (DCs)-, become activated and migrate as “passenger leukocytes” via lymphatic or blood vessels to the draining lymph nodes or spleen, respectively, where they supposedly present by themselves donor intact MHC molecules to directly allo-reactive T cells [1,2]. This concept, which to our knowledge has not been formally proven, has been challenged in recent years. Indeed, donor-derived DCs are undetectable or found at extremely low numbers in graft-draining lymphoid organs during the first week after transplantation of fully H2-mismatched skin, pancreatic islets, or heart allografts in mice [3,4]. Besides, once they leave the graft, donor migrating DCs have a short lifespan and, in fully allogeneic models, they could become targets of recipient NK cells and cytotoxic T cells (CTLs) [5–8]. Importantly, the lymphatic vessels of the grafts – one of the main exit routes of passenger leukocytes- are cut off during surgery, and they fully reconnect with the recipient lymphatic vasculature days after transplantation, once the anti-donor T cell response has been already elicited [4]. Therefore, if no or extremely low number of donor migrating APCs traffic to the graft-draining lymphoid organs, how directly allo-reactive T cells become activated so rapidly and efficiently after transplantation? Two recent studies have independently demonstrated in mouse models that, although donor migrating APCs are difficult to detect in graft-draining lymphoid organs after heart or skin transplantation, a relatively high number of recipient APCs resident in the draining lymphoid organs carry on their cell surface donor intact MHC class-I and class-II molecules [3,4]. These recipient APCs cross-dressed with donor MHC molecules triggered, via the semi-direct pathway, activation and effector cell-differentiation of directly allo-reactive T cells in the graft-draining lymphoid organs.

Passage of intact MHC molecules between allogeneic cells

The fact that APCs acquire allogeneic MHC molecules from other leukocytes or endothelial cells is not novel. It is well established that leukocytes transfer surface molecules, including MHC molecules, through one or multiple mechanisms that have been termed cross-dressing, trogocytosis, or cell nibbling, based on the experimental model analyzed and cell types involved in the molecular transfer. Indeed, Lechler and colleagues originally postulated

cross-dressing of recipient conventional DCs with donor MHC molecules as the basis of the semi-direct pathway of T-cell allo-recognition in transplantation [9]. Experiments where donor and recipient (acceptor) DCs were separated by 0.4µm pore size membranes suggested that although not essential, cell-to-cell contact increases substantially transfer of intact MHC molecules between DCs [9]. Since this seminal study, donor MHC molecules have been detected on the surface of recipient APCs resident in graft-draining lymphoid organs following heart and kidney transplantation in mice [10,11]. After bone marrow transplantation in mice, donor DCs acquire recipient MHC molecules, which indicates that the passage of MHC molecules between donor and recipient APCs in vivo is bi-directional [12]. Despite these relevant findings, the ultimate mechanism(s) by which recipient APCs acquire and retain on the cell surface donor MHC molecules with the right topology for presentation to directly allo-reactive T cells, and the impact that the resulting semi-direct pathway of allo-recognition exerts in graft rejection have remained relatively unexplored until recently. The new and rapidly expanding field of extracellular vesicles (EVs) - in particular exosomes-, as mediators of inter-cellular communication, is currently providing new answers to these long-standing questions in the transplantation field [13].

Extracellular vesicles: membrane couriers between cells

Cells communicate with each other through release of soluble mediators, cell-to-cell contact via surface receptor-ligand interactions and, as more recently discovered, by exchanging EVs, which differ in biogenesis, vesicle size, and molecular composition [14]. It seems likely that during evolution transfer of EVs was one of the primordial mechanisms of cell-to-cell communication before the development of more sophisticated mechanisms of cellular interaction such as binding of surface membrane receptors with its ligands [15]. The term EVs include microvesicles, nanovesicles (i.e. exosomes), vesicles released by cells undergoing apoptosis (i.e. apoptotic cell blebs and apoptotic bodies), and other EVs not well defined yet [16,17] (Table 1). Microvesicles are released by shedding of the plasma membrane of living cells, range in size between 0.2–1 µm, and are also known as microparticles or ectosomes [14]. Exosomes are produced within the endocytic compartment of living cells and are between 30–120 nm in diameter [13,14]. Exosomes are generated as intraluminal vesicles by reverse budding of the limiting membrane of early endosomes, which are then termed multivesicular bodies. Exosomes are released to the extracellular milieu when the limiting membrane of the multivesicular body fuses with the cell membrane. Exosomes can also originate within Golgi-apparatus derived vesicles generated for exocytosis. Once secreted by the parent cells, exosomes bind or are internalized by acceptor cells, attach to the extracellular matrix, or traffic passively through lymph, blood or other bodily fluids. Because there is very limited information regarding the role of microvesicles and apoptotic cell-derived EVs on recognition of non-self MHC molecules in transplantation, the rest of the review will be focused mainly on exosomes.

Exosomes, as other EVs, function as carriers for horizontal propagation of nucleic acids, proteins, and likely lipids and carbohydrates between cells. The composition of exosomes depends on the lineage, and stage of activation/differentiation, infection, and transformation of the parent cells. Exosomes also carry proteins that are preferentially enriched on the surface or in the lumen of the EVs (e.g. the tetraspanins CD9 and CD63, tumor

susceptibility gene 101, syntenin-1), which are commonly used as exosome markers [17]. Exosomes also contain in their lumen functional mRNAs, non-coding RNAs (e.g. microRNAs), and even extra-chromosomal DNA (e.g. amplified *MYC*). Exosomes have cell-independent ability to process precursor miRNAs into mature miRNAs within the EVs [18]. By fusing with the target cells, exosomes deliver their cargo of functional mRNAs, regulatory RNAs, and proteins into the cytosol of the acceptor cells [19–21]. Transfer of miRNAs and proteins through exosomes and other EVs, between cancer cells and the tumor microenvironment and draining lymphoid tissues, participate in tumor initiation, invasion, angiogenesis, pre-metastasis niche formation, drug resistance, and immune escape [22]. Because, to some extent, the RNA and protein content of EVs reflects that of the parent cells, EVs isolated from bodily fluids (e.g. plasma, urine) could be used as potential diagnostic and prognostic biomarkers in cancer, and in infectious or metabolic diseases, and as predictive biomarkers of transplant rejection [23,24]. The potential use of EVs as platforms to deliver biomolecules to target cells for therapeutic applications is an active area of research [25,26]. Exosomes derived from mature and immature DCs have been used, in combination with other therapies, to stimulate or suppress the immune response in humans and murine models [27,28].

Interestingly for the field of transplantation, the surface membrane of exosomes released by activated professional APCs are highly enriched in Ag-presenting molecules (e.g. MHC class-I and –II), T-cell costimulatory molecules (CD86) and APC-T-cell adhesion molecules (e.g. CD54) with their binding domains topologically oriented towards the outer side of the EVs [13]. Although APC-derived exosomes, at relatively high concentrations, can function as Ag-presenting vesicles for T-cell clones and memory T cells, their capability to stimulate by themselves naïve T cells is rather weak [13]. In contrast, the potency of APC-derived exosomes to stimulate naïve T cells increases substantially when the EVs are attached to APCs [13]. Exosomes released by activated/matured APCs (i.e. mature exosomes) carry higher content of surface MHC Ag, CD54 and CD86, and are more potent T-cell stimulators, compared to exosomes secreted by quiescent APCs (i.e. immature exosomes) [29,30]. Moreover, non-professional APCs cross-dressed with mature exosomes released by professional APCs acquire the ability to stimulate naïve T cells [29]. The capacity of graft-derived exosomes -and likely other types of EVs- to transfer non-self MHC Ag and APC-activating mediators to the recipient APCs seems to be behind the elicitation of the rapid and efficient adaptive immune response that leads to acute rejection of allografts.

Transfer of extracellular vesicles boosts direct allo-recognition in transplantation

As mentioned in the Introduction, the concept that donor APCs mobilized from allografts (as passenger leukocytes) present by themselves donor MHC molecules to directly alloreactive T cells has been questioned in recent years. Two recent studies have demonstrated in mice that no or extremely few donor passenger APCs are detected in the graft-draining lymphoid organs after transplantation of fully-mismatch skin or heterotopic (abdomen) heart grafts, respectively [3,4]. However, despite the absence or paucity of donor APCs in the draining lymphoid organs during the first week after transplantation, it is well known that skin and

heart allografts elicit potent activation of directly alloreactive T cells. Interestingly, recent evidence indicates that although no or very few donor passenger APCs mobilize to the draining lymphoid organs within the first week after transplantation of skin or heart allografts in mice, donor MHC molecules are detected in small clusters of EVs attached to recipient conventional DCs and B cells in the graft-draining lymphoid organs [3,4] (Figure 1). The EVs transferred from the donor cells were identified as exosomes based on their size (76 ± 32 nm in diameter) and positivity for the exosome markers CD63 and CD9 [3]. The donor-derived exosomes remained bound to the surface of the recipient DCs for hours, or were endocytosed by the recipient APCs for further processing of the donor Ag for presentation via the indirect pathway, or for degradation [3]. The donor exosomes did not seem to fuse with the surface membrane of the recipient DCs, which explains the observation that the donor MHC molecules were not detected embedded in the plasma membrane of the recipient DCs [3]. Importantly, the donor MHC molecules transferred to recipient APCs were functional. Indeed, within 3 days of heart transplantation, FACS-sorted recipient splenic conventional DCs cross-dressed in vivo with donor exosomes triggered ex vivo proliferation and effector cell differentiation of directly alloreactive CD8 T cells in mice [3]. In vivo, passage of red fluorescent protein-tagged exosomes from i.v. administered BALB/c fully-mature DCs to yellow fluorescent protein-tagged conventional DCs resident in the spleen of C57Bl/6 mice, increased expression of host MHC class-II molecules, CD40, CD80 and CD86, but not PD-L1, by the host DCs [3]. Thus, exosomes released by donor migrating DCs transfer donor MHC molecules to the recipient APCs through exosomes, which also augment the T-cell allo-stimulatory capacity of the acceptor DCs. Interestingly, exosomes released by mature DCs carry functional membrane-bound TNF- α , a potent DC-activating cytokine [31]. In support of a role of the recipient APCs in presentation of donor MHC molecules to donor-reactive T cells in graft-draining lymphoid organs, ablation of recipient conventional DCs after heart transplantation decreased severely presentation of donor intact MHC molecules to directly alloreactive T cells via the semi-direct pathway, and delayed significantly acute allograft rejection in mice [3].

The available evidence indicates that donor-derived EVs that cross-dress the recipient APCs in draining lymphoid organs are released by the passenger leukocytes, donor cells within the graft, or both [3,4] (Figure 1). It is likely that in those allografts where donor passenger leukocytes are unable to migrate out of the transplant (i.e. non-vascularized skin allografts in untreated mice), donor-derived EVs leaked in the graft bed traffic passively through the severed openings of recipient lymphatic capillaries towards the graft-draining lymphoid organs [4]. These EVs could derive from donor passenger cells trapped in the transplant, or endothelial, parenchymal or non-migratory stromal cells from the allograft. A recent study has shown that exosomes are efficiently carried across the lymphatic endothelium in vitro, and are rapidly transported within lymphatic vessels in vivo [32]. Interestingly, exosomes by themselves can stimulate lymphatic vessel formation [33]. Besides the cellular source of the donor EVs, the type of donor EVs secreted, and the relevance of cross-dressing with donor EVs on the semi-direct pathway, may differ depending on the type of organs, tissues, or cells transplanted. EVs may also transfer autoantigens that promote graft rejection. Indeed, serum-deprived endothelial cells release exosome-like EVs enriched in the LG3 fragment of the autoantigen perlecan, a vascular extracellular matrix protein [34]. The LG3-enriched

EVs trigger anti-LG3 antibody production and accelerate rejection of aorta allografts in mice [34].

Exosome transfer and split-tolerance to non-inherited maternal antigens

The idea that no or extremely few donor passenger APCs can have an enormous impact on acute rejection of the allograft by means of a semi-direct, exosome-based mechanism led us to inquire whether such a mechanism could account for the enormous impact of maternal microchimerism. It has long been known that very few maternal cells (on the order of 1 in 10,000 to 1 in 1,000,000) persist in the offspring of a normal mammalian pregnancy. This extremely low proportion of allogeneic cells have a profound impact on the offspring's immune system, as it has been associated with development of tolerance toward non-inherited maternal Ags (NIMAs) [35–37]. An interesting quality of this tolerogenic impact has been described: it is a split tolerance phenomenon, in which direct allorecognition is functional [38,39] and associated with increase in episodes of acute rejection [40], whereas the indirect pathway is regulated/anergized [41] in such a way that, once the acute episodes are overcome, a chronic metastable allo-tolerance is developed [40]. Because of its “split” quality, the impact of NIMAs on the T cells of the offspring, can play a role in both the prevention of chronic GVHD [42], as well as the graft versus leukemia effect [43] in hematopoietic stem cell transplantation.

The presence of rare maternal cells can be detected directly by PCR, and indirectly by some unusual features of the host myeloid dendritic cells (mDCs). These mDCs are the ones that are known from the work of Herrera et al [9] to retain the allogeneic MHC molecules as intact Ag-presenting molecules after exposure to allogeneic cells. The same study demonstrated that such MHC molecule acquisition occurs, to some extent, independently of cell-cell contact. This phenomenon was first noticed in a murine model of NIMA effect by Zhang and Miller in 1993 [44] although it was then misinterpreted as retention of intact maternal cells rather than “cross-dressing” of host cells. Dutta et al [35], using the same F1 backcross model (B6 ♂ x BDF1 ♀), reported that splenocytes and peripheral blood cells in half of the H2^{bxb} offspring retained a low level of NIMAs of the “d” haplotype on the surface of MHC class-II⁺ cells (IA^{b+}). These “cross-dressed” cells, were sometimes in quite high frequency— as much as 20% of the total CD11c⁺ cell compartment [35].

This observation of acquired Ag in NIMA^d-exposed offspring led us to investigate the phenomenon of semi-direct pathway and its impacts on allograft rejection and tolerance. First, we noticed that there were differences in the level, the organ distribution, and the quality of maternal microchimerism (MMc) amongst the NIMA^d offspring after DBA/2 (H-2^d) heart transplantation [45]. When the backcross breeding was done in such a way that maternal cells could be tagged by GFP, we found that higher levels of MMc were associated with 1) tolerance to a subsequent DBA-2 heart allograft; 2) the presence of immune regulatory cells specific for the NIMA^d Ag in spleen and lymph nodes; and 3) the presence of GFP⁺ maternal cells in CD11b⁺ and CD11c⁺ myeloid cell populations [45]. Since CD11c⁺ DCs are also known to be capable of secreting large numbers of EVs/exosomes, we tested whether the serum of the offspring contained MHC Ags in the form of EVs [46]. We found that we could divide the NIMA^d-exposed offspring into 2 types – one type, with low

levels of MMc, had no membrane-bound alloantigen acquired on the surface of their mDCs (hereafter referred to as “non-mAAQ” mice). ELISA for IE, a MHC class-II molecule not expressed in H2^{bxb} mice, but produced by MMc cells of BDF1 origin, showed that it was present only in the EV-free (100,000 × *g* supernatant) fraction of serum in non-mAAQ mice. In mice with membrane alloantigen acquisition (mAAQ⁺), by contrast, the IE molecule was present both in the EV-free serum fraction and the ultracentrifuge pellet containing EVs. Analyses of EV-enriched fractions showed the presence of exosome-sized vesicles (50–110 nm diameter), enriched for the exosome marker CD9, with low content of Golgi, endoplasmic reticulum, and histone proteins [46]. Furthermore, when EVs from the mAAQ⁺ serum were tested in a short-term culture with B6 (H2^{d-negative}) cells, both plasmacytoid DCs (pDCs) and mDCs were found to acquire surface H2-K^d (MHC class-I) and IA^d (MHC class-II) molecules of maternal origin. EVs from serum of non-mAAQ mice had no such effect. However, this did not mean that non-mAAQ DCs lacked NIMA-presenting capacity. Indeed, the non-mAAQ mice, when analyzed for the expression of the IEα_{52–68} allopeptide / IA^b complex (i.e. the YAE epitope), had even greater expression on the surface of pDCs, as compared with pDCs of mAAQ⁺ offspring. In addition, there was a significantly lower expression of the co-inhibitory molecule, PD-L1 on the surface of non-mAAQ pDCs vs. pDCs from mAAQ⁺ mice. To determine whether the non-mAAQ mice might therefore have the capacity to stimulate indirect pathway T cells recognizing the YAE epitope, TEa TcR transgenic CD4 T cells were introduced into the mice. Remarkably, TEa cells only proliferated in the non-mAAQ mice. In contrast, transfer into the mAAQ⁺ mice caused TEa cells to undergo abortive activation, followed by anergy [46].

We then analyzed the proliferative capacity of semi-direct pathway in the NIMA^d mice. The 4C TcR transgenic CD4 T cell was chosen, as it was known to directly recognize the intact IA^d molecule. As expected, 4C cells were not stimulated to proliferate in the non-mAAQ host. Yet, despite the pattern of abortive activation of TEa CD4⁺ indirect pathway T cells, 4C T cells proliferated well in mAAQ⁺ mice. We then looked at the surface expression of acquired IA^d Ag on DCs within the mAAQ⁺ host. Using imaging flow cytometry with two different fluorophores, it was clear that both the IA^d and the K^d molecules were present in precisely the same sector on the surface of the mDCs that had acquired Ag. However, PD-L1, a major co-inhibitory molecule, was prominent over the rest of the surface of the mDCs, but was not associated with the acquired MHC alloantigens. In contrast, the CD86 co-stimulating molecule was associated with the patches of K^d and IA^d.

There remained the puzzle of how a TEa cell specific for the indirect pathway epitope recognized by the YAE antibody could be so strongly inhibited at the same time that the 4C cell was being activated. Close examination of the YAE epitope using imaging flow cytometry relation to PD-L1 showed that there were zones of overlap between the YAE and the PD-L1 signal. This led us to postulate that the reason for the inability of mAAQ⁺ mDCs to productively activate the TEa indirect pathway T helper cell was the result of a PD-L1-dependent anergy. To test this, mDCs and pDCs were isolated from the spleens of non-mAAQ and mAAQ⁺ mice. As expected, non-mAAQ DCs caused proliferation of the TEa cells, with or without the addition of PD-L1 blocking antibody, whereas no proliferation above background was seen with the 4C direct pathway responders. When mAAQ⁺ mDCs and pDCs were used, we detected strong proliferation of the 4C cells, and no division of the

TEa cells, as predicted by the *in vivo* proliferation results. However, the addition of an antibody against the PD-L1 co-inhibitory ligand restored a strong proliferative response to both pDCs and mDCs of mAAQ⁺ offspring [46].

We propose that the MMc-derived EVs, upon interaction with host DCs, have opposite impacts upon the functionality of the indirect and semi-direct pathways of T cell allorecognition, which become anergized and primed, respectively. Exactly how maternal EVs perform this “trick”, so different from the strong expression of positive co-stimulation, with no PD-L1, on host DCs cross-dressed by passenger leukocyte-derived EVs in acute rejection [3], is still to be determined.

Conclusion

These findings identify cross-dressing via clusters of exosomes as an explanation for the potency of alloimmunity, unveil a new role for EVs in the elicitation of the semi-direct pathway of allorecognition, and open new possibilities for development of therapies to treat transplant rejection or to reinforce tolerance that may be based on microchimerism. The semi-direct pathway was originally proposed by Lechler [47] as a solution to the so-called “4 cell problem” in transplantation, i.e. how indirect pathway CD4 T “help” could be provided to a direct pathway CD8 CTL, something that had been clearly demonstrated 10 years earlier [48]. The phenomenon of alloantigen acquisition by host DCs was able to reduce this to a 3 cell (T helper cell-DC-CTL) interaction. Table 2 summarizes the two types of semi-direct pathway outlined in this manuscript, and how what the EVs do to alter indirect pathway T cell “help” impacts acute rejection and maternally-induced split tolerance. The mechanistic basis of MMc-associated split tolerance, and that of alloactivation leading to acute rejection, appear to both revolve around EVs and the semi-direct pathway, opening venues for new therapeutic and diagnostic approaches in transplantation.

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Key points

- In some transplant models (e.g. fully-mismatch skin or heart allografts in untreated mice), donor passenger leukocytes are absent or detected at very low numbers in the draining lymphoid organs.
- Donor passenger leukocytes and likely non-migratory cells from the graft, transfer donor EVs (i.e. exosomes) carrying donor intact MHC molecules and APC-activating signals to recipient APCs resident in the graft-draining lymphoid organs.
- Recipient DCs cross-dressed with donor-derived exosomes promote proliferation and effector cell differentiation of directly allo-reactive T cells through the semi-direct pathway of allo-recognition.
- Depletion of recipient DCs cross-dressed with donor-derived exosomes decreases substantially the T-cell response against donor intact MHC molecules, and delays significantly cardiac allograft rejection in mice.
- Natural conditions like MMC are associated with the release of allogeneic exosomes with specific properties, capable of generating a “split tolerance” condition implying priming and functionality of the semi-direct recognition but anergization of the indirect pathway.

Figure 1. Biological relevance of extracellular vesicles in transplant biology, and their potential clinical applications

Donor tissues/cells/EVs are illustrated in grey and recipient's in black. Recipient DCs (and other APCs) located in graft-draining lymphoid organs acquired donor intact MHC molecules, for presentation to T cells via the semi-direct pathway, through capture of donor-derived exosomes and likely other types of EVs (e.g. microvesicles), and donor MHC Ag released as free molecules. The donor-derived EVs carrying donor MHC molecules and APC-activating signals are released by cells from the graft or by passenger leukocytes mobilized to secondary lymphoid organs. Transfer of donor MHC molecules via EVs to the recipient APCs amplifies elicitation of the anti-donor response through directly alloreactive T cells, even in the absence or extremely few numbers of passenger leukocytes in the graft-draining lymphoid organs. EVs released into bodily fluids (e.g. plasma, urine) could be used as predictive biomarkers of graft rejection. EVs, genetically-engineered or loaded with drugs or Ags, offer multiple possibilities for therapeutic applications.

Table 1

Comparison of different types of extracellular vesicles (EVs)

	Exosomes	Microvesicles	Apoptotic cell-derived EVs
Size	30–120 nm	0.2–1 μ m	0.2–1 μ m (apoptotic cell blebs). 3–5 μ m (apoptotic bodies).
Biogenesis	As intraluminal vesicles inside multivesicular bodies (endosomes), or within Golgi-derived vesicles.	Shedding of the plasma membrane.	Shedding of the plasma membrane (apoptotic cell blebs). Cell fragmentation (apoptotic cell bodies).
Markers	Tetraspanins (CD9, CD63), tumor susceptibility gene 101 (TSG101, syntenin-1). Externalized phosphatidyl- serine.	Surface markers of the plasma membrane of the parent cells. Externalized phosphatidyl- serine.	Externalized phosphatidyl- serine (apoptotic cell blebs). Presence of nuclear fragments and cell organelles (apoptotic cell bodies).
Content	Nuclei acids (mRNA, miRNA, non-coding RNAs). Transmembrane or membrane-bound proteins (MHC Ag, adhesion and costimulatory molecules, tetraspanins, MFGES /lactadherin, growth factor receptors). Cytoplasmic molecules (TSG101, transcription factors, heat shock proteins).	Nuclei acids (mRNA, miRNA). Different surface and intracellular components depending on the parent cell.	Externalized phosphatidyl- serine, ribonucleoproteins and chromatin complexes (apoptotic cell blebs). Nuclear fragments and cell organelles (apoptotic cell bodies)
Potential clinical use	Biomarkers. Platforms for drug delivery. Positive or negative vaccination.	Biomarkers. Platforms for drug delivery.	Positive or negative vaccination.

Table 2

Proposed impact of EVs / exosomes in allorecognition pathways

Source of EVs	Context	Semi-direct pathway	Indirect pathway	Outcome
Graft, passenger leukocytes	Transplantation	Co-Stimulation (CD86, CD40)	Co-Stimulation (CD86)	Coordinate indirect pathway T helper cell help → CTL
				Efficient allo-sensitization of direct pathway T cells against donor MHC molecules → acute rejection
Maternal cells (microchimerism)	Chronic exposure	Co-Stimulation (CD86)	Co-Inhibition (PD-L1)	“Un-helped” CTL
				Mild acute graft rejection, no chronic rejection