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REVIEW

# T regulatory cells-derived extracellular vesicles and their contribution to the generation of immune tolerance

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## Abstract

T regulatory (Treg) cells have a major role in the maintenance of immune tolerance against self and foreign antigens through the control of harmful inflammation. Treg cells exert immunosuppressive function by several mechanisms, which can be distinguished as contact dependent or independent. Recently, the secretion of extracellular vesicles (EVs) by Treg cells has been reported as a novel suppressive mechanism capable of modulating immunity in a cell-contact independent and targeted manner, which has been identified in different pathologic scenarios. EVs are cell-derived membranous structures involved in physiologic and pathologic processes through protein, lipid, and genetic material exchange, which allow intercellular communication. In this review, we revise and discuss current knowledge on Treg cells-mediated immune tolerance giving special attention to the production and release of EVs. Multiple studies support that Treg cells-derived EVs represent a refined intercellular exchange device with the capacity of modulating immune responses, thus creating a tolerogenic microenvironment in a cell-free manner. The mechanisms proposed encompass miRNAs-induced gene silencing, the action of surface proteins and the transmission of enzymes. These observations gain relevance by the fact that Treg cells are susceptible to converting into effector T cells after exposition to inflammatory environments. Yet, in contrast to their cells of origin, EVs are unlikely to be modified under inflammatory conditions, highlighting the advantage of their use. Moreover, we speculate in the possibility that Treg cells may contribute to infectious tolerance via vesicle secretion, intervening with $CD4^+$ T cells differentiation and/or stability.

KEYWORDS
extracellular vesicles, T regulatory cells, tolerance

## 1 | INTRODUCTION

The immune system is responsible for the remarkable function of maintaining a maximally diverse group of T cells, among other leucocytes, capable of defending against various pathogens and constantly

Abbreviations: APCs, antigen presenting cells; BM-DCs, bone marrow-derived dendritic cells (DCs); CD73, ecto-5-nucleotide enzyme; Cox-2, cyclooxygenase-2; CTLA-4, cytotoxic T lymphocyte antigen-4; CTLs, cytotoxic T lymphocytes; d3Tx, thymectomized on the third day of life; $DC_{OVA}$, OVA-pulsed DCs; ESCRT, endosomal sorting complex required for transport; EVs, extracellular vesicles; FasL, Fas ligand; Foxp3, Forkhead box P3; $Foxp3^+$, Treg cells; H3K27, histone H3 lysine 27; H3K4, Histone H3 lysine K4; iDCs, immature DCs; IKK2, inhibitor kappa B kinase 2; ILVs, intraluminal vesicles; IPEX, immune dysregulation, polyendocrinopathy, enteropathy X-linked; LAG-3, lymphocyte activation gene-3; M2, Type-2 macrophages; mDCs, mature DCs; MDSC, myeloid-derived suppressor cells; MVBs, multivesicular bodies; Nrp-1, neuropilin-1; PD-1, programmed cell death protein 1; PLC$\gamma$, phospholipase C gamma; Rab27, Ras-related protein Rab-27; ROR$\gamma$t, retinoic acid receptor-related-orphan-receptor-gamma t; T-bet, T-box transcription factor; tDCs, tolerogenic dendritic cells; TIEG1, TGF-$\beta$ inducible early gene 1; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIM-3, T cell immunoglobulin and mucin domain 3; Treg, T regulatory cells; TSG101, tumor susceptibility gene 101; Tx, thymectomy.

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changing neoplastic cells, while rigorously preventing immune responses against healthy tissues. The latter process is broadly categorized as "immune tolerance" and when the mechanisms that promote this state fail, autoimmunity, cancer, or infections can take place.[1-4] The immune system maintains self-tolerance through several mechanisms. One of them is the suppression of effector cells mediated by subsets of regulatory cells such as tolerogenic dendritic cells (tDCs), regulatory B cells, myeloid-derived suppressor cells (MDSC), type-2 macrophages (M2), and vastly studied, T regulatory cells (Treg) cells. Several advances have allowed the identification, analysis, and experimental manipulation of Treg cells subpopulations, which have been identified based on their expression of molecular markers, cytokine production, and mechanisms of action.[5-11] The existence of Treg cells was first postulated in the late 1960s by Nishizuka and Sakakura, in a report in which they were studying the pathogenesis of autoimmune oophoritis developed after neonatal thymectomy (Tx). They noted that mice thymectomized on the third day of life (d3Tx) experienced organ-specific autoimmune signs, which was not observed in mice thymectomized on day 1 or 7 of life. Interestingly, disease could be completely prevented if d3Tx animals received a thymus transplant between days 10 and 15 after birth. They concluded that d3Tx prevented suppressor cells from reaching the periphery, resulting in autoimmune disease. This idea led to the hypothesis that auto-reactive T cells were exported from the thymus during the first three days of life and, somewhat later in ontogeny, a population of suppressor T cells may emigrate from the thymus to control auto-reactive T cells.[12] Later, studies of Gershon and Kondo using thymectomized, lethally irradiated, and bone marrow-reconstituted mice described that a subset of T cells exert immune suppression to induce tolerance. Applying this experimental setting, which permits to identify thymus and bone marrow-derived cells, the authors could conclude that bone marrow precursors require the cooperation of thymus-derived cells to induce tolerance.[13] Nowadays there is no doubt of the functional contribution of Treg cells in the process of generating and maintaining tolerance, although the mechanisms used to exert such activities are still a matter of study. A few years ago, a novel mechanism of suppression was attributed to Treg cells: extracellular vesicles (EVs) production. Although this process was identified early on, recent evidence indicates that Treg cells may control the immune response through the export of several factors packaged in EVs.[14-17] This discovery has significant potential as these vesicles could be used, in a targeted manner, to modulate immunity in different pathologic scenarios. In this review, we will revise and discuss current knowledge on Treg cells-mediated immune tolerance giving special attention to the production and release of EVs.

## 1.1 | Immune tolerance

The immune system protects the host from a broad range of pathogenic microorganisms while avoiding excessive immune reactions that would cause critical damage to the host.[2] Thus, "immune tolerance" is considered as the control of harmful inflammation, including processes to enhance wound healing and tissue repair.[18] The mechanisms that control potentially dangerous self-reactive (auto-reactive) lymphocytes have been coined as "central" and "peripheral tolerance."

"Central tolerance" is conceived as the process of removing self-reactive T and B cells during their development along with the generation of thymic-derived Treg cells.[19]

### 1.1.1 | Peripheral tolerance

Central tolerance is an imperfect process, in which some self-reactive cells may escape thymic negative selection.[20] In addition, not all antigens that T cells need to be tolerant to are expressed in the thymus.[21] Therefore, additional tolerance mechanisms exist to restrain the number and/or function of peripheral T cells that may react to self-antigens in addition to food antigens.,[21] a concept known as "peripheral tolerance," which includes the proceeded of "anergy," defined as a state of unresponsiveness, and "cell deletion."

## 1.2 | Treg cells and their products

In mouse, Treg cells are characterized by the constitutive expression of the transcription factor Forkhead box P3 (Foxp3), which gene is coded in the X-chromosome.[22,23]; however, on human Treg cells Foxp3 is not a specific marker due to the transient expression of Foxp3 during early T cell activation.[24] Mutations in Foxp3 gene results in severe autoimmune diseases such as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) in humans and the scurfy phenotype in mice.[25] demonstrating that Treg cells are indispensable in preventing autoimmunity through the lifespan of the organism. In general, there are two pathways of $Foxp3^+$ Treg cells development: natural-occurring, thymus-derived Treg cells (nTreg or tTreg cells) and peripheral-derived or induced (pTreg, iTreg) cells that generate from naïve T cells that encounter its antigen and increase Foxp3 expression in tolerogenic conditions such as commensal microbiota antigens in mucosal surfaces.[26]

Treg cells exert immunosuppressive function by several described mechanisms, which can be distinguished as contact dependent (cell-to-cell interaction) and contact independent (soluble factors).

### 1.2.1 | Contact-dependent mechanisms

Treg cells express a myriad of surface membrane inhibitory receptors that mediate down-regulation of the immune responses. In this context, some of the best characterized inhibitory molecules include immune checkpoints regulators, such as: (i) CTLA-4 (cytotoxic T-lymphocyte antigen-4), a receptor that inhibits the interaction of the T cell expressed co-stimulatory receptor CD28 with its cognate receptors (CD80/CD86, or B7) expressed on antigen presenting cells (APC).[27] CTLA-4 affinity for CD80/CD86 receptors is ~100 times higher than CD28 receptor affinity, and its interaction with these ligands induces their trans-endocytosis and lysosomal degradation, leading to decreasing effector T cells activation and function.[28] (ii) PD-1 (programmed cell death-1), which upon ligation with its ligands PD-L1 and PD-L2 drives the inhibition of TCR signaling-related kinases, leading to attenuation of T cell activation and expansion.[29]

Even more, PD-L1 ligation can induce Foxp3 expression and pTreg cells generation, and loss of PD-1 expression on Treg cells contribute to Treg cells phenotype unstability.[30] (iii) LAG3 (lymphocyte activation gene 3) a homolog of CD4 protein that binds MHC-II with high affinity, preventing the maturation and the ability of APCs to activate effector T cells.[31] (iv) TIGIT (T cell immunoreceptor with Ig and ITIM domains), which is an inhibitory receptor constitutively expressed by Treg cells that regulates effector T cell activation binding to the poliovirus receptor expressed on APCs with around 100 times higher affinity than the costimulatory molecule CD226, competing for receptor binding or preventing the dimerization of CD226.[32]

Other contact-dependent mechanisms include the induction of cell death and modulation of the immune synapse and cell phenotype. Treg cells expressing Fas ligand (FasL) may induce apoptosis of $Fas^+$ cells.[33] Neuropilin-1 (Nrp1) is a membrane protein with affinity for a variety of ligands, and is involved in several physiologic processes such as angiogenesis, neuronal guidance, and immune synapse.[34] It was initially described that the contribution of Nrp1 to Treg cells function was to stabilize the interaction between Treg cells and APCs during antigen presentation, dampening the proper activation of conventional T cells and thus inhibiting the immune response.[35] However, subsequent reports also showed that Nrp1 is necessary to maintain Foxp3 expression and suppressive function, due to phenotypic instability and increased production of proinflammatory cytokines in Nrp1-deficient $Foxp3^+$ Tregs.[36,37]

### 1.2.2 | Contact-independent mechanisms

On the other hand, Treg cells are capable of secreting or inducing the secretion of soluble factors (cytokines, metabolites, vesicles) that contribute to their suppressive activity, and may act together with the aforementioned proteins depending on the context.[38]

Treg cells express molecules that are involved in the metabolic disruption of target cells. For example, Treg cells have a constitutively high expression of CD25, the $\alpha$-chain of the IL-2 receptor because they do not produce but need IL-2 for cell survival, proliferation, and proper suppressive function.[39] This feature promotes IL-2 consumption by Treg cells, which plays an essential role in controlling effector T cell function by causing death of activated $CD4^+$ T cells via IL-2 deprivation.[40,41] Also, Treg cells promote metabolic disruption through the action of the enzymes CD39 and CD73 (ecto-5-nucleotide enzyme), surface ectonucleotidases that mediate the conversion of proinflammatory extracellular ATP into immunosuppressive extracellular adenosine.[42] Adenosine may interact with any of its receptors (A1, A2a, A2b, or A3) expressed on immune and nonimmune cells, mainly promoting immune suppression. Particularly, on effector T cells, the interaction with A2aR inhibits TCR signaling, decreasing their proliferation and proinflammatory cytokine secretion.[43-45]

Finally, the production of anti-inflammatory cytokines, such as IL-10, TGF-$\beta$, and IL-35, has also been strongly evidenced.[46-50] IL-10 is an immunosuppressive cytokine commonly associated to Treg cells able to decrease the production of proinflammatory cytokines from APCs, such as IL-12, IFN-$\gamma$, or TNF-$\alpha$.[51] It has been reported that IL-10 decreases the expression of MHC-II and the expression of B7 family molecules (CD80/CD86) in DCs, endowing DCs with an immature phenotype, and thus, a tolerogenic function.[52] Different immune cell types secrete IL-10; however, the specific deletion of IL-10 gene in $Foxp3^+$ Treg cells is sufficient to generate several auto-immune pathologies in animals, such as spontaneous colitis,[46,47] highlighting the physiologic relevance of $Foxp3^+$ Treg cells-derived IL-10.

TGF-$\beta$ is a pleiotropic cytokine that serves several roles in the immune response and affects all T cell subpopulations. In peripheral tissues, TGF-$\beta$ promotes the conversion of naïve T cells into iTregs, which also occurs during in vitro differentiation.[26,53] In mice models, $Foxp3^+$ Treg cells-derived TGF-$\beta$ have shown to inhibit the activation and proliferation of effector T cells and modulate the immune response during colitis, tumor, type 1 diabetes, allergy, organ transplantation, and experimental autoimmune encephalomyelitis, among others.[48,54-58]

IL-35 is a member of the IL-12 cytokine family, primarily secreted by $Foxp3^+$ Treg cells as a potent immunosuppressive cytokine that is required by Treg cells to fully exert suppressive function both in vitro and in vivo.[49] Treg cells secrete IL-35 to inhibit the activation and differentiation of naïve $CD4^+$ T cells into a proinflammatory phenotype and favors the transformation of these naïve cells into IL-35-secreting $CD4^+Foxp3^-$ regulatory cells with suppressive function.[49] Treg cells-derived IL-35 have also shown to inhibit the production of IL-17 in effector T cells and decrease inflammation symptoms in mice models of arthritis.[50]

Few years ago, the secretion of EVs by Treg cells was first reported,[14] and since then, multiple studies have investigated their role in many experimental settings, which will be described in detail in the next sections. Figure 1 depicts most of the molecules and factors involved in Treg cells-mediated suppression.

## 1.3 | Extracellular vesicles

EVs comprise a heterogeneous group of naturally occurring membranous structures containing cytosol enclosed in a lipid bilayer, released from essentially all cell types.[59] This term includes a wide variety of particles that can be classified according to their size (micro or nanovesicles), their cellular origin, proposed functions, or their formation inside or at the surface of cells (prefix ecto or exo).[60] EVs are considered mediators of intercellular communication, acting as carriers of lipids, proteins, and nucleic acids, such as mRNA, miRNA, noncoding RNA, and even DNA fragments. This feature converts them into potential vectors of genetic information, able to modify gene expression in recipient cells. In this context, EVs participate in different physiologic and pathologic processes such as membrane exchange between cells, immune modulation, angiogenesis, regeneration, and tumor microenvironment sensitization, among others.[61,62]

## 1.4 | EVs biogenesis

Based on the current biogenesis mechanisms, EVs can be broadly differentiated into two main categories: microvesicles and exosomes.[59]

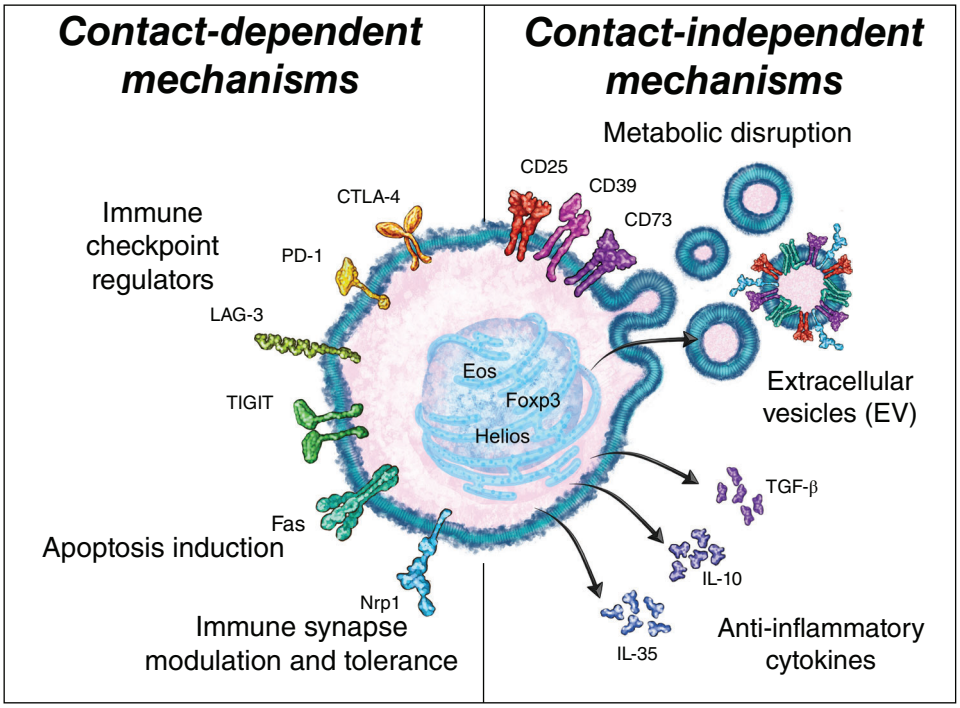


**FIGURE 1** **Treg cells immune suppressive mechanisms.** Among the contact-dependent mechanisms (left), we found the action of immune checkpoint regulators such as CTLA-4, an inhibitor molecule of the interaction between T cell expressed co-stimulatory receptor CD28 with its cognate receptors CD80/86 expressed on APCs; PD-1, a TCR signaling-related kinases inhibitor that leads to the attenuation of T cell activation and expansion after the interaction with its ligands PD-L1 and PD-L2; LAG3, an homolog of the CD4 protein that binds MHC class II with high affinity, preventing the maturation and the ability of APCs to activate effector T cells; TIGIT, a receptor binding competitor that binds to the poliovirus receptor expressed on APCs with around 100 times higher affinity than the co-stimulatory molecule CD226. Moreover, molecules such as Fas promote apoptosis after the interaction with its ligand (FasL). Likewise, Neuropilin-1 (Nrp1) has been identified as an immune synapse modulator that promotes the interaction between Treg cells and APCs during antigen presentation, dampening the proper activation of conventional T cells and thus inhibiting the immune response. On the other hand, contact-independent mechanisms (right) involve molecules such as CD25, the high affinity $\alpha$-chain of the IL-2 receptor, highly expressed on Treg cells. This causes the sequestration of IL-2 from the environment, leading to IL-2 deprivation and therefore, death of $CD4^+$ T cells. CD39 and CD73 are surface ectonucleotidases that mediate the conversion of proinflammatory extracellular ATP into immunosuppressive extracellular adenosine. Furthermore, anti-inflammatory cytokines such as TGF-$\beta$, IL-10, and IL-35 may inhibit the activation and proliferation of effector T cells. Finally, the transmission of extracellular vesicles (EVs) that deliver a wide variety of immune-modulating molecules has been identified as a novel suppressive mechanism. The molecules identified in Treg cells-derived EVs are showed in Figure 2

Both categories involve membrane-trafficking processes, even though they occur at distinct sites and have different biogenesis mechanisms. EVs can be originated by outward budding of the plasma membrane or by an intracellular endocytic trafficking pathway involving the fusion of multivesicular late endocytic compartments. Particularly, EVs called microvesicles, microparticles, or ectosomes, directly shed from the plasma membrane into extracellular space and have a wide range of sizes (up to 1 μm).[63] On the counter side, "exosomes" are small vesicles (50–150 nm) of endosomal origin formed as intraluminal vesicles (ILVs) molded by inward budding and released from multivesicular bodies (MVBs).[64] Exosomes where first described on the early 1980s as small vesicles (~50 nm) associated with the recycling and release of transferrin receptor in reticulocytes.[65] But it was not until 1987 when Rose Johnstone coined the term "exosome" to describe small membrane vesicles formed by vesiculation of intracellular endosomes and released by exocytosis as a consequence of multivesicular endosome fusion with the plasma membrane.[66] Exosomes are generated within the endosomal system as ILVs secreted during the fusion of MVBs with the cell surface. This process requires particular sorting machineries that segregate cargoes into micro-domains with consequent inward budding and fission of small membrane vesicles containing sequestered cytosol. In this context, the endosomal sorting complex required for transport (ESCRT) machinery has been demonstrated to play a fundamental role. The ESCRT is a family of proteins that associate in successive complexes (ESCRT-0-I, II, and III) at the membrane of MVB to regulate cargo targeting into the formation of ILVs.[67]

Exosomes may also be formed in an ESCRT-independent manner through the generation of ceramide by neutral type II sphingomyelinase, which imposes a spontaneous negative curvature on membranes,[68] or by the action of proteins of the tetraspanin family.

In sum, EVs biogenesis is an intricate phenomenon that involves complex machinery and is cell type specific and directly influenced by the physiologic or pathologic state of the donor cell. Moreover, even though microvesicles and exosomes have different origins, the

overlapping range of size, similar morphology, and variable composition challenges the possibility of having a rigid and accurate classification and nomenclature of EVs. Hence, in this review, we will refer indistinctly to exosomes as EVs because technical accuracy and standardization in their isolation in all studies revised is limited.

## 1.5 | Role of EVs in immune tolerance

Immune cell populations, as all cell types, release EVs with specific cargo, which appears to be crucially involved in the regulation of immune responses.[69] From this perspective, EVs may act as mediators of intercellular communication, antigen presentation, opsonization, among others. EVs released by immune cells may have bidirectional functions promoting either the activation or suppression of immune responses.[69,70] In addition, EVs from nonimmune cell origins, such as cells present in the blood, mammary glands, or tumors may also modulate immune responses.[71]

The first studies that described EVs from immune cells showed that APCs-derived EVs, particularly from B cells and DCs, were able of carrying MHC class I, MHC class II, and T cell co-stimulatory molecules.[72,73] In this context, EVs can act as antigen-presenting platforms and participate in T cell priming and activation. APC-derived EVs have shown both in vitro and in vivo to effectively stimulate T cell responses.[74]

As for T cells, it is essential to highlight that these cells have shown to increase their secretory capacity upon TCR activation.[75] Besides EVs-enriched proteins, others related to T cells immune functions, such as HLA-I, $\beta$2-microglobulin, components of the TCR/CD3 complex, among others, have been identified on these vesicles.[75] T cells are able to generate EVs directly from the cell surface, probably by exploiting molecular components and mechanisms at the plasma membrane that are usually associated with the endosomal biogenesis of ILVs.[76]

## 1.6 | Treg cells derived EVs

Among the wide variety of T cells, Treg cells have been shown to actively release immunosuppressive EVs capable of acting in a cell-contact independent manner. Smyth et al. first described that the immune modulation-associated molecules CD25, CD73, and CTLA-4 were present on Foxp3$^+$ Treg cells-derived EVs and among them only CD73 seemed to be essential for Treg cells-mediated suppressive function.[14] The expression of CD73 promotes the conversion of extracellular AMP to adenosine, which, as mentioned before, following interaction with adenosine receptors on target T cells inhibits cytokine release, leading to immune modulation. Thus, in Smyth's study, the incubation of Treg cells-derived EVs with AMP showed adenosine production, proving that this mechanism was viable in a cell-independent way. Most importantly, the authors suggested that CD73 expression on Treg cells-derived EVs was essential for their suppressive function as EVs from CD73KO Treg cells did not display suppressive activity.[14]

The same year, Yu et al. evaluated the effect of Treg cells-derived EVs collected from recipient or donor mice using an in vitro approximation and a kidney transplantation model. In this study Treg cells-derived EVs showed effective suppression of T cell proliferation in a dose-dependent manner. Interestingly, EVs obtained from donor Treg cells result more effective in favoring long-term allograft tolerance than recipient-derived Treg cells.[77] Subsequently, in a very elegant study, Okoye et al. demonstrated that Treg cells were not only able to release EVs upon activation, but they also released substantially more CD63$^+$ EVs per cell than any other lymphocyte analyzed.[78] In addition, they reported that Treg cells-derived EVs are able to suppress T cell-mediated responses through the transference of micro-RNAs. Particularly, they identified that Treg cells-derived EVs contained and transferred miR-155, Let-7b, and Let-7d RNAs into co-cultured T cells, and that this process was Ras-related protein Rab-27 (Rab27) dependent.[78] Interestingly, they observed that Let-7d miRNA was preferentially packaged and transferred to Th1 cells, suppressing their proliferation and IFN-$\gamma$ secretion through cyclooxygenase-2 (Cox-2) inhibition. Moreover, this effect was capable of suppressing Th1 activation and inflammation in a colitis murine model. Taking these results into account, the authors suggest that even though isolated Treg cells-derived EVs were able to suppress conventional T cells, this was not as efficient as Treg cells, indicating that additional mechanisms are indeed required for optimal suppression.[78]

In 2017, Aiello et al. evaluated EVs release and immunoregulatory properties of dnIKK2-Treg cells, a cell line generated after stimulation with allogeneic immature DCs (iDCs) expressing a dominant-negative form of IKK2 (dnIKK2), which previously showed to inhibit T cell response in vitro in a contact-independent manner. It was found that dnIKK2-Treg cells release EVs, which were taken up by target T cells and exerted an anti-proliferative effect on them.[16] Furthermore, these EVs were able to convert T cells into regulatory cells and prolonged kidney allograft survival in vivo. This immune-modulating capacity was attributed to specific miRNAs and iNOS enzyme, which, once delivered into naïve T cells, blocked cell cycle progression and induced apoptosis. The authors identified three miRNAs, miR-503, miR-330, and miR-9, which affect the transcription of crucial genes involved in the regulation of cell cycle, such as cyclin E and cyclin D1. Nevertheless, miRNAs partially participated in the anti-proliferative effect of dnIKK2-Treg cells-derived EVs. The enzyme iNOS was also concentrated in dnIKK2-Treg cells-derived EVs, suggesting that this protein could be delivered into target cells, thus mediating NO-dependent anti-proliferative, cytotoxic, and apoptotic effects.[16] As mentioned earlier, dnIKK2-Treg cells-derived EVs induced regulatory function on target T cells, which is independent on Foxp3. T cells exposed to dnIKK2-Treg cells-derived EVs release high amounts of IL-10 and express Tim3. Furthermore, dnIKK2-Treg cells-derived EVs were also able to reduce the differentiation of IFN-$\gamma^+$ T cells, not exclusively Th1 cells, as in Okoye's report.[16]

The following year, Azimi et. al evaluated whether Treg cells-derived exosomes from patients with relapsing-remitting multiple sclerosis (RRMS), an autoimmune disease characterized by neuroaxonal degeneration in the central nervous system, had impaired suppressive function based on previous reports that detected insufficient control of autoreactive T cells due to defective functioning of CD4$^+$CD25$^{high}$ Treg cells.[79,80] To evaluate the latter, they isolated exosomes obtained from MS patients or control- derived Treg cells supernatant an

compared their effects on the proliferative capacity and survival of conventional $CD4^+$ T cells from patients with RRMS. In this context, they found that RRMS patient's Treg cells-derived exosomes exerted impaired suppressive function and induced less apoptosis on Tconv cells compared to Treg cells-derived exosomes obtained from healthy controls, proposing, for the first time, the dysfunction of MS patient's exosomes.[81]

Finally, Tung et al. described that Treg cells-derived EVs were able to modulate DCs function, inducing tDCs. In this study, a murine Treg cell line (dTreg cells) was generated upon stimulation of C57BL/6 $Foxp3^+$Treg cells with allogeneic BALB/c DCs in vitro, which has a direct allo-specificity for BALB/c MHC class II molecule I-Ad antigens. dTreg cells produced EVs of around 100 nm in size following TCR activation, which were acquired by BM-DCs upon co-culture. In contrast to Treg cells, dTreg cells-derived EVs did not influence the expression of the co-stimulatory molecule CD80 on BM-DCs. However, the authors observed that following LPS activation, dTreg cells-derived EVs-treated DCs significantly reduced IL-6 and increased IL-10 secretion.[17] This effect was associated to the transfer of genetic material from EVs to DCs. miRNA content analysis showed that miR-150-5p and miR-142-3p were differentially detected in dTreg cells-derived EVs compared with control $CD4^+Foxp3^-$ T cells-derived EVs. Moreover, miR-142-3p expression was significantly increased in DCs co-cultured with dTreg cells-derived EVs, suggesting that these EVs may deliver their miRNA contents to DCs. Surprisingly, miR-142-3p expression has been associated with decreased IL-6 production, and miR150-5p has shown to regulate IL-10. Therefore, these data indicate that dTreg cells-derived EVs can certainly modify DCs phenotype and function, leading to the generation of tDCs.[17]

In addition to $CD4^+$ Treg cells, other murine T cells with regulatory capacities have been found to release EVs following activation. Xie et al. observed that $CD8^+$ Treg cells secreted exosomes (named $EXO_{Tr}$) capable of inhibiting DC-induced $CD8^+$ cytotoxic T lymphocyte (CTL) responses.[82] To assess their immune suppressive capacity, the authors immunized C57BL/6 mice with OVA-pulsed DCs ($DC_{OVA}$) plus $EXO_{Tr}$. Then, evaluated OVA-specific CD8 T cells response and anti-tumor immunity in mice THAT were challenged with OVA-expressing BL6-$10_{OVA}$ melanoma cells. The results demonstrated that $DC_{OVA}$-stimulated $CD8^+$ T cell responses and protective antitumor immunity significantly dropped in immunized mice receiving co-injection of $EXO_{Tr,}$ proving that these vesicles were capable of suppressing immune responses.[82]

In conclusion, recent studies suggest that Treg cells-derived EVs could represent a refined intercellular exchange device with the capacity of modulating immune responses, thus creating a tolerogenic microenvironment in a cell-free manner. The proposed mechanisms in which Treg cells-derived EVs could be mediating immune responses encompass miRNAs-induced gene silencing, the action of surface proteins and the transmission of enzymes (Fig. 2). Therefore, these studies open up endless questions and possibilities regarding Treg cells-derived EVs role in multiple immune scenarios. How much of the suppressive activity of Treg cells is due to EVs vs. other mechanisms? Are there any differences in the cargo of EVs coming from natural or peripherally induced Treg cells? Which kind of technologies would help overcome the technical difficulties of isolating both Treg cells and EVs for a wider development of this research field? Could Treg cells-derived EVs be of potential interest in an immunotherapeutic clinical context?

It is important to point out that EVs-mediated suppression does not account for the whole modulatory capacity of Treg cells. Thus, considering the growing repertoire of molecules that have shown to prevent immune activation,[83] it is more likely that the combination of cell contact and secreted factors would account for the optimal Treg cell-mediated suppression. However, the blockade of the ability of Treg cells to release EVs seen in Rab27-DKO mice,[15] diminished the capacity of Treg cells to suppress $CD4^+CD25^-$ conventional T cells proliferation to approximatively half of what is observed in wild-type controls, allowing us to speculate the relevance of exosome-mediated suppression.

In this context, it would not be farfetched to wonder about their immunotherapeutic potential as an alternative or complementary therapy in conditions where restoration of immune tolerance is required. Currently, clinical trials that evaluate the use of $Foxp3^+$ Treg cells as tolerance promoters are being tested in safety and efficacy.[84] Nevertheless, it has been extensively recognized that inflammatory environments may promote the conversion of human $Foxp3^+$ Treg cells into effector T cells in vivo. If we consider this scenario, in which cells are being administered into patients suffering inflammatory pathologies, one could expect that the phenotype of the cells may vary when facing this new environment. In this regard, EVs could serve as a suitable cell product to be administered into patients because they are unlikely to be modified (or change their composition) under inflammatory conditions, in contrast to their cells of origin. Nevertheless, it is important to remark that cell type, environmental context, and physiologic state are decisive in the cargo and function of these vesicles; thus, they should be taken into account in all experimental scenarios.

The studies reviewed in this report show heterogeneity in the type of Treg cells investigated, method of EVs isolation, criteria used for their characterization, and concentrations administered in vivo (Table 1). To this, we could add the fact that Treg cells have shown different phenotypic and functional characteristics according to their origin (thymic or peripheral) and location (lymphoid or tissue resident).[85] Hence, additional studies considering the aforementioned may be very advantageous, in order to advance toward a safe, standardized, and plausible clinical application of these vesicles.

## 1.7 | Treg cells plasticity, stability, and infectious tolerance: could EVs be playing a role?

As we know, Treg cells carry out their regulatory functions using diverse strategies, which largely depend on the transcription factor FoxP3. This transcription factor, in turn, is regulated by different mechanisms. Due to the Treg cells constitutive expression of CD25, these cells have a high affinity for IL-2, which can activate several pathways. Among them the activation of JAK3 stands out because it phosphorylates STAT proteins, particularly STAT3 and STAT5, favoring

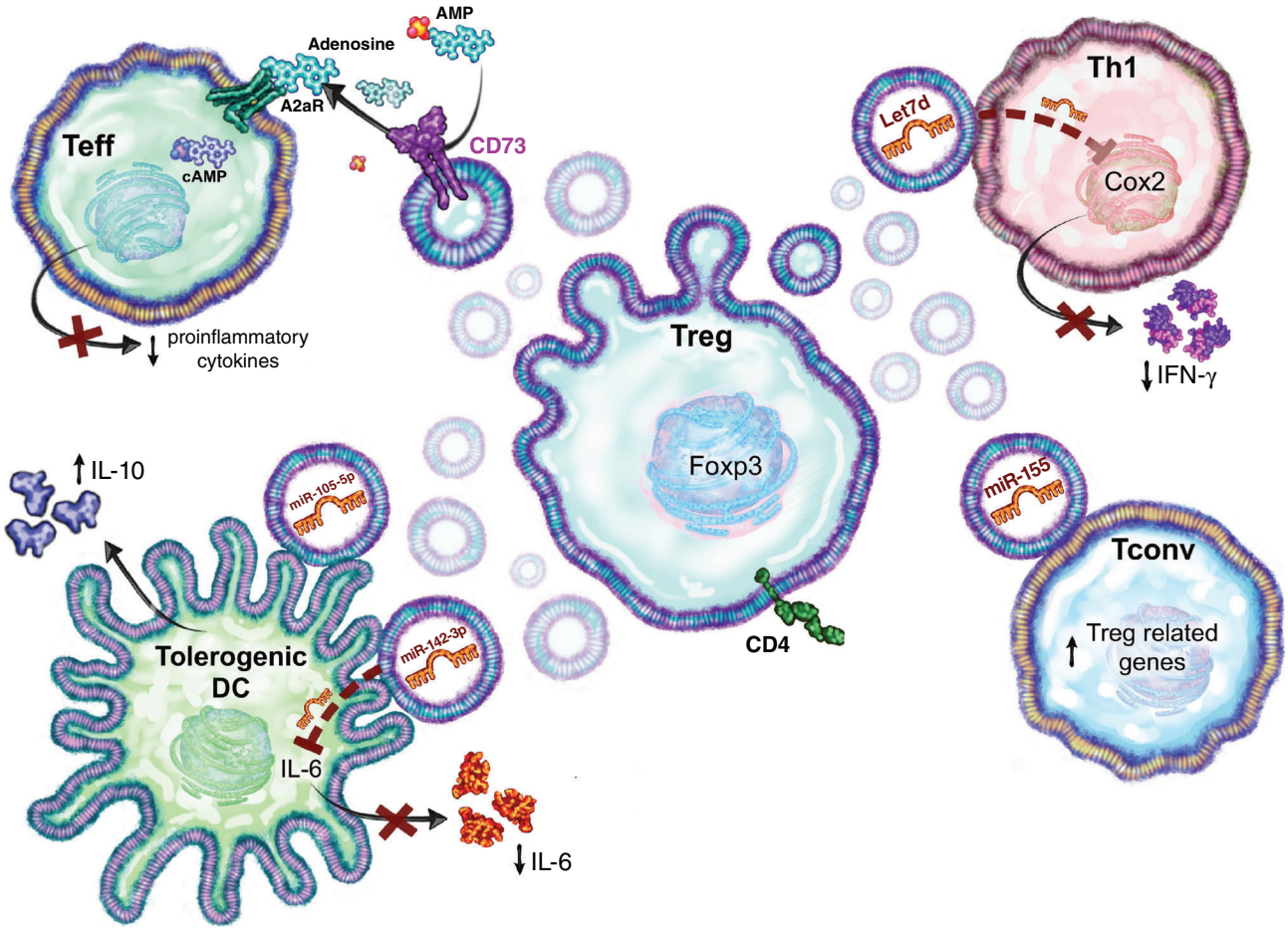


**FIGURE 2** **Treg cells-derived EVs suppressive mechanisms.** CD73-expressing Treg cells-derived EVs contribute to Treg cells suppressive activity through the production of adenosine, which interacts with its receptors on target cells. On effector T cells, the interaction between adenosine and A2aR increases intracellular levels of cAMP leading to inhibition of cytokine production, including IL-2 and IFN-$\gamma$. In contrast, nonautonomous gene silencing mediated by miRNA-containing Treg cells-derived EVs is described as another suppressive mechanism. Delivery of EVs containing Let-7d to Th1 effector cells results in suppression of proliferation and cytokine secretion through the targeting of Cox-2. Furthermore, delivery of miR-155 into conventional T cells promotes the up-regulation of several Treg cells-associated genes in recipient cells. Likewise, Treg cells-derived EVs can modify dendritic cells (DCs) phenotype and function inducing a tolerogenic phenotype through miRNA transference. Particularly, the amount of miR150-5p and miR-142-3p increases on DCs co-cultured with Treg cells derived EVs, which promoted the up-regulation of IL-10 and down-regulation of IL-6

the expression of FoxP3 by binding at a STAT-binding site in the FoxP3 gene.[86] Other molecules have also a collateral role in the development of Treg cells, such as CD28, which, not only enhance the signaling of TCR and NF-AT pathway in Treg cells through the activation of phospholipase C gamma (PLC$\gamma$)[87] but also allows the survival of these cells indirectly by stimulating the release of IL-2 by other T cells.[88] On the other hand, the activation of TCR in human Treg cells leads to the activation of the NF-AT pathway allowing the formation of an NF-AT/AP-1 complex that binds in specific places to the FoxP3 promoter, stimulating its expression.[89] TGF-$\beta$ would also play a role in the activation of the FoxP3 gene in naïve Treg cells through the activation of the transcription factor TIEG1, which binds to the promoter of the FoxP3 gene boosted by the E3 ubiquitin ligase ITCH.[90] Furthermore, TGF-$\beta$ activates the fusion of Caenorhabditis elegans Sma genes and the Drosophila Mad, Mothers against decapentaplegic pathway, activating SMAD3, a protein that in conjunction with NF-AT, acts on an enhancer of the *foxp3* gene.[87] If we consider that EVs contain distinct molecules, of different cellular origin and function, it is acceptable to presume that Treg cells could release EVs harboring cytokines, their receptors, among other proteins, that could intervene with signaling pathways involved in Treg cells differentiation and/or stability. In other words, Treg cells may secrete EVs to self-maintain and not only to act or modulate on other target cells. In this regard, we have observed in our laboratory that Treg cells-derived EVs contain molecules such as CD25, neuropilin-1 (Nrp-1), granzyme B and CD73, among multiple proteins that are components of the TCR complex and of key signaling pathways (JAK/STAT, TGF-$\beta$) (unpublished results). All these molecules are linked to Treg cells biology and their EVs could behave as communicators between Treg cells to assure their permanence during an ongoing immune response.

As mentioned earlier, FoxP3 expression can be regulated through "epigenetic modifications" as well. In this case, it has been postulated

**TABLE 1** Heterogeneity in Treg cells-derived EVs studies

| Author | Year | Treg cellsorigin | Purification of Tregs | Purification of EVs | Markers, visualization | Concluding remarks |
|---|---|---|---|---|---|---|
| Lesley Ann Smyth | 2013 | (i) Natural Treg cells; (ii) Auto-Treg cell line with self-specificity | CD4+CD25+ isolation kit (natural Treg cells) | Serial centrifugation and ultracentrifugation (100,000× *g*) | Tetraspanins LAMP-1/CD63 and CD81. Electron microcopy | Treg cells secrete exosomes following TCR activation. Treg cells-derived exosomes suppress CD4+ T cell proliferation and IFN-$\gamma$ secretion. CD73 expression is necessary for their suppressive function through adenosine generation. |
| Xuesong Yu | 2013 | Natural Treg cells | Flow cytometric cell sorting (CD4+CD25+) | Ultracentrifugation (110,000× *g*) and 30% sucrose/D2O density cushion + ultracentrifugation. | Transmission electron microscopy (TEM) | Treg cells-derived exosomes can suppress T cell proliferation (in vitro) and prolonged graft survival in a kidney transplantation rat model. |
| Isobel S. Okoye | 2014 | Natural Treg cells | Flow cytometric cell sorting (CD4+CD25+Foxp3rfp+) | Combination of serial centrifugation, ultracentrifugation and ExoQuick solution | Dynamic light scatter (size), CD9, CD81 or CD63 ELISA (quantification) | Treg cells release substantially more exosomes than other lymphocytes. Treg cells-derived exosomes transfer microRNAs to suppress certain genes. Particularly, Th1 proliferation, IFN-$\gamma$ secretion and inflammation in a colitis murine model were observed through transfer of Let-7d. |
| Sistiana Aiello | 2017 | DnIKK2-Treg (CD4+CD25-) cells | Sorted CD4+ T cells after stimulation with immature dendritic cells (DCs) (transfected with adenovirus-encoding dnIKK2) | Serial centrifugation and Ultracentrifugation (100,000× *g*) | Electron microscopy, FACS and Western blot (CD63, TSG101 and Calnexin) | DnIKK2-Treg cells -EVs contain a unique cargo of miRNAs and iNOS. Once delivered, blocked cell cycle progression and induced apoptosis. DnIKK2-EVsexposed T cells converted into Treg cells and prolonged kidney allograft survival. |
| Sim L. Tung | 2018 | BALB/c specific Treg cell line (dTregs). | Cell Line | Serial centrifugation and ultracentrifugation and ExoQuick-TC | Electron microscopy and NanoSight | dTreg-derived EVs induce tolerogenic DCs with reduced IL-6 and increased IL-10 secretion. Effect associated to the transmission of miR-142-3p and miR-150-5p, respectively. |
| Maryam Azimi | 2018 | Treg cells derived from human PBMC. | Dyna beads Regulatory CD4+CD25+ T cell kit | Total exosome isolation Kit | CD63 ELISA kit (quantification) | Treg-derived exosomes from multiple sclerosis (MS) patients and healthy controls suppressed proliferation and induced apoptosis of Tconv. The effect of MS-derived exosomes was significantly less potent than healthy controls. |

the great importance of DNA hypomethylation for the transcription of FoxP3 as for other molecules characteristic of Treg cells, such as CTLA-4.[91] For instance, it has been shown that there are differences in the degree of DNA methylation in the CpG islands of the FoxP3 proximal promoter when comparing Treg cells with naïve T cells. Specifically, greater methylation has been found in naïve T cells, whereas Treg cells are characterized by hypomethylation of the CpG islands.[92] Other important modifications are at histone level through acetylations and methylations. Among the most important are the modifications on the histone 3 or H3, such as the trimethylation of lysine 4 (H3K4), which generates a permissive state in the FoxP3 promoter for transcription, as found in Tregs, whereas in conventional T cells other configurations predominate, such as histone H3 lysine 27 (H3K27).[93] All these epigenetic modifications have high relevance particularly in the differentiation of iTreg cells.

For a long time, it was thought that Treg cells maintain their suppressive function regardless of the context. However, robust investigation has identified the transformation of these cells toward a Th17-like effector phenotype under inflammatory conditions. In detail, Xu et al. were able to produce the differentiation of Foxp3$^+$ Treg cells in IL-17 producing cells in the presence of IL-6, inferring that mouse Treg cells can be induced toward a Th17-like phenotype.[94] Having this as a base, Koenen et al. could induce IL-17 producing cells from FoxP3$^+$ Treg cells in the presence of APCs and the cytokines IL-2 or IL-15.[95] Similarly, many other studies have studied the effect of (de)-differentiation of Treg cells toward a proinflammatory phenotype in specific contexts, which is known as "Treg cells plasticity." This concept has become very important due to its involvement in various pathologies, such as allergies, chronic and autoimmune diseases, cancer, transplant rejection, and others.[96] For instance, Chen et al. showed that overexpression of the E3 ubiquitin ligase STUB1, highly present in inflammation or infection, may result in degradation of Foxp3, with the subsequent loss of Treg cells suppressive function, and the appearance of Treg cells with Th1-like phenotype.[97] In another model, Dominguez-Villar et al. evaluated the influence of IL-12 on the Treg cells, culturing the cells with and without IL-12, and showing that a percentage of the group exposed to IL-12 expressed IFN-$\gamma$, getting a Th1-like phenotype.[98] Thus, in response to certain scenarios, Treg cells may acquire effector phenotypes recognizable by the expression of canonical markers, such as T-box transcription factor (T-bet; Th1-like Treg cells), GATA3 (Th2-like), or retinoic acid receptor-related-orphan-receptor-gamma t (ROR$\gamma$t; Th17-like).[99] The report by Okoye et al. supports the conception that Treg cells-derived EVs could positively impact on immune tolerance by specifically affecting T cell differentiation. It is widely evidenced that miRNAs can shape the phenotype of immune cells for favoring immune tolerance; thus, the transfer of miRNA from Treg cells-derived EVs is an additional strategy of these cells to control overt inflammation.

"Infectious tolerance" is a form of peripheral immune regulation, dependent on CD4$^+$ T cells that can suppress the generation of any effector CD4$^+$ T cells, resulting in that these CD4$^+$ T cells also become tolerant and gain the ability to suppress through further generations of cells.[100] One of the first studies in this field was done by Qin et al. in 1993, who performing transplant experiments in mice, found that CD4$^+$ T cells were necessary to induce and maintain long-term transplantation tolerance.[101] This phenomenon was then explained by the infectious tolerance that suppressive CD4$^+$ T cells exerted on other naïve T cells, guiding them to a similar state of tolerance, even in the absence of the original tolerogenic stimulus (which was, in this case, anti-CD4 and -CD8 antibodies).[101] After that, various experiments where long-term suppression was observed demonstrated that tolerance could be transferred through many generations of naïve secondary recipients.[101-106] The latter findings can now be supported, at least partly, by the fact that Treg cells release EVs (loaded with immune regulatory factors) upon TCR engagement; therefore, Treg cells may contribute to infectious tolerance via vesicle secretion. These observations supported the theory that infectious tolerance must be a normal self-tolerance process. Then, the question that then arose was how, because most of these studies were conducted using artificial monoclonal antibodies, tolerance was induced. Later on, in 2002, Jonuleit et al. demonstrated that the process of infectious tolerance could occur naturally. Whereas, co-culture of human CD25$^+$ Treg cells with CD25$^-$CD4$^+$ T cells resulted in the conversion of CD25$^-$CD4$^+$ T cells into cells with suppressive activity (Thsup) with suppressive activity, which have emerged from the initial CD25$^-$CD4$^+$ T cell population. The mechanism by which this happens was found to be contact dependent and partially mediated by membrane-bound TGF-$\beta$. However, this new generation of Thsup cells exerts their suppressive function over a new generation of naïve CD4$^+$ T cells in a cell contact independent fashion, partly through the secretion of TGF-$\beta$, but not of IL-10.[107] In 2003, similar results were found by Walker et al. who found that the expression of Foxp3 could be induced in CD4$^+$CD25$^-$ T cells upon TCR stimulation. However, they also found that this new subset of Treg cells exerted their immune suppressive function in a contact-dependent and cytokine-independent manner.[108] Furthermore, murine studies have demonstrated that the process of tolerance induction requires TCR engagement in the presence of soluble TGF-$\beta$, which favors the appearance of a Thsup population displaying de novo expression of Foxp3.[26] Contrary to what was seen in humans, this new Thsup population exerted its suppressive function in a cell contact dependent fashion, suppressed T cell proliferation, and inhibited the production of Th1- and Th2-like cytokines in vitro, and displayed suppressive function in vivo.[26] It is important to note that most of the studies analyzing whether suppression takes place in a cell-contact dependent or independent manner have been carried out using transwell settings, in which the size of the membrane pore used could (or could not) permit the transport of EVs between wells. Therefore, the possibility that Treg cells-derived EVs play a role in "infecting" T cells to become Treg cells is still open. In this regard, we have observed that Foxp3$^-$ T cells incubated with Treg cells-derived EVs up-regulate Foxp3 mRNA expression and decrease those of IFN-$\gamma$ and IL-17 (unpublished results), suggesting that the secretion of EVs could be a factor involved in the process of infectious tolerance. Even more, a very recent paper by Sullivan et al. suggests that EVs obtained from spleens of tolerized animals contain IL-35 in their membrane, which could mediate infectious tolerance by targeting Tconv cells and inducing the expression of the immune regulators PD-1, Tim3, and LAG3.[109] Thus, the mechanism by which

infectious tolerance can occur naturally can vary depending on the suppressive cell and the technique used to evaluate the role of "soluble factors."

## 2 | CONCLUDING REMARKS

Altogether, we believe that all the evidence obtained up to date, including in vitro and in vivo studies, supports the production of EVs by Treg cells as an additional mechanism to induce tolerance. The development of new techniques will allow us in the near future to fully characterize these vesicles and identify their cargoes to further dissect how Treg cells-derived EVs exert immune suppression. Additionally, by discovering key elements present in these EVs, one could either implement the tracking of them from individual's samples (as new biomarkers) and/or design "hand-made" vesicles for therapeutic applications.

### AUTHORSHIP

C.R., M.C-M., I.C., N.V., A.E., P.C-K., A.R., F.G-J., I.E., R.V., and K.P-L. conceived and wrote the manuscript. A.M-R. created the figures.

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### DISCLOSURES

The authors declare no conflicts of interest.

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