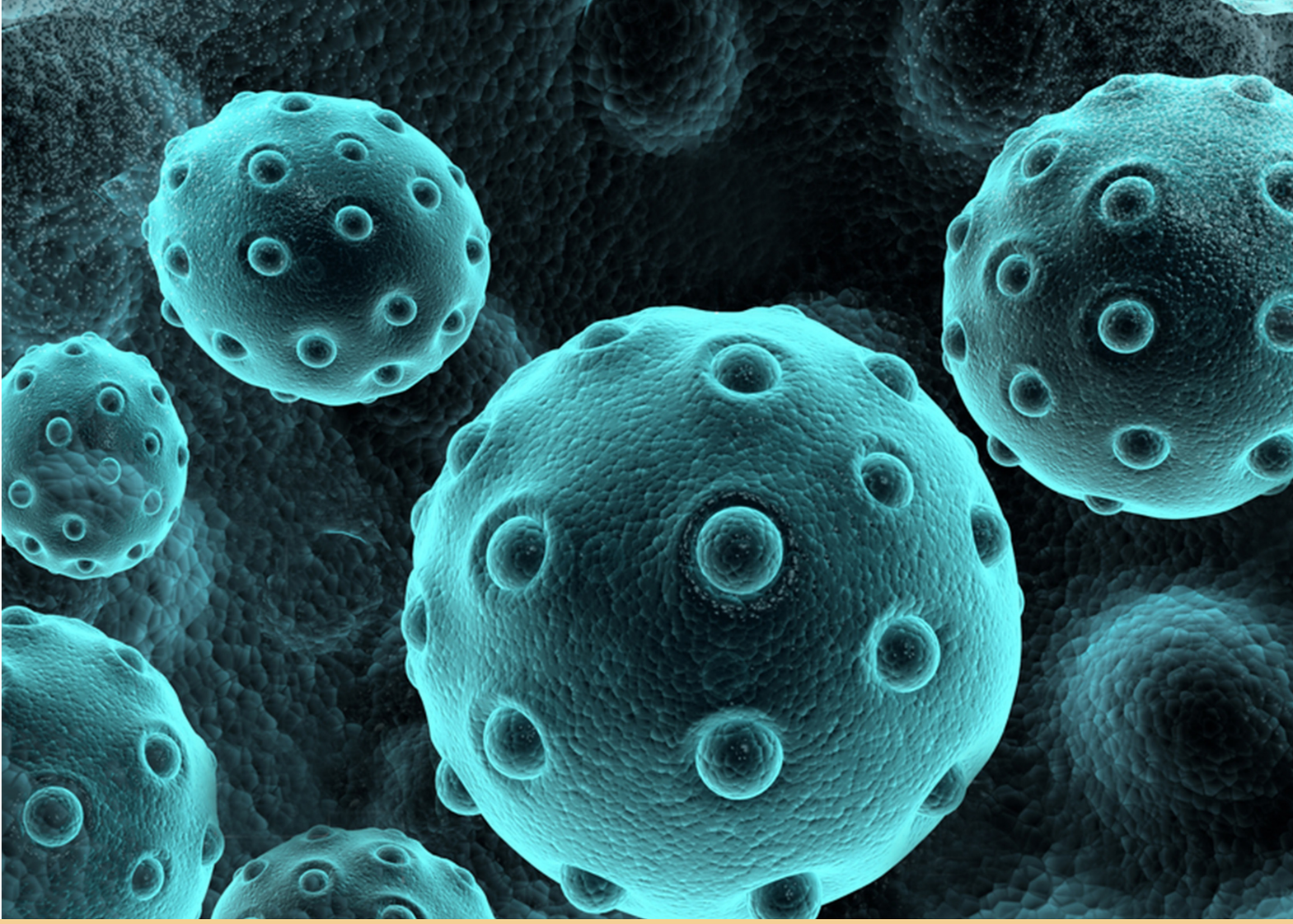

Carmelita Pestana Sousa

Exosomes: an emerging strategy to treat Autoimmune and Inflammatory diseases

Monografia realizada no âmbito da unidade de Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientada pela Professora Doutora Eliana Maria Barbosa Souto e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2016

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Coimbra, 15 de Setembro de 2016.

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Carmelita Pestana Sousa

Monografia realizada no âmbito do Mestrado Integrado em Ciências Farmacêuticas da Faculdade de Farmácia da Universidade de Coimbra

15 de Setembro de 2016

O Tutor da Monografia:

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“To be great, be whole;
Exclude nothing, exaggerate nothing that is not you.
Be whole in everything. Put all you are
Into the smallest thing you do.

So, in each lake, the moon shines with splendor
Because it blooms up above.”

Fernando Pessoa

Content

Abstract

Current treatment options for autoimmune and inflammatory diseases are scarce and consist of broad immunosuppressive agents that have no impact on reverting the already existing damage, and might even ultimately lead to severe side effects. To avert these drawbacks, some progress has been made on developing new treatment strategies by exploiting host cells, nanoparticles, genes. In this context, exosomes have recently emerged as potential new treatment modality in the management of autoimmune and inflammatory diseases. They are nano-sized vesicles that are secreted by most of cell types. It has been shown that these vesicles are keyplayers in cell communication and preserve the cell phenotype from which they derived. Indeed, increasing evidence supports that exosomes can convey important molecules to recipient cells and modulate their function. Additionally, because they can easily overcome biological barriers, have natural homing specificities, are better tolerated, and easily engineered to comprise other molecules such as drugs, besides being considered as cellular surrogates they can be exploited as drug delivery carriers. Although their role is better understood in cancer setting, only modest progress has been made in gaining better knowledge of exosomes in autoimmune diseases. The present review is an attempt to summarize the current status of the application of exosome-based drug delivery systems as well as cell-derived exosomes to treat autoimmune diseases, what are their advantages and main challenges that this approach might pose during its clinical translation. Our aim is that, with this review, researchers can quickly be brought up to speed with, and improve, the state of the art.

Keywords: autoimmune diseases, exosomes, inflammatory diseases

Resumo

Os atuais tratamentos para doenças autoimunes e inflamatórias são escassos e consistem em agentes imunosupressores gerais, que não têm qualquer impacto em reverter danos, e até podem originar graves efeitos adversos. Para culmatar estas desvantagens, algum progresso tem sido feito para desenvolver novas estratégias terapêuticas recorrendo para tal a células, nanopartículas, genes. Neste contexto, os exossomas surgiram recentemente como potencial nova modalidade terapêutica para a gestão de doenças autoimunes e inflamatórias. Exossomas são nano-vesículas que transportam moleculas importantes para as células alvo e modulam a sua função. Adicionalmente, uma vez que conseguem facilmente atravessar barreiras biológicas, têm preferências intrínsicas de transporte, são melhor tolerados, e facilmente alterados para transportar outras moleculas como fármacos, para além de serem considerados como substitutos celulares, estes podem ser explorados como sistemas de transporte para fármacos. Apesar do seu papel ser melhor conhecido no cancro, apenas um modesto progresso foi feito para conhecer melhor os exosomes nas doenças autoimunes. Esta *review* é uma tentativa de sumarizar o conhecimento atual sobre a aplicação dos exosomas como sistemas de transporte de fármacos, bem como dos exosomas derivados de células para tratar as doenças autoimunes, quais são as suas vantagens e desafios que a implementação desta abordagem na clínica pode trazer. O nosso objetivo, com esta review, é que outros cientistas fiquem a conhecer melhor esta abordagem, de modo a que possam também eles contribuir futuramente para o estado da arte.

Palavras-chave: Doenças autoimunes, exossomas, doenças inflamatórias

Abbreviations

Acetycholine receptor AchR

APCs Antigen presenting cells

Arg Arginase

B-LCL B lymphoblastoid cell lines

CIA Collagen-induced arthritis

CTL Cytotoxic T lymphocytes

DCs Dendritic cells

Dex Dendritic cells-derived exosomes

DiR Dialkylcarbocyanine membrane dye

DSS Dextran sulfate sodium

DTH Murine delayed-type hypersensitivity

EAE Experimental autoimmune encephalomyelitis

EAMG Experimental autoimmune myasthenia gravis

EBV Epstein-Barr virus

ER Endoplasmic reticulum

ESC Embryonic stem cells

EVs Extracellular vesicles

GATA Gene transcription of trans-acting T-cell specific transcription factor

GI Gastrointestinal

GVHD Graft-versus-host disease

HEK Human embryonic kidney cells

IBD Inflammatory bowel disease

iDCs immature DCs

iDex Immature DC-derived exosomes

IDO Indoleamin-2,3-dioxygenase

IFN Interferon

iNOS inducible nitric oxide synthase

LAMP Lysosome-associated membrane protein

Let Lethal

LPS Lipopolysaccaride

MDSCs Myeloid-derived suppressor cells

MHC Major histocompatibility complex

MIIC MHC class II containing compartment

MOG Myelin oligodendrocyte glycoprotein

mOVA Membrana-bound OVA strain

MPS Mononuclear phagocytic system

MS Multiple sclerosis

MSC Mesenchymal stromal cells

MVB multi vesicular bodies

NO Nitric oxide

OTI ovalbumin-specific $CD8^{+}$T cell

OVA ovalbumin

PC Pancreatic cancer

PEG Polyethylene glycol

PGE Prostaglandin E

PI3K Phosphatidyl-inositol 3-kinase

PS Phosphatidylserine

rmIL-10 recombinant murine IL-10

RVG Rabies viral glycoprotein

SLE Systemic lupus erythematosus

sMVs Microvesicles

STAT Signal transducer and activation of transcription

TCR T cell receptor

TLR Toll-like receptor

TNBS Trinitrobenzene sulfonic acid

TNF Tumor necrosis factor

Treg Regulatory T cells

vIL-10 viral IL-10

1 Introduction

Epidemiological studies have provided evidence of a constant rise in incidence and prevalence of autoimmune diseases over the last 30 years (1). However, the number of approved new therapies for this disease has not followed this tendency. Therapy with nonsteroidal anti-inflammatory, steroids, and immunosuppressive drugs were common in order to curtail the relapses and prevent organ damage by suppressing chronic inflammation. Nonetheless, serious side-effects had undermined the long-term use of these agents (2). Monoclonal antibodies, receptor-immunoglobulin fusion proteins, interferons despite their efficacy and better safety profile, they are not able to revert the already existing damage and frequently loose their efficacy due to the development of neutralizing antibodies. Therefore, curative therapy is still a major unfulfilled medical need and it is mandatory to develop new target-specific therapeutic strategies in order to reduce overall immunosuppressive and off-target effects, while reverting the already existing damage and dampen the progression of the disease. To avert the previous reported drawbacks of current therapies, some progress has been made on developing new treatment strategies by exploiting host cells, nanoparticles, genes. In this context, exosomes have recently emerged as a potential new treatment modality in the management of autoimmune and inflammatory diseases.

Extracellular vesicles (EVs) is the collective term for cell-secreted phospholipid bilayer-bound structures which are not homogeneous and can be classified according to their morphologic, biochemical and biogenic parameters (3). Within this heterogeneous group apoptotic vesicles, microparticles and exosomes have been described. Apoptotic vesicles have an approximate size of 1-2 μm but are rapidly engulfed by phagocytic cells and readily eliminated and therefore are less well characterized. Microvesicles (sMVs) (100-1000 nm) and exosomes (30-150 nm) are more commonly referred. It has been proposed that sMVs and exosomes arise from different biogenesis mechanisms, with sMVs coming through direct budding from plasma membranes, while exosomes have endocytic origins (4).

The process of exosomes formation starts with the inverse membrane budding into the lumen of an endocytic compartment, leading to the formation of multi vesicular intracellular structures or multi vesicular bodies (MVB). MVB undergo maturation that results in gradual changes in content and composition of the membrane. There are two possible fates for the MVB, either they are targeted to lysosomal degradation or fused with the plasma membrane. Fusion of the MVB with the plasma membrane drive the release of internal vesicles into the extracellular milieu. Alternatively, it might exist a direct formation and release from the plasma

membrane which did not affect the exosome composition since these exosomes continued to express molecules assigned to the traditional exosomes derived from the fusion of the MVB to the plasma membrane.

Contrary of what was first assumed, the content of exosomes is not random but instead the result of a highly selective packaging process. Although the sorting mechanisms are mostly not well understood, some conditions are known to influence this process namely the parental cell status/type and/or the microenvironmental conditions yielding exosomes with a different expression profile. The content of exosomes comprises both membrane-associated oriented in a similar manner as the parental cell as well as cytosolic molecules (5). Their structure is similar to nanospheres, containing a lipid bilayer membrane enriched in cholesterol, phosphatidylcholins, and diglycerides. In addition, they express proteins involved in numerous cell biology functions such as proteins from the tetraspanin family (CD9, CD63, CD81 and CD82), integrins, cell adhesion molecules, growth factor receptors, heterotrimeric G proteins, and phosphatidilserine-biding MFG-E8/lactadherin, endosome or membrane-biding proteins, annexins, Rabs, MHC class I and II. Although abundantly present in exosomes, making some of these proteins sometimes useful as exosome markers, they are not exosome specific since other extracellular vesicles, with endosomal origin, also contain these proteins (5, 6). The parental cell type, the content of exosomes, the surface molecules are some elements that can influence the fate of exosomes.

Exosomes are present in cell culture supernatants and in different biological fluids such as plasma, lymph liquid, malignant pleural effusion, amniotic liquid, breast milk, semen, saliva and urine (7). These nano-sized membrane vesicles can be secreted by most of the cells like for example reticulocytes, dendritic cells (DCs), B cells, T cells, mast cells, platelets, epithelial cells, neurons, oligodendrocytes, Schwann cells and tumour cells. The most commonly detected exosomes are the platelet derived. The exosomes secretion can be a constitutive process, such as in DCs and epithelial cells, or an inducible process depending on the cell type and activation state (7). In antigen presenting cells (APCs), exosomes are thought to arise from the inward invagination and budding off of the endosomal limiting membrane of the major histocompatibility complex (MHC) class II containing compartment (MIIC), in a process that has been shown to be dependent on phosphatidyl-inositol 3-kinase (PI3K). The internal vesicles within the multivesicular endosomes express MIIC specific markers but did not express calnexin, present in endoplasmic reticulum (ER), nor the transferrin receptor characteristic of the plasma membrane and early endosomes. The expression of MIIC markers, especially the MHC II in a peptide-binding conformation, suggested that exosomes could interact with T cells

(8). Indeed, EVs isolated from human blood were shown to induce $CD4^+$ T cell apoptosis in vitro (9).

In the last decades, new evidence suggests that these vesicles are not only cell debris, with no biological role, but instead a key component for information exchange between cells either in health and disease conditions (10, 11). They exert their biological role in a pleiotropic manner. They can modulate immune responses through the activation, transfer or removal of surface receptors on target cells, the removal of cytolytic components such as membrane attack complexes, and the transfer of signaling molecules/effectors such as nucleic acid species, infectious particles, oncogenes. The efficiency of the fusion of the exosome with the recipient cell was shown to depend on the environment conditions as well as the state of the recipient cell. Acidic conditions, as it is seen within tumor mass, or immature DCs (iDCs), that have a higher capture capacity, are factors that favor this process (7).

Among the nature derived nanoparticles that have been developed, exosomes drew special attention since they intrinsically possess many attributes of a desirable drug delivery vehicle. Their small size allows them to bypass the mononuclear phagocytic system (MPS) clearance, thereby prolonging their circulation time for passive targeting to inflammatory tissues. Moreover, they can deliver their cargoes directly into the cytosol, avoiding the lysosomal/endosomal pathway and thus, increasing the transfection efficiency when they are used as gene delivery systems (12). Importantly, after transfer of mouse exosomal RNA to human mast cells, new mouse proteins were found in the recipient cells, proving that some of these carried molecules might be functional and that exosomes can be used to carry and deliver these same molecules (13). Indeed, it was recently reported that endogenous miR-155 and miR-146a, are released from DCs within exosomes and are subsequently taken up by recipient DCs. Delivery of miR-155 enhances while miR-146a reduces inflammatory gene expression consequently reprogramming the cellular response to endotoxin (14).

Despite the potential of exosomes to be used to treat autoimmune and inflammatory diseases, only modest progress has been made in this context. Therefore, our goal is to summarize the current status of the application of exosomes in these diseases. We will begin by showing that despite the potential of exosomes to treat, exosomes can also play an important role in disease. Therefore, a better knowledge of exosomes biology is also highly needed to unveil the dichotomy. In the next sections, we will show evidence of the therapeutic action of exosomes either as cell-derived exosomes or as exosomes-based drug delivery system. Finally, we will address the main challenges that this strategy might pose during its clinical translation.

2 Role of Exosomes in disease

Cancer derived exosomes were shown to promote Treg expansion and maintain the immunosuppressive effect by expressing high quantities of TGF-βon their surfaces. Stimulation of effector T cells were also decreased due to inhibition of DC maturation by upregulating IL-6 and phosphorylating signal transducer and activation of transcription(STAT)-3. The natural killer cells cytotoxicity is also decreased through janus kinase-3 downregulation (15). Accordingly, pancreatic cancer (PC) DCs-derived exosomes, exhibited a down regulation of Toll-like receptor(TLR)-4 and downstream cytokines via miR-203 transported by exosomes (16). Other genes targeted by PC-derived miRNAs were discovered, namely the regulatory factor X-associated protein by mi-212-3p resulting in a decrease of MHC II expression and the promotion of immune tolerance (17). Thus, cargoes carried by EVs are at least partially responsible for tumor expansion and metastasis.

Accumulating reports have also suggested a pathological role in triggering inflammation and autoimmune responses. EVs carrying damage associated molecular patterns have substantial role in the induction and persistence of inflammation. Molecules such heat shock proteins, S100 proteins, and high mobility group protein B-1, found in high concentrations in apoptotic vesicles prompt the release of adenosine triphosphate from autophagic, dying cells that are phagocytosed and activate the inflamasome in macrophages (3). Studies in non-neoplastic salivary gland epithelial cells have found exosomes containing ribonucleoproteins, which are major autoantigens in systemic rheumatic diseases (18). Other autoantigens that have been reported are E3 ubiquitin-protein ligase TRIM21 (or Ro(SS-A)) and Lupus la protein, and the Smith antigen involved in the Systemic Lupus Erythematosus (SLE) and Sjögrens syndrome, respectively (3). Moreover, data also supports a role for EVs in the formation of immune complexes in SLE owing to their RNA and DNA content (3). Thus, EVs may be involved in autoimmunity by efficiently presenting autoantigens to APCs which can subsequently activate effector cells. In the inflammatory diseases setting, EVs were shown to alter cell lipid metabolism through the delivery of metabolites and key enzymes that are part of the arachidonic acid cascade to adjacent cells, thereby inducing the production of pro-inflammatory molecules (3). For a comprehensive review of the EV role in autoimmune diseases see (19). Indeed, global blockade of exosome production with GW4869, a neutral sphingomyelinase inhibitor that inhibits the ceramide-mediated inward budding of multivesicular bodies, attenuated sepsis-induced inflammation, improved cardiac function and prolonged animal survival (20).

3 Dendritic cells-derived exosomes

Aside from the evidences that exosomes highly contribute for cancer and autoimmunity, they also can contribute to blunt inflammatory signaling. Indeed, suppressor of cytokine signaling proteins – a family of the STAT-induced STAT inhibitors – were shown to be present within extracelular vesicles secreted by alveolar macrophages. The vesicles are taken up by alveolar endothelial cells that inhibit cytokine-induced STAT activation (21). This intercellular comunication can however be dysregulated during inflammation.

In recent years, dendritic cells-derived exosomes (Dex) have gained much attention in autoimmune diseases and tumors because they resemble the biology of the cell from which they were derived. For instance, APC-derived exosomes express on their surface the MHC I and II, and CD86, CD80 and CD40, as well as adhesion molecules that may target exosomes to their acceptor cells (22). However, exosomes derived from more iDCs, which have MHC II endocytosis upregulated, the MHCII-peptide complexes are only transiently expressed on their surface and consequently, exosomes might have a low level of MHC-II transcripts and lack of MHC II expression on their surfaces (23). More importantly, they have been shown to cross biological barriers such as the synovial membrane and the blood brain barrier. Since they can gather different molecules within themselves, they are likely to trigger a more nature and long lasting therapeutic effect (each molecule can interfere with different pathways). Moreover, they possess natural stability and homing specificities. Thus, cell surface modification is not a requirement for active targeting (7).

Given the fact that exosomes are unable to change phenotype, Dex represent a novel therapeutic approach for the treatment of inflammatory and autoimmune diseases. There are several ways through which DC and Dex can be rendered immunosuppressive.

iDCs either expressing viral IL-10 (vIL-10) from adenoviral mediated gene transfer, or treated with recombinant murine IL-10 (rmIL-10) suppressed paw inflammation both in the injected and untreated contralateral footpads in a murine delayed-type hypersensitivity (DTH) model. Similarly, periarticular administration of exosomes derived from bone marrow (BM)-DCs either previously transduced with adenovirus expressing vIL-10 or treated with rmIL-10 had shown similar therapeutic outcomes as modified-DCs. The exact mechanism through which exosomes function to suppress inflammation and responses against self is unclear. It was hypothesized that exosomes derived from DCs expressing vIL-10 act by downregulating costimulatory molecules such as B7.1 and B7.2 thereby modulating T cell function. Nevertheless, this modulation was shown to be less effective in vitro suggesting that other indirect

mechanisms might be involved. Interestingly, exosomes from DCs expressing vIL-10 showed decreased levels of heat shock cognate protein 70 which was reported to convert T cell tolerance to autoimmunity (24). Likewise, due to the essential role of IL-10 in the development of normal mucosal immunity, it was explored whether exosomes derived from DC treated with IL-10 could suppress trinitrobenzene sulfonic acid (TNBS)-induced colitis. The severity of diarrhea and colon inflammation was attenuated as well as the levels of Th1 type cytokines such as interferon (IFN)-γ, IL-2, and tumor necrosis factor (TNF)-α. However, these effects were not reproduced with the exosomes derived from iDCs. Comparative flow cytometry analysis revealed that exosomes derived from IL-10-treated DCs exhibited a higher immunosuppressive phenotype as well as membrane-associated IL-10 (25).

IL-4, a mediator of Th2 cell commitment, also exhibits anti-inflammatory effects such as the suppression of IL-1 and TNF-α. In fact, injection or gene transfer of this cytokine has been shown to be therapeutic in different murine models of autoimmune diseases, including collagen-induced arthritis (CIA), type I diabetes, and experimental autoimmune encephalomyelitis (EAE) (26). Indeed, systemic administration of BM-derived, myeloid DC, genetically modified to express IL-4 was able to reverse established murine autoimmune arthritis as well as significantly reduce the swelling in a murine DTH model. Similarly, exosomes derived from these DCs were also as or more suppressive than the parental DCs in CIA and DTH models. It was suggested that DC/IL-4 and DC/IL-4-derived exosomes reduce inflammation in the DTH model through a MHC and partly Fas-dependent mechanism suggesting that T cell function is modulated either in- or directly. Since adoptive transfer of $CD3^+$, splenic T lymphocytes from DC/IL-4 and DC/IL-4 derived exosomes can partially suppress the DTH response suggests that this modulation is likely due to the induction of Treg (26). Previous studies with DC from FasL-deficient mice and Fas-deficient recipient mice underlined that the immunosuppressive action is due to the presence of FasL in exosomes and not due to a modulation of FasL-expressing parental DCs. However, how the immunosuppressive function of exosomes is mediated by FasL/Fas is unknown because extensive T cell apoptosis was not observed in the draining lymph node and spleen. Nonetheless, apoptosis was mediated by both syngeneic and allogeneic exosomes whereas immunosuppression was only observed with syngeneic Exo/FasL, suggesting a MHC class II dependency(27).

Indoleamine-2,3-dioxygenase (IDO) is a tryptophan degrading enzyme, only present in certain subsets of cells, that is important in host defense and immunosuppression. It is transcriptionally induced by a variety of inflammatory stimuli such as IFN-γ, IFN-α, CD40L, Glucocorticoid induced TNF receptor-related and TNF-α. Endogenous IDO is involved in tolerance

maintenance in a number of settings, possible by depleting T cells of essential tryptophan rendering T cells anergic or in the other hand by enhancing T cell apoptosis. IDO is also able to produce metabolites of tryptophan termed kynurenines that can also modulate T cell function. IDO-expressing DC and respective exosomes are anti-inflammatory and therapeutic in both CIA and DTH models. Surprisingly, the Exo/IDO were as effective as the DC/IDO. Maturation assessment suggested that in this case, the therapeutic effect was not due to change in DC maturity. Instead, it was shown to be partially dependent on B7-1 and B7-2 costimulatory molecules expression in both DC and exosomes. Thus, exo/IDO may interact directly with T cells or with endogenous APCs to alter their function (28).

The immunomodulatory effects of statins have been increasingly reported. DC treatment with statins was shown to render tolerogenic functions to DC. It inhibits expression and secretion of pro-inflammatory cytokines and therefore further T cell activation and proliferation. After injection, statin-Dex were detected in the spleen, thymus, and popliteal and inguinal lymph nodes and successfully induced immune tolerance in experimental autoimmune myasthenia gravis (EAMG) rats thereby ameliorating clinical symptoms. Statin-DEX were able to down regulate the expression of CD80, CD86 and MHC class II molecules on endogenous DCs. The lower numbers of antigen specific T cells also contributed for a lower production of anti-Acetylcholine receptor(AchR) antibodies by B cells. The therapeutic effects were also associated with increased Treg cells in the thymus and lymph nodes and an alteration of the transcriptional profile. Compared with control-Dex, statin-Dex carried lower levels of MHC class II and higher levels of FasL and IDO. Although it is still unknown if exosomes over-carrying IDO could activate Treg cells, EAMG rats after treatment exhibited a significantly increase in numbers of $Foxp3^+$ cells in thymus and $CD4^+$ $Foxp3^+$ T cells in mononuclear cells ăfrom the lymph nodes when compared with the control. Therefore, statin-DEX over carrying IDO may increase the numbers or function of Treg cells. As seen with other exosomes-based approaches to treat autoimmunity and chronic inflammation, the effects of statin-Dex on EAMG were partially dependent on FasL/Fas pathway. Accordingly, despite the treatment with statin Dex with higher FasL had result in higher number of Fas^+ cells, the number of apoptotic cells was not different between this type of exosomes and the control or statin-Dex. Rats treated with statin-Dex with anti-FasL antibody treatment exhibited more severe clinical symptoms (29).

TGF-β1 is a negative regulator of pro-inflammatory immune responses that may be responsible for the DCs development modification towards a regulatory phenotype, thereby inhibiting inflammation. Accordingly, systemic administration of TGF-β1 gene modified iDCs delayed the development of dextran sulfate sodium(DSS)-induced murine inflammatory bowel

disease(IBD) . Treatment with exosomes secreted by TGF-β1 gene modified DCs attenuates Th17 responses which are crucial for the development of tissue specific autoimmune diseases. This action, along with an increase of the Treg cells number, leads to inhibition of murine IBD development. However, this treatment was not effective after the disease onset (30). The immunosuppressive activities of TGF-β1 can be mediated via secreting soluble or membrane-associated. Further studies with recombinant adenovirus that expresses membrane-associated TGF-β1 and with secreted TGF-β1 gene-modified DCs were administered in an EAE model to know whether these two different TGF-β1 forms triggered different outcomes. Indeed, membrane-associated TGF-β1 exosomes have stronger immunoregulatory activity to inhibit EAE even after disease onset compared with the secreted ones. It prevented the *de novo* differentiation of Th17 cells via inhibiting IL-6 production by DCs. Although exosomes secreted by TGF-β1 gene-modified DCs were able to inhibit T cell proliferation, they failed to induce Treg in vitro. Only with a higher dose - 10 μg/mL - was observed Treg induction. Thus, treatment with secreted exosomes was not sufficient to control the pro-inflammatory responses. Importantly, data from the membrane-associated exosomes experiments has shown that they are effective in inhibiting the development and progression of EAE, independently of MHC types (31). This result contradicts the aforementioned results with Exo/FasL.

Exosomes were also shown to be important in suppressing immune inflammation in the allograft heart. For example, cardiovascular exosomes were found to carry integrin αvβ6 to promote the generation of the donor antigen specific immune tolerance. Immunization with donor-derived exosomes induced plenty of Treg in the recipient mice. More important is that these induced Treg were donor antigen specific and maintained in long-term by the allograft itself. Integrin MM1Pa present in these exosomes which binds to phDAC1 inhibiting the gene transcription of trans-acting T-cell specific transcription factor(GATA)-3 in polarized Th2 cells. GATA3, the key transcription factor of Th2 cells, does not bind to Foxp3 to inhibit Foxp3 transcription, and therefore polarized Th2 cells are induced to differentiate into Treg. Hence, the data showed by this study suggest that donor-derived peripheral exosomes have the potential to be used to inhibit the immune inflammation in the heart transplantation (32).

iDCs derived exosomes were also reported to promote the allograft heart survival. This approach has several advantages: iDCs derived exosomes are readily source of donor antigens which can be frozen without compromising the therapeutic effect, and be prepared quickly and used when necessary. Moreover, large doses of immature DC-derived exosomes (iDex) (50 μg) do not accelerate immune rejection nor sensitize recipients against donor antigens. However, when injected prior to transplantation, exosomes from mature DCs can trigger effector T cells

responses. Exosomes secreted by B cells, mast cells can also be used as a source of donor MHC class II antigens. Even though small amounts of iDCs derived exosomes were sufficient to prolong allograft survival, this effect was insufficient to induce immune tolerance. Rapamycin, a non-specific immunosuppressive therapy, had to be used in combination with iDCs derived exosomes, to favor the presentation of alloantigens in a tolerogenic fashion. Importantly, the tolerance persisted long term and was transferred to naive syngenetic recipients by injecting tolerogenic immunocytes (33).

In other cardiac allograft transplant model using congenic rats fully mismatched for the class I and II genes of the MHC region, iDCs derived exosomes administered post-transplantation were also able to prolong graft survival but this effect was moderate. To ensure the presentation of alloantigens in a tolerogenic manner, it was used LF 15-0195 drug, a new desoxypergualin analog, which has been recently reported to inhibit DCs maturation. This short term treatment promoted donor-specific cardiac allograft tolerance along with a considerably delay of chronic rejection (34). Similarly, in vivo injection of the 20 μg of donor iDex have shown to prolong the survival of intestinal allografts. It was observed an increase of IL-10 secretion, $CD4^{+}CD25^{+}$T cells percentage and FOXP3mRNA expression in splenic T-cells (35).

In a mouse model of EAMG, mouse BM-derived iDex had induced tolerance. Disease progression was ameliorated by reducing AChR-reactive lymphocyte proliferation, AChR antibody levels and pro-inflammatory cytokine levels (36).

Serum exosomes produced by young or environmental enrichment-exposed rats significantly increased oligodendrocyte precursor cell, myelin content and neural stem cell levels and reduced oxidative stress either in hippocampal slice cultures and in vivo when nasally administered to naive rats. This study endeavor the production of similar pro-myelinating exosomes and aroused the interest to use exosomes to revert the damage in autoimmune and inflammatory diseases (37).

Stimulation of primary DC cultures with low levels of IFN-γ, to mimic environmental stimulation, has result in IFN-γstimulated Dex that were able to increase myelination, by targeting multiple steps of the oligodendrocytes differentiation, and oxidative tolerance. In addition, in vivo work confirmed that when nasally administered these Dex had effectively increased brain myelination. Tracking assays performed in vitro revealed that these exosomes were mostly taken up by oligodendrocytes. Although others have shown that DCs treatment with IFN-γhad yielded exosomes expressing surface markers responsible to activate T cells, others have reported that exosomes found in the periphery could impact brain myelination by miR-219 delivery. The miR-219 was reported to be essential and sufficient in the formation

and maintenance of compact myelin, and for the oligodendrocytes-committed percursors differentiation by reducing the expression of inhibitory regulators of differentiation (38). Other molecules that may acount for the observed increase of myelin include miR-9 and miR-17-92 cluster. Regarding the oxidative tolerance, this effect was linked to the presence of several anti-inflammatory miRNAs. Indeed, miR-181a and miR-124 were found in higher levels in these exosomes, and are associated with the regulation of monocytes/macrophages responses by dampening inflammatory signaling and the production of reactive oxygen, or with the down-regulation of M1-associated pro-inflammatory IL-6, TNF-$\alpha$and inducible nitric oxide synthase (iNOS) , respectively (39).

More recently, the same group attempted to define which cell type was responsible for the pro-myelinating effect in exosomes derived from environmental enriched rats. miRNA expression profiling of exosomes secreted by each type of blood-cell have shown that all of them contained miRNA-219. Other miRNA species were also found in high quantities, such as miR-9 and miR-17 responsible for oligodendrocyte differentiation, and miR181a with anti-inflammatory function. In addition, exosomes were also shown have high quantities of miR-665 which is reduced in multiple sclerosis (MS) lesions (40).

Although some agents to treat autoimmune diseases, and in this case MS, have minor remyelinating potential, none adequately regenerate myelin sheets without coming with an array of harmful immune sequelae. Therefore, exosomes can be of use to revert the already existing damage in these diseases.

4 Myeloid-derived suppressor cells derived exosomes

Myeloid-derived suppressor cells (MDSCs) is a heterogeneous population of cells defined by their myeloid origin, immature state and the ability to mediate immunosuppressive responses through T cell inhibition. Granulocytic-MDSC derived exosomes isolated from the culture supernatant of the granulocytic-MDSC spleen's of tumor bearing mice were shown that could attenuate DSS-induced colitis and restore intestinal immune balance. The suppression of the $CD4^+$ T cell proliferation and the DTH response were partially attributed to arginase (Arg)-1 activity . Arg-1 has been suggested to interfere with CD3 ξ- chain biosynthesis and to down regulate the expression of T cell receptor (TCR) on T cell surface. Consequently, it renders T cells unresponsive without MHC II restriction and in an antigen-non-specific manner. Moreover, the formation of peroxynitrite through the cooperative activity of reactive oxygen species and nitric oxide (NO) leads to the nitration of tyrosines in TCR. As a result, the flexibility of the

TCR complex is compromised and the binding of peptide loaded MHC is inhibited therefore blocking T cell's response to an antigen specific stimulation (41).

5 Regulatory T cells-derived exosomes

Although transfer of molecules have been mostly reported as a unidirectional process, from DC to T cells, it was found that the transfer of biomolecules can also occur in the opposite way. Indeed, ovalbumin-specific $CD8^+$T cell (OTI)-released EXOs induced, both in vitro and in vivo, ovalbumin (OVA)-specific pMHC I downregulation on DC_{OVA} and in vitro DC apoptosis in a Fas/FasL dependent manner, after EXOs absorption by DCs via MHC-TCR and CD54-leukocyte function-associted LFA interaction. As a result, DC lost their stimulatory effect on $CD8^+$ T cell proliferation and exhibited less survival. Apoptosis rate was dependent of the dose. Additionally, in this study was also demonstrated that Ag-specific OTI mouse $CD8^+$ T cells EXOs can also inhibit OVA-specific DC-mediated $CD8^+$ cytotoxic T lymphocytes (CTL) responses and diabetes in transgenic mice expressing membrane-bound OVA strain (mOVA) under the control of rat insulin promoter (42). Exosomes from $CD8^+CD25^+$ Treg cells have been isolated. These exosomes expressed exosomal proteins such as lysosome-associated membrane protein (LAMP)-1ăand CD9 as well as Treg markers albeit in much less amount than cells themselves. C57BL/6 mice were intravenously immunized with EXO-derived or $CD8^+CD25^+$ Treg cells plus DC_{OVA} to assess the immunoinhibitory effect of each approach. Among the total $CD8^+$T cell population, 2,52 % were OVA-positive. This number decreased for 1,81% and 1,08% when DC_{OVA} were co-injected with EXO-derived or $CD8^+CD25^+$ Treg cells, respectively. Thus, natural $CD8^+CD25^+$T cell-secreted EXOs are able to inhibit in vivo DC-induced CTL responses (43). Several studies have been reported the use of Dex in the prevention of allograft rejection. A study, aimed to assess whether exosomes released from Treg could also participate in transplantation tolerance has found that exosomes released by $CD4^+CD25^+$T cell, especially from donor Treg, could be used to prolong the survival time of the kidney transplant and inhibit Treg proliferation(44). The responsible mechanism and molecules are unknown, however previous studies found that $CD4^+CD25^+$ Treg suppression is associated with surface molecules such as lymphocyte-activation gene 3, galanin subtype 1, and neuropilin-1 (43).

Thus, T cell EXOs may be useful to treat autoimmune diseases as well.

6 Mesenchymal stromal cells derived exosomes

In the field of regenerative medicine, there is an ever-growing number of reports evidencing the tissue repair functionalities of mesenchymal stromal cells (MSC) and the secreted EVs. Their cytoprotective properties are the result of apoptosis inhibition, stimulation of the proliferation of residing cells, and promotion of neovascularization (45). In addition, they can also alter the behavior of both adaptive and innate immune cells in order to induce a more anti-inflammatory or tolerant phenotype and thus, they have emerged in recent years as a promising approach to treat autoimmunity and inflammation. Indeed, MSC may inhibit natural killer cells proliferation and activity, suppress T lymphocyte proliferation, DCs maturation and B lymphocyte proliferation and activation, as well as induce Treg expansion. This anti-proliferative action is induced either in a cell-to-cell contact or paracrine manner by secreting factors such as IL-6, IL-10, Prostaglandin E(PGE)-2 , hepatocyte growth factor, IDO, NO, TGF-β1 and human leukocyte antigen G (46). They express a wide range of regulatory molecules such as programmed death-ligand 1, membrane-bound TGF-β, galactins, MHC-Q2a among others (47). Owing to the unique immunosuppressive capacity of MSCs, it was hypothesized whether MSCs-derived exosomes harboring regulatory molecules could modulate the phenotype of auto-reactive cells by function as nano-shuttles for inserting these molecules in target cells. This interaction between exosomes-target cells has been demonstrated upon different studies and apart specific ligand-receptor interactions, the presence of phosphatidylserine (PS) enables exosomes to be captured by different cells. Moreover, the higher expression of its ligand, T cell immunoglobulin mucin protein-4, accelerates the capture of exosomes. This hypothesis was tested in a EAE model and it was observed MSC-derived exosomes incorporated in lymphocytes' membrane where they display their surface regulatory molecules. However, horizontal transferring of other regulatory molecules may be involved and responsible for the expression or increased of the aforementioned molecules. Functional bioactivity of the exosomes was confirmed by the significant decrease in cytokine secretion of Th1 and Th17 phenotypes upon stimulation with Myelin oligodendrocyte glycoprotein$(MOG)_{35\text{-}55}$ peptide (47).

sMVs from heterologous human BM-MSCs have a similar immunomodulatory function as MSCs in T1D, which is observed not only with islet antigen glutamic acid decarboxylase but also with a recall antigen. They consistently downregulated Th1 responses and proliferation of Th17 cells, inhibiting their pathological effects on beta cell function. This therapeutic effect was not observed with sMVs derived from fibroblasts, suggesting that the immunomodulatory effect seems to be specific of MSC-derived sMVs. Integrin blockade reduced the immunomodulatory

effects and thus, is likely to depend at least in part on the internalization of sMV. In addition to dampening the inflammatory response through the inhibition of effector T cells, sMVs also induce a regulatory phenotype by increasing the production of IL-10. PGE2 and TGF-βwere produced by T cells upon contact with sMVs. It was observed that TGF-βconveyed as mRNA and protein within and on the surface of sMVs function as a sustained signal on recipient cells. Depletion of the RNA content decreased the TGF-βtranscripts highlighting the importance of mRNA and miRNA transfer to target cells. Indeed, miRNA21 was previously shown to enhance TGF-βsignaling. Similarly, IL-6 production also increased in presence of sMVs. IL-6 has been shown to be crucial to suppress mDCs and may have an important role in restoring beta cell function (48).

Several studies have suggested that MSCs down regulate the expression of CCR7 and CD49dβ1, involved in DC homing to lymph nodes, therefore hindering DC's ability to prime antigen-specific T cells. Exo-conditioned DCs, taken from patients with recent onset of T1D, induced Treg proliferation and increased T cell secretion of IL-10, TGF-βand IL-6., with IL-6 likely contributing for beta cell function. Thus, MSC and their exosomes are a promising approach to locally control autoimmune pancreas inflammation and halt the progression of T1D (49).

Impaired wound healing because of high levels of inflammation that inhibits the regeneration of skin architecture is a frequent problem of diabetes patients, leading to regression to a chronic wound state. In these conditions macrophages, the primary effectors of inflammation in tissue injury, have a tendency to be polarized towards the M1 phenotype which triggers proinflammatory responses. In addition, M2 polarization is impaired and consequently, there is no recruitment of STAT3 or other transcriptional factors that promote tissue remodeling and dampen inflammation. Studies with MSCs have found that pre-exposure to lipopolysaccaride (LPS) can increase trophic effects and a superior capacity to preserve skin flap survival in a diabetic rat model. Although the exact mechanism is unknown, it has been suggested that MSCs create an optimal environment by secreting molecules that will function in a paracrine dependent manner. Despite the role of exosome-derived LPS pre-MSCs (LPS pre-Exo) in macrophage polarization and suppression of inflammation not being fully elucidated, it was recently reported that lethal (Let)-7b in LPS pre-Exo is crucial for macrophage plasticity by targeting TLR4. Down regulation of TLR4 resulted in the fine-tuning of the inflammatory response and wound healing. It was also observed that this treatment resulted in a increase of exosomes production by MSCs. Thus, LPS pre-exo besides the greater regulatory properties, let-7B released from these exosomes also concomitantly activate feedback inhibitor mechanisms

that will restrain the magnitude of inflammatory responses (50).

In a colitis rats model induced by TNBS, the expression of TNF-α, IL-1β, cyclo-oxygenase-2, and iNOS in colonic tissues, was lower in bone BMSC-EV treated group compared with the control. In addition, expression of nuclear factor χ-light-chain-enhancer of activated B cells-p65ăhad also decreased. Thus, BMSC-EV treatment blocked either the downstream and upstream signals therefore ameliorating inflammation. These effects were complemented with an increase of the IL-10 levels. Oxidative stress and lipid peroxidation are also involved in the pathogenesis of the inflammatory bowel disease triggering the recruitment and activation of neutrophils and membrane barrier dysfunction. In this regard, biochemical markers of neutrophil accumulation such as myeloperoxidade, and malandialdehyde, which correlates with oxidative damage and lipid peroxidation, were reduced. In the other hand, enzymes known by their role in the defense against oxidation such as superoxide dismutase and glutathione were increased. The excess of oxidative stress and inflammation usually have an apoptotic effect on intestinal epithelial cells. BMSC-EV were able to decrease apoptosis through inhibition of both c-caspase9 and c-caspase8, consequently blocking either the intrinsic as well as the extrinsic pathway (51).

Exosomes have also been exploited to treat therapy refractory graft-versus-host disease (GVHD) . EVs isolated from four different unrelated BM-donors, were submitted to a filtration process,to isolate the bigger particles, and undergone a polyethylenoglycol precipitation to eliminate any virus. The fraction with higher content of anti-inflammatory molecules were first tested in vitro in allogeneic target cells and owing to the favorable results, subsequently infused into a severe ill patient. The applications were well tolerated and no side effects were detected. In line with the reduced profile of pro-inflammatory cytokines the clinical GVHD symptoms improved soon after the start of the MSC-based therapy (52).

Currently, a clinical trial is undergoing aiming at evaluate the effect of microvesicles and exosomes therapy on β- cell mass in T1D (NCT02138331). These exosomes have exosome-associated proteins such as tetraspanin proteins, CD9 and CD81, Anti-human-lymphocyte globulin-2-interacting protein X, tumor susceptibility 101, and RNA species, predominantly short RNAs, most of them are microRNAs. Indeed, several studies have reported the role of miRNAs in maintenance of the islet and beta-cell functions, and regulating the pathways involved in insulin expression, processing and secretion being either positive or negative regulators. Among miRNAs, miR-375, miR-338-3p, miR-184, miR-124a2, miR-184, miR-200 are key miRNAs affecting beta cell mass or islet morphology (53).

7 Exosomes as drug carriers

Curcumin is a polyphenol derived from the rhizomes of Curcuma longa, with anti-inflammatory, antineoplastic, antioxidant, and chemopreventive activity. Despite this highly appreciated functions, its poor solubility and preferential interaction with lipid membranes resulted in problems with bioavailabity and clinical efficacy. To overcome these problems, several attempts have been made to encapsulate curcumin in different artificial systems. However, exosomes as natural carriers exhibit increase biocompatibility and are preferred drug carriers. Indeed, host-derived exosomes are able to form complexes with curcumin which subsequently are taken up by activated myeloid cells and induce apoptosis of these monocytes. The stability and biocompatibility of cur cumin increased while its bystander and off-target effects have reduced. This approach successfully protects mice from LPS-induced septic shock. In addition, it was also demonstrated that exosomes could target not only $CD11b^{+}$ $Gr\text{-}1^{+}$ but also increase the delivery of curcumin to these cells, thereby increasing the cell death rate. Nevertheless, multiple mechanisms are likely to be involved in the therapeutic effect of curcumin since it is known to participate in a number of pathways (54).

Moreover, exosomes containing curcumin or STAT3 inhibitor, cucurbitacin I administered intranasally proved to reach brain microglia cells and exert a therapeutic value in three independent models: for curcumin complexed exosomes in LPS-induced brain inflammation model and MOG-induced EAE autoimmune disease; and for STAT3 complexes exosomes in a bliobastoma model established by injecting GL26 expressing luciferase intracranially. Indeed, exosomes were able to delay EAE disease and inhibit tumor growth in vivo. Thus, these results indicate that direct intranasal-to-brain transport is feasible and that this might be a promising noninvasing approach for the treatment of brain inflammatory-related diseases since exosomes provide a way to bypass the blood brain barrier. Distribution studies have shown that within 1 hour of intranasal administration the exosomes started to inward to brain. This finding is consistent with the extraneuronal pathways that has been reported for transport of therapeutic agent from the nasal cavity to brain which requires only a few minutes for a drug to reach the brain, whereas transport through the intraneuronal pathway involves several days. Other transportation routes are likely to contribute for exosomes to reach the brain (e.g. transportation along the trigeminal nerve). Thus, more studies are needed to pinpoint the route through which exosomes translocate to the brain and ultimately to nervous system and address potential parameters that affect brain homing in order to increase the quality of exosomes for this purpose (55). Furthermore, microglial cells were identified as the preferentially targeted cells by

exosomes without any observed side effect, which adds even more therapeutic value. This is because microglial cells have been shown to contribute significantly to inflammation by producing IL-1 and IL-6. However, the mechanisms for this selectivity have not been addressed (56). Induction of apoptosis is one of the mechanisms underlying the therapeutic effects. Nevertheless, others can not be excluded as curcumin is a pleiotropic agent.

Besides the ability to transfer drugs, exosomes were also shown to be amenable vectors of siRNA. Targeting was achieved by engineering the DCs to express Lamp-2b, an exosomal surface membrane protein, which the extra-exosomal N terminus was fused to the following peptide moieties: rabies viral glycoprotein (RVG) peptide that specifically binds to the AchR, a muscle-specific peptide and a FLAG epitope. The possibility of loading exosomes with exogenous cargoes have been explored and was observed that among the nanoscale electroporation protocols, electroporation at 400 V and 125 uF resulted in the greatest retention of siRNA. This method did not alter the physical properties of RVG exosomes and they were able to successfully deliver their cargoes in vitro. Importantly, quantitive polymerase chain reaction analysis revealed that siRNA delivery with targeted exosomes achieves comparable gene knockdown as transfection reagent. This effect was cell-specific. Thus, modified Lamp2b constructs successfully endowed exosomes with cell targeting capabilities. Results from the MTT toxicity assay and carboxyfluorescein succinimidyl ester assay imply that exosomes may be well tolerated in vivo both from the toxicological and immunological point of view, respectively. Moreover, no significant changes were observed in the levels of IL-6, IFN-γ-inducible-protein-10, TNF-α, IFN-αconfirming the inert profile of exosomes. Contrary of what is observed with viruses, re-administration of exosomes did not decrease the transgene delivery efficiency demonstrating that exosomes can be re-administered multiple times without losing their delivery efficacy. Moreover, exosomes only encapsulated 20% of the siRNA however they still exhibited greater protein knockdown compared to RVG-9R delivery. Thus, this treatment is potentially suited for long-term silencing of genes. Despite the success of these approaches without any over side effects, DC-derived nucleic acids and proteins need to be further characterized, in order to assess their impact in recipient cells. Moreover, it is currently unknown whether other cargoes will be loaded and delivered as efficiently as unmodified siRNA (57).

Drug delivery using exosomes as vehicles has been exploited to treat other conditions (58). It has been recently reviewed in (59, 60).

8 Other types of exosomes

EVs are widely distributed in different organisms such as bacteria and yeast, and they were shown to be able to communicate with eukaryotic cells (61). In fact, there are already some studies highlighting the role of pathogen-derived exosomes in the modulation of the immune system (62, 63). Helminth-derived exosomes have been shown to modulate host inflammatory responses suggesting that the responsible regulatory molecules within these exosomes could be delivered in artificial exosomes with the aim of regulating pathological inflammatory conditions. Importantly, it has already been demonstrated that helminthic exosomes secreted at the host-parasite interphase are actively captured by host cells. In addition, several miRNA found within helminth-secreted exosomes seem to be homologous to human miRNAs, and possess broad effects in T-cell function and phenotype. Thus, despite some redundant therapeutic effects and the need of in-depth research regarding the effects of specific helminth molecules stimulating different immune mechanisms, there might also be some chances of these exosomes to be useful (63).

Exosomes also participate in the multistage apoptosis process by carrying chemokines that recruit phagocytes to sites for cell apoptosis. Indeed, microparticles released by apoptotic Burkitt lymphoma cells, have been reported to be important in monocyte recruitment to B-cell apoptosis as they transport chemokines such as fractalkine and intercellular adhesion molecule-3 that despite being adhesion molecules seem to have a dual role participating in apoptosis as well (64). Those findings also suggest that apoptotic cells may release microparticles with anti-inflammatory effects on macrophages (65).

9 Advantages of this delivery system

Exosomes have come up as a solution to overcome the drawbacks of cell therapy and side effects of non-specific immunosuppressive drugs. However, there are still several challenges that need to be addressed before cell-derived exosomes can be approved for the treatment of certain diseases. Nonetheless, their advantages compared with other strategies are well recognized.

First, since exosomes are biological nanovesicles they have shown excellent biodistribution and biocompatibility.

Another advantage is the static phenotype of exosomes compared with the parental cell, which can undergo phenotypic changes following injection. Their phenotype is likely to reflect

the phenotype of the cell at the time of release. Thus this strategy may be safer and more effective than the use of modified cells (24).

In contrast with cells, exosomes can be sterilized by filtration through 0.22 μm filters and afterwards be handled, stored, and characterized more easily. Nevertheless, any given exosome samples may provide a heterogeneous mixture with different compositions. This heterogeneity might be useful for the biological activity, since multiple signals not only act synergistically to the same outcome, but also provide a challenge to guarantee the standardization of exosomes based preparations (66). Moreover, the complex molecules on their surface offer potential mechanisms of homing to specific target organs and micro environments (41). Nonetheless, if a better tissue specificity or delivery efficacy is needed, ligands are genetically fused to the extra-exosomal termini of exosomal membrane proteins(67). This process is relatively easy when compared with synthetic lipid nanoparticles, whose stability, material properties and synthesis complexity might be potentially affected after adding a different ligand on their surface. Importantly, some of these proteins such as lactadherin or milk fat globule-epidermal growth (MFGE)-8 naturally enhance exosomes uptake by binding PS and with cell surface integrin proteins CD51 and CD61 through a second domain (68). MFGE8 was shown to be reduced in sepsis leading to problems in apoptotic cells clearance. Indeed, adoptive transfer of exosomes enriched in this protein reduced TNF-$\alpha$and IL-6 levels, and improved survival (69). Tetraspanins are also great anchoring scaffolds for drug packaging and tissue targeting since they contain both intra- and extra-vesicular domains. In this regard, human embryonic kidney cells (HEK)-293 were transfected with a mammalian expression vector encoding the human CD63, and the resulted protein was fused to a fluorescent green protein or red fluorescent protein. This chimeric protein was used to track the genesis and secretion of exosomes. It was observed a correct intracellular partitioning of the engineered protein into the proper endosomal compartment underlining the potential of the tetraspanins family to be used as molecular scaffold to direct fused peptides to endocytic compartments. Moreover, the peptide was shown to be functionally and structurally preserved (70).

Recently, it was proposed an approach to prepare engineered hybrid exosomes by fusing the membranes of liposomes, embedded with peptides or antibodies as targeting moieties or Polyethylene glycol (PEG), with exosomes employing the freeze thaw method, in order to modify or fine tune the surface features of the exosomes. This physical technique yielded exosomes free of contamination with unwanted chemicals such as calcium or PEG. Cellular uptake studies also shown that cellular uptake of PEG-modified exosomes was significantly increased compared with cationic lipids modified. Concomitantly, it was also verified an increase of the

circulation time in blood as well as a reduction in their uptake by mononuclear phagocytes (71). However, efficient exosome-mediated delivery is highly dependent on the target-binding peptide that was used owing to different affinities or display of the ligand on the exosome surface. For example, some peptides expressed for example on the N terminus of Lamp2b are more prone to acid-dependent proteolytic degradation. Recently, it was reported that introducing glycosylated motifs at particular locations within the fusion protein, protect the targeting peptides from degradation without afecting the display and binding to their cognate protein targets (67).

Their ability to cross biologic barriers such as the blood brain barrier, together with the cell targeting specificity enable the exosomal delivery system to transport pharmaceutically active substances and genetic materials into the CNS. In fact, exosomes were observed to be involved in myelin membrane biogenesis and contributed for the elimination of overproduced myelin membranes, and currently this regenerative role is being exploited to ameliorate neurological diseases such as MS and Alzheimer.

10 Hurdles in the translation of this approach

One of the difficulties in translating this strategy into clinic lies on the fact that vesicle subpopulations are not thoroughly defined and may overlap in size and density. Ranging from the ultracentrifugation, the gold standard for EV isolation, or ultrafiltration membranes techniques to immunoprecipitation using antibodies to typical surface antigens or high performance liquid chromatography, none have been found to be an ideal, scalable and cost effective method to isolate and obtain purified exosomes. Moreover, the existence of exosomes with unique cargoes and mechanisms of formation hinder the production of pure isolations. Therefore, the development of biocompatible, economically viable source and methods for harvesting exosomes are highly needed. It is important to note that intact exosomes are required for conferring the therapeutic effects rather than only membranes. Moreover, cycles of freeze/thaw or sonication, were shown to abolish the therapeutic capacity of the exosomes (24). Hence, the effectivity and tolerability of the aforemention steps must be demonstrated. The isolation techniques and the correspondent advantages and disadvantages have been recently reviewed in (4, 61, 72)

Recently, a new method to enrich and purify exosomes was reported. Since exosomes and virus particles have similar biophysical properties it was hypothesized that a PEG-based approach could be used. Indeed, using this protocol yielded exosomes highly comparable to the gold standard differential centrifugation method, and superior to commercially available meth-

ods. Harvesting exosomes with PEG-based method was then compared with the differential centrifugation technique as well with commercial kits. The resulted exosomes were similar to DC derived ones, but with much less protein contamination when compared with commercial methods. Importantly, this technique is also easier and more cost effective to execute and easily adapted for other purposes (e.g. by adding a filtration step). This method did not affect the quality of RNA suggesting that it does not affect the stability of small RNA species. Modifications to this protocol can be made to refine the enrichments, such as centrifugation force and time, nano-filtration, and percentage of PEG used (73).

Another problem is that any microbial contamination will suffer sedimentation during exosomes isolation. This may affect cellular responses and, in this case, the study of exosomes. For example, Mycoplasma contaminated exosome fractions are known to induce polyclonal B cell responses (74).

One of the difficulties in studying exosomes is that, generally, cells produce small amounts of exosomes. For instance, DCs secret 1-2 μg of protein/ 10^{6} DCs (8). Moreover, regarding exosomes secreted from DCs, the majority of the data is obtained from studies with monocytes, DCs precursors from BM, or DCs lines (8). In addition, the composition of exosomes widely varies depending on the culture conditions and activation state of the cells, which can lead to contradictory results and pose a challenge to crossing data. For example, Epstein-Barr virus (EBV)-transformed human B cell lines and granulocyte-macrophage colony-stimulating factor-propagated monocyte-derived DC were reported to give rise to small and homogeneous exosomes while long term culture Dex, although isolated with the same techniques, were heterogeneous in size. DCs isolated from different species also lead to discrepancies in the size range and composition of the exosomes. For example, exosomes derived from mouse BM-DCs were shown to be more heterogeneous than the human cell derived counterpart.

Tian Chen and colleagues developed a high scale method to produce exosomes therefore bypassing costs per new batch of human embryonic stem cells (ESC)-derived MSC. They transfected human ESC-MSCs with a lentivirus carrying a MYC gene and subsequently studied their characteristics such as MYC transgene integration, protein levels, surface markers, differentiation potential among others. Despite MYC-transformed MSCs largely resembled the parental cells, they exhibited differences regarding their differentiation potential, plastic adherence, growth rate. However, exosomes, isolated through HPLC fractionation continued to exert therapeutic effect, highlighting the potential of MYC transformation to be used as a practical strategy to ensure an infinite supply of cells for the production of therapeutically efficacious exosomes. In addition, the increased proliferative rate also reduced the time and

costs associated with this production (75).

Recalling the aforementioned study of Stickney et al, engineered HEK293 was able to continuously produce, secret and uptake displayed exosomes with minimal effects on normal cell biology, functioning as cellular factories for permanently production of surface displayed exosomes (70). Recently, Wonju Jo and colleagues also proposed a new method that consists in the extrusion of cells through a hydrophilic microchannels to generate artificial nanovesicles. The lipid membrane in the fabricated nanovesicles comes from the living ESC membranes, and exhibit intact membrane proteins. The content of these nanovesicles is similar with the cytoplasmic content of the parental cell, and these molecules were shown to be successfully delivered to target cells through the plasma membrane (76).

EBV-transformed B lymphoblastoid cell lines (B-LCL) were found to produce and release $FasL^+$ $MHCII^+$ which could be used as cellular factories for producing exosomes for use in patient-customized treatments of many hyper-inflammatory conditions (77). This system was proved to be efficient at transforming human B cells of any human donor, and did not perturb their natural ability to express FasL. Furthermore, it requires little labor to maintain the cells healthy and to continue producing exosomes. They can be frozen and stored without losing their abilities to grow in cell culture. This allows a long term storage that can be readily expanded and scalling up if needed. In addition, the plethora of lines that have been transformed and properly storage could provide a repository of B-LCL that might closely match the MHC of the recipient patients. Importantly, the fact that this is a non-replicating B95-8 laboratory strain there are no active virions in the B-LCL culture and therefore the risk in transferring replicative EBV along with the exosome infusion is little or null (77). Genetically modifying the B95-8 strain can also be of use to improve the loading of cells or exosomes with the exogenous peptides. For instance, by using a lysosomal sorting sequence fused with the protein of interest the gene products could be actively targeted to the lysosomal compartment where the exosomes are assembled (77). Similarly, in order to increase the specificity for a target cell or tissue, one could engineer a recombined immunoglobulin gene to be expressed on the exosome surface. IgM and IgD have been found on the surface of B-cell derived exosomes (77).

Raw mature milk has been proposed as a biocompatible and cost effective source for harvesting bulk quantities of exosomes. Milk exosomes exhibited cross species tolerance with no adverse immune and inflammatory response (12).Indeed, it was recently found in exosomes and milk fat globule membrane, the butyrophilin, a protein that shares cross-reactive epitopes with MOG. Transdermal delivery of bovine milk vesicles, either through topical administration

or by using epicutaneous administration techniques, could be considered as a strategy to induce MOG-specific tolerance (78). Oral delivery of bovine milk derived extracellular vesicles were also shown to delay the onset of arthritis and reduced cartilage pathology and bone marrow inflammation in IL-1Ra-deficient mice and collagen-induced arthritis (79).

Many researchers have successfully packaged a cargo of interest within the exosomes using a variety of methods raging from genetic modification of the parental cell to overexpress a specific gene product, to non-genetic methods by simply incubating or mixing the isolated exosomes with the chosen cargo. Transfection with cationic lipids or electroporation have also been used. However, these techniques might disturb exosomes or cargo stability.

Despite the therapeutic potential of exosomes, their biodistribution has been less explored. Questions still remain regarding the route, dose, source, timing, and duration of treatments with exosomes. Recently, biodistribution of EVs was carried to answer some of these questions. HEK293T EVs labelled with dialkylcarbocyanine membrane dye (DiR), a near-infrared dye, were analyzed to assess their distribution in mice. DiR-labelled EVs experiments revealed that EVs generally distribute to organs of the MPS with highest accumulation in liver, followed by spleen, gastrointestinal(GI)-tract, and lungs. Tissue fluorescence exhibited a dose-dependent response, however the relative distribution among organs shifted. Relative liver accumulation decreased which might be due to a low signal or the result of a saturation of the MPS. When injected intravenously, many compounds were shown to be taken up by patrolling macrophages. This process is known to be size dependent and thus, nanoparticles are naturally less prone to opsonization. This means that they can easily penetrate the fenestrations in the hepatic sinusoidal endothelium, increasing their uptake and accumulation. Similarly in spleen, they can avoid the filtration process at the interendothelial slits and spill out due to the discontinuous endothelium (80). Notwithstanding, PS expressing EVs, a membrane protein that undergoes externalization during the EV formation, have a short-life of less than 10 minutes (81). This result corroborates with the previous report in which incorporation of as little as 3% in the outer layer of intact cells results in a signal for triggering in vivo recognition and concomitant elimination from the circulation (82). These findings suggested a role for PS in extracellular vesicle phagocytosis. Indeed, incubation of macrophages with anti-Tim4, a PS-receptor involved in the engulfment of apoptotic cells, reduced the uptake of PS-expressing exosomes by macrophages (81). Despite this well recognized mechanism of PS-dependent phagocytosis, other pathways are likely to contribute to the clearance of exosomes. So far, the wide range of phagocyte receptors that has been defined, contrasts with the limited number of apoptotic cell-surface molecules that are known. Hence, it is highly needed to investigate

other molecular mechanisms that underlie phagocyte recognition and clearance in order to better understand the biological fate of exosomes.

Regarding the influence of different injection routes in the distribution patterns of exosomes, it was observed that, compared with intravenous injections, intraperitoneal and subcutaneous lead to lower EV accumulation in liver but higher in spleen and GI. Nevertheless, intraperitoneal injections exhibited higher total tissue fluorescence.

Cell source also seems to have a huge impact in biodistribution of EVs since exosomes secreted from muscle cells, melanoma and primary immature BM-derived DCs exhibited different accumulation patterns. Generally EVs exhibited a similar mode size of 100 nm apart from the MSC-EVs which size were homogeneous and the mode size 60 nm. C2C12-derived EVs displayed greater liver accumulation in liver, while DC preferentially accumulated in spleen and B16F10-EVs were easily found in GI-tract. Potential cross-species differencies were excluded by means of a xenotransplant of EVs from rat cells (oligodendrocytes) and from cells of human origin (HEK293T and primary human MSCs). The xenotransplanted EVs have shown the same distribution trend as mouse-derived EVs. In these experiments, MSC-EVs highly accumulated in liver compared with the other two types. Thus, differences in EV distribution and natural tropism should be taken into account when choosing the most appropriate cell source. Unexpectedly, species origin did not seem to affect the distribution (80).

If the development of therapeutic strategies that avoid the administration of cells could be foreseen, circumventing part of the safety issues related to the use of living cells, such as the risk of transformation of the cells, more systematic comparisons to assess how the immunomodulatory effects of exosomes approximate to their parental cell are needed. Recently, a systematic comparison of in vitro immunomodulatory effects of BM-derived MSCs with the sMVs generated from BM-derived MSCs had drawn some useful conclusions about the use of MVs as surrogates of MCS in cellular therapy approaches (83). In this regard, it is necessary to do more in vivo comparisons in order to define optimal conditions to generate exosomes with the required phenotype (84). Despite the use of purified exosome preparations, the heterogeneity of this population should not be ignored. This leads to problems with identification, isolation, and characterization of the exact subset responsible for the therapeutic effect. Thus, a better understanding of the complexity of these population, and the full appreciation of each exosomal components would certainly help to better characterize, isolate, and maybe determine the rational design of effective exosome-based or exosome-mimicking drug carriers (5). In this regard, high-resolution flow cytometry, Immunocapture-based methods, Laser Tweezers Raman Spectroscopy, Atomic Force Microscopy, Electron Imaging Methods, nanoparticle

tracking analysis, and tunable resistive pulse sensing might help to better characterize and sort exosomes subsets (5, 19). Moreover, a deeper understanding of each exosomal component is also needed to characterize how exosomes exert their therapeutic effect (5). Hence, further investigations are needed to standardize exosomes production and evaluation of their quality control, bacteriological testing, oncogenic potential, and viability.

11 Conclusion

Although the field of exosome-based therapeutics is in its infancy, the ability to engineer exosomes to display specific ligands for cell targeting and their subsequently uptake, incorporate specific nucleic acids and protein cargoes, load therapeutic agents, and bee well tolerated in vivo has been demonstrated to some extent. These works are the first steps in proof-of-concept that show the feasibility of using exosomes, not only in cancer and infectious diseases, but also in autoimmune and chronic inflammation diseases. Because they are not toxic and can easily cross biological barriers, exosomes can easily deliver regulatory molecules to target cells. This feature is likely to be useful for the adjunct use of exosomes with immunomodulatory therapies owing to their regenerative potential.

Currently, several vaccines composed by outer membrane vesicles, which are secreted by bacteria, are in the market (the most recent one, Bexsero®, developed by Novartis). Moreover, several clinical trials are underway and the treatment of a range of diseases has been considered as potentially profiting from exosomes therapy.

However, progress in this area has been hampered by the fact that little is known about the biology of exosomes. More studies are needed to address the differences between exosomes that are secreted by different cell types, the composition pattern of these vesicles, and their biological fate. In addition, challenges such as developing high quantities of exosomes, finding efficient methods to package exosomes with cargoes of interest, and finally guaranteeing that all the steps of exosomes' manufacturing comply with quality guidelines, need to be overcome. Overall, the purpose of this thesis was to highlight and support the need for more research in exosomes with a focus on therapeutics.

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