Journal of Advanced Research 31 (2021) 61–74

Contents lists available at ScienceDirect

Journal of Advanced Research

journal homepage: www.elsevier.com/locate/jare

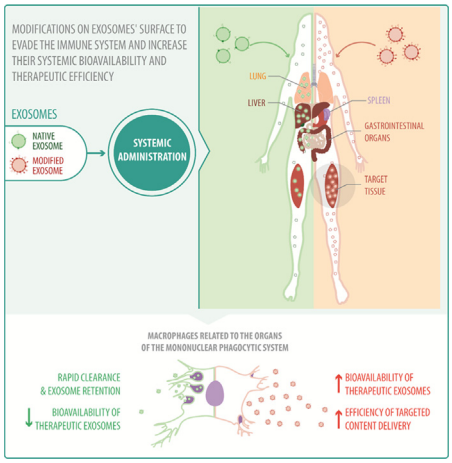
Review

Camouflage strategies for therapeutic exosomes evasion from phagocytosis

Nicol Parada [a,b], Alfonso Romero-Trujillo [a,b], Nicolás Georges [a,b], Francisca Alcayaga-Miranda [a,b,c,d,*]

[a] School of Medicine, Faculty of Medicine, Universidad de los Andes, Santiago, Chile
[b] Laboratory of Nano-Regenerative Medicine, Centro de Investigación e Innovación Biomédica (CIIB), Faculty of Medicine, Universidad de los Andes, Santiago, Chile
[c] Cells for Cells, Santiago, Chile
[d] Consorcio Regenero, Chilean Consortium for Regenerative Medicine, Santiago, Chile

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 September 2020
Revised 29 December 2020
Accepted 1 January 2021
Available online 8 January 2021

Keywords:
Exosomes
Small extracellular vesicles
Biodistribution and pharmacokinetics
Evasion immune system
Evasion phagocytosis
Drug delivery

ABSTRACT

Background: Even though exosome-based therapy has been shown to be able to control the progression of different pathologies, the data revealed by pharmacokinetic studies warn of the low residence time of exogenous exosomes in circulation that can hinder the clinical translation of therapeutic exosomes. The macrophages related to the organs of the mononuclear phagocytic system are responsible primarily for the rapid clearance and retention of exosomes, which strongly limits the amount of exosomal particles available to reach the target tissue, accumulate in it and release with high efficiency its therapeutic cargo in acceptor target cells to exert the desired biological effect.

Aim of review: Endowing exosomes with surface modifications to evade the immune system is a plausible strategy to contribute to the suppression of exosomal clearance and increase the efficiency of their targeted content delivery. Here, we summarize the current evidence about the mechanisms underlying the recognition and sequestration of therapeutic exosomes by phagocytic cells. Also, we propose different strategies to generate 'invisible' exosomes for the immune system, through the incorporation of different anti-phagocytic molecules on the exosomes' surface that allow increasing the circulating half-life of therapeutic exosomes with the purpose to increase their bioavailability to reach the target tissue, transfer their therapeutic molecular cargo and improve their efficacy profile.

Peer review under responsibility of Cairo University.

* Corresponding author.
E-mail address: falcayaga@uandes.cl (F. Alcayaga-Miranda).

https://doi.org/10.1016/j.jare.2021.01.001

Key scientific concepts of review: Macrophage-mediated phagocytosis are the main responsible behind the short half-life in circulation of systemically injected exosomes, hindering their therapeutic effect. Exosomes 'Camouflage Cloak' strategy using antiphagocytic molecules can contribute to the inhibition of exosomal clearance, hence, increasing the on-target effect. Some candidate molecules that could exert an antiphagocytic role are CD47, CD24, CD44, CD31, β2M, PD-L1, App1, and DHMEQ. Pre- and post-isolation methods for exosome engineering are compatible with the loading of therapeutic cargo and the expression of antiphagocytic surface molecules.

Introduction

Exosomes are nanometer-sized membrane vesicles released to the extracellular milieu after fusion of multivesicular endosomes with the cell membrane [1]. Released exosomes can either be taken up by neighboring or distant cells when they travel *via* body fluids and thus modulate their biological function [2]. Lipids, proteins, nucleic acids, amino acids and metabolites are the main components of the exosome cargo whose profile content largely depends on the function and metabolic state of their parent cell, since the exosomal cargo in part mimics the contents of their cell of origin [3]. Even though in some cases unmodified (or native) exosomes have consistently proven to be useful therapeutic tools in animal models [4–7] the focus today is on using these nanovesicles as a delivery system for a controlled artificial cargo utilizing specific proteins or RNA molecules as siRNA or miRNA [8]. Due to their unique lipid and protein membrane composition, exosomes are efficiently internalized by acceptor cells with relatively few undesirable immune reactions [5], which has been confirmed in different *in vivo* and clinical studies concluding that exosomes injections are well tolerated even at repeated doses [10–12]. In addition, there is evidence that exosomes can interact with specific target cells depending on their composition and origin [13–15] and release with high efficiency its cargo in acceptor target cells to exert phenotypic changes [16]. These properties have earned exosomes the reputation of being "natural carriers" for the distribution of their endogenous or artificially loaded molecular cargo. However, the rapid clearance rate of circulating exogenous exosomes after systemic injection show no differences with respect to liposomes or liposomes synthesized from lipid extracts of exosomes, indicating that the protein and lipid composition of the exosomal membrane does not favor or promote the permanence of the exosomes in circulation [16]. According to the literature, the blood concentration–time profiles revealed by pharmacokinetic analyses show that circulating exogenous exosomes quickly disappear in a half-time of ~2–30 min, mainly taken up by macrophages associated with the organs of the mononuclear phagocyte systems (liver, spleen and lung), regardless of the parent cell from which these exosomes are derived [9,18–21]. It is evident that these findings raise concern about the translational perspective of exosomes, at least in pathologies that require their systemic administration, such as metastatic cancer. This retention of exogenous exosomes in organs is clearly an important issue to consider since it limits the total number of exosomes reaching the target tissue, as was corroborated by Smyth et al. [17]. In their research work, the rapid clearance of exogenous exosomes administered intravenously limits their accumulation in tumor tissue, as has also been previously described with liposomal formulations [17]. Due to these nonspecific interactions with non-target cells, the development of strategies that promote an immune evasion to increase a longer circulation time of therapeutic exosomes could increase their chances of reaching the target tissue, being internalized by acceptor target cells and transferring the molecular message encoded in the exosomal cargo and finally increasing their therapeutic efficacy (see Fig. 1).

A good example of the strategy mentioned above is the research carried out by Karmerkar et al. [22] which demonstrated the regulation of exosome interaction with macrophages through the incorporation of the CD47 protein on the exosomes surface, a molecule that contains an immunoglobulin-like domain that serves as a "don't eat me" signal. This modification allows phagocytosis evasion by monocytes circulating, increasing the half-life of exosomes in the circulation which consequently results in a more efficient targeting of these exosomes to cancer cells to deliver their therapeutic cargo. Certainly, modifying exosomes to give them the ability to keep them out of sight from the immune system is not an easy task. A possible approximation could be to mimic the mechanisms used by cancer cells to hide from the immune system, through the use of surface molecules that activate the´don't eat mé signals, such as CD47, PD-L1, CD31 and CD24 [22–26].

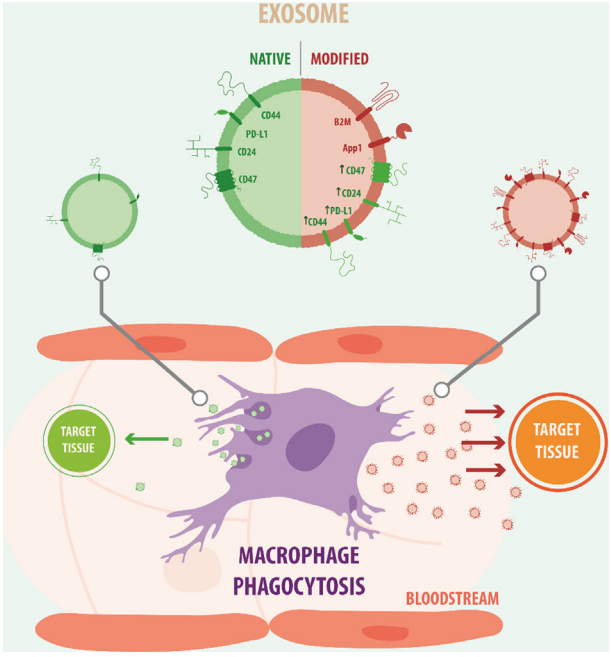


Fig. 1. Interaction of exosomes with macrophages. Once administered systemically, pharmacokinetic studies have shown that exogenous exosomes remain in circulation for ~2–30 min. Macrophages are primarily responsible for the rapid clearance of exosomes from the bloodstream, which drastically limits the amount of exosomal particles that are available to reach the target tissue and exert their therapeutic effect. The integration of anti-phagocytic molecules in the surface membrane of exosomes, such as CD47, CD24, CD31, CD44, PD-L1, β2M, App1 and DHMQ, would allow the evasion of phagocytosis and consequently a longer residence time of the modified exosomes in circulation, which would ultimately induce a greater systemic bioavailability of these modified exosomes with the ability to reach the target tissue and concentrate on it in a shorter time.

In this review, we summarize and discuss current evidence about the relevant role of phagocytic cells in the pharmacokinetics of therapeutic exosomes, the mechanisms involved in their recognition by immune cells, and consequently how they are distributed in the body according to the cellular source from which exosomes derived or the route of administration used. Also, we explore different candidate molecules for an adjusted "camouflage cloak" capable to protects therapeutics exosomes from macrophage-mediated phagocytosis. This will allow to extend the half-life in circulation of exogenous exosomes while shortening the time necessary to achieve the therapeutic concentration in targeted tissues. These improvements will result in increasing the safety and efficacy of exosome by limiting their adverse effect. Finally, we address the different methodologies that currently exist that allow modifying the original structure of the surface membrane of exosomes with the different anti-phagocytic molecules, to provide them with the ability to evade the innate immune system. These different strategies are analyzed in their methodological aspects and in the experimental results described with exosomes in the literature.

Exosomes interaction with innate immune system and their relationship with the clearance

In phagocytic cells, more specifically macrophages, exosomes internalization occurs mainly in an energy-dependent pathway [27,28], *via* phagocytosis and is dependent on the actin cytoskeleton (not on pathways involving caveolae, macropinocytosis or clathrin-coated vesicles as occurs in other non-phagocytic cells) and PI3-kinase (PI3K)-dependent phagocytic pathway [29]. Once phagocytosed, exosomes move to phagosomes and further sorted into phagolysosomes [29]. Although the active role of macrophages in the blood clearance of exogenous exosomes injected intravenously has been demonstrated in different studies, the uptake of exosomes is affected by the differentiation status of myeloid cells [30]. According to the *in vitro* data published by Czernek et al. [30], macrophages and mature dendritic cells are more efficient at internalizing exosomes than monocytes or immature dendritic cells. However, this efficiency of macrophages in capturing exosomes does not appear to be affected by the cellular origin from which they are derived [19,21,31,32], or from the type of exosomes subpopulation [21], or from the isolation method used [20]. It has been shown that macrophages play a key role in the hepatic and splenic uptake of exosomes derived from B16-BL6 murine melanoma cells [19,28,31], C2C12 murine myoblast cells [19], NIH3T3 murine fibroblasts cells [19], MAEC murine aortic endothelial cells [19], RAW264.7 murine macrophage-like cells [19] and mouse dendritic cells [33]. Also, macrophages are involved in the uptake of MDA-MB-231 breast cancer cells derived exosomes in the lung and brain after intravenous injection [32]. Studies in macrophages-depleted mice corroborate the key role of these immune cells in the clearance of exosomes. Using liposomes containing clodronate to induce irreversible functional damage and apoptosis in macrophages [34], exosomes clearance from the systemic circulation showed a significant delay, raising its circulating half-life from minutes to hours [21,31].

Macrophages take exosomes out of circulation through the recognition of the negative charge of phosphatidylserine (PS) enriched in their surface membrane [28,35] just as they recognize apoptotic cells [36]. The enrichment of PS in exosomal membrane has been reported either in exosomes derived from cell culture [28,31] or blood [21,37,38]. In a series of experiments, Matsumoto et al. [28] investigated PS involvement in the recognition and uptake of exosomes by macrophages. Using annexin V (AnV), a PS-binding protein, the authors confirmed the existence of PS on the surface of exosomes. Furthermore, following incubation with AnV they observed a great reduction in the uptake of PKH67-labeled exosomes by macrophages. In additional experiments, the authors observed that negatively charged PS- or phosphatidylglycerol-loaded liposomes suppressed the cellular uptake of PKH67-labeled exosomes by macrophages, whereas phosphatidylcholine-containing liposome did not affect the uptake. Subsequently, in an *in vivo* analysis, the blood clearance of Gaussia luciferase-labeled exosomes after intravenous injection into mice was significantly delayed by the preinjection of PS- or phosphatidylglycerol-containing liposomes, confirming that the negative charge of PS is involved in the recognition and clearance of intravenously injected exogenous exosomes by macrophages. Interestingly, this pre-injection strategy of PS-containing liposomes allowed a less *in vivo* accumulation of exosomes in the liver.

Scavenger receptor class A family (SR-A) expressed on macrophages, vascular smooth muscle and endothelial tissues also recognize the negative charge of molecules [39]. Watson et al. [40] identified SR-A as a major receptor for the clearance of exosomes by monocyte/macrophage. In a breast cancer murine model, the authors evaluated the implication of SR-A in the exosomes clearance through blocking this receptor using dextran sulfate. After the systemic injection of HEK293 cell-derived exosomes, a decrease in liver clearance was observed which ultimately resulted in a good strategy to achieve a greater accumulation of exosomes in breast tumor tissue. The presence of Galectin-5, a galactose-specific lectins, on the exosome surface derived from rat reticulocyte has also been involved in modulating the exosome uptake by macrophages [27]. Barres et al. [27] demonstrated a decrease of exosomes' phagocytosis by adding galectin-5 possibly through masking β-galactosides on the surface of exosomes. Also, because the uptake of PKH67-labeled exosomes by macrophages was markedly decreased in the presence of EDTA, the authors conclude that the binding of exosomes to macrophages could be mediated by their C-type lectin receptor. Other lectins have also been involved in exosome uptake by dendritic cells [41].

Sialic acid enriched on B cell-derived exosomes has also been involved in the recognition and uptake by lymphoid and spleen tissue resident CD169+ macrophages *in vivo* [42]. As was described by Saunderson et al. [43], after intravenous injection of B cell-derived exosomes expressing α2,3-linked sialic acid, they are mostly internalized by CD169+ macrophages in the marginal zone of the spleen and in the subcapsular sinus of the lymph node in wild type mice. However, in $CD169^{-/-}$ mice, exosome access to the lymphoid system is dysregulated, resulting in aberrant trafficking of exosomes into the splenic red pulp or lymph node cortex, suggesting that the sialoadhesin CD169 (Siglec-1) is required for the capture of B cell-derived exosomes *via* their surface-expressed α2,3-linked sialic acid.

The activation of the complement system also show an important role in exosome clearance and biodistribution [17], such as has also been widely reported to liposomal formulation [44–46]. The involvement of the complement system in the exosomes clearance was first reported by Smith et al. [17], who in a study of biodistribution and clearance of 4 T1 exosomes utilizing mice with impaired adaptive or innate immune system, observed the significance of the innate immune system along with the complement protein C5 on exosomes' rate of clearance. In others studies, exosomes have been shown to bind complement proteins *in vitro* and *in vivo* [47,48].

It is important to note that the pharmacokinetic parameters related to the clearance of exogenous exosomes and their biodistribution in the body have been determined in immunocompetent murine models. Undoubtedly, these data must be confirmed in experimental settings closer to the human, evaluating – for example – in a humanized murine model reconstituted with a human immune system, the circulating half-life of exogenous exosomes and their pattern of biodistribution in the body.

Table 1
Comparative summary of the biodistribution of systemically administered exogenous exosomes.

Type of sEV	Cell source	Labeling method	Dose	Strain of mouse	Route	Time of detection [Tissue distribution]	References
Exosomes	4 T1	DiR 0.5%	60 μg protein/mouse	BALB/c	IV	2 h Liver > Spleen > Lungs	[17]
Exosomes	PC3	DiR 0.5%	60 μg protein/mouse	BALB/c	IV	24 h Liver > Spleen > Kidneys	[17]
Exosomes	MCF-7	DiR 0.5%	60 μg protein/mouse	BALB/c	IV	24 h Spleen > Liver > Kidney	[17]
Exosomes	B16-BL6	gLuc-lactadherin	5 μg protein/mouse	BALB/c	IV	4 h Lung > Spleen > Liver > Kidney	[18]
Exosomes	B16-BL6	SAV-LA-coupled/ 125-labeled	4 μg protein/mouse	BALB/c	IV	4 h Liver > Spleen > Lung	[49]
sEV > 200 nm	HEK293T, C2C12, B16-F10, and BMDCs	1 μM DiR	1×10^{10} part/gram mouse	C57BL/6	IV	24 h Liver > Spleen > GI tract > Lungs	[14]
sEV > 200 nm	HEK293T	1 μM DiR	1×10^{10} part/gram mouse	C57BL/6	IP	24 h Liver > Spleen > Pancreas > GI tract	[14]
sEV > 200 nm	HEK293T	1 μM DiR	1×10^{10} part/gram mouse	C57BL/6	SC	24 h Liver > Spleen > Pancreas > GI tract	[14]
Exosomes	MSC	1 μM DilC18	8×10^{9} part/mouse	C57BL/6	IP	6 h Pancreas > Liver > Spleen > Lung 24 h Liver > Spleen > Stomach > Lung > Ovary > Bowel48 h Liver > Stomach	[53]
Exosomes	HCT116 and HT29	CD63Nluc/ mCherry	Continuous dose	BALB/c -nu/ nu	SC	7 weeks Stomach > GI tract	[136]

Abbreviations: sEV, small extracellular vesicles; part, particles; h, hour; IV, Intravenous; SC, Subcutaneous; IP, Intraperitoneal; MSC, mesenchymal stem cells; BMDCs, Dendritic cells derived from bone marrow; GI tract, Gastrointestinal tract.

Current bioavailability problems

Short-half-life and accumulation in organs

From the exosomes' pharmacokinetic profile reported in the literature, exogenous exosomes administered intravenously have a circulating half-life of ~2–30 min [11], variations that can be attributed to the labeling methods for the *in vivo* tracking and the sensitivity and quantitative capacity of each method used. After systemic administration, exogenous exosomes are mainly distributed or accumulated in the liver, followed by spleen, lungs and gastrointestinal tract [14,17,49], uptake attributed to macrophages resident in these organs. Table 1 summarizes the different studies that have been published on the biodistribution of exogenous exosomes administered systemically.

Findings reported by Yang et al. [11] demonstrate that there is a logarithmic correlation between time and fluorescence after intravenous injection of PKH26-labeled exosomes in a mouse glioma model. After ~30 min, there was an approximately 200-fold reduction in fluorescence intensity, which represents practically the total number of exosomes administered. Corroborating the above, Lai et al. [50] also performed *in vivo* imaging of GlucB-labeled HEK293T exosomes, reporting a similar half-life time of ~30 min with a predominant accumulation in the spleen followed by the liver. However, various studies indicated an even shorter half-life, reaching times of just ~2 min in circulation. Thus, Takahashi et al. [18] determined that the exosome blood concentration–time profile of B16-BL6 exosomes traced with gLuc-lactadherin disappeared from the blood circulation in approximately ~2 min with a reduction of almost 95% of the dose at 5 min and with little luciferase activity detected in the serum at 4 h after intravenous injection. Also, the authors reported that sequential *in vivo* imaging showed that the B16-BL6 exosome-derived signals distributed mainly in the liver and then in the lungs. Morishita et al. [49] also ratified these data in a subsequent study. Importantly, pharmacokinetic evaluation of exosomes from splenocytes and 4 different types of mouse cell lines: C2C12 murine myoblast cells, NIH3T3 murine fibroblasts cells, murine aortic endothelial cells (MAEC) and RAW264.7 murine macrophage-like cells also showed a very short half-life of ~2–4 min, mainly accumulated in the liver and spleen, after intravenous injection into wild type mice [19,43].

Due to this very short half-time in circulation minimal amounts of exosomes have been detected in organs or tissues other than those associated with the mononuclear phagocytic system. Despite the above, various research works have reported that unmodified exosomes manage to reach the tumor tissue in a variable percentage depending on the source of the parent cell, the tumor type and its anatomical location, and the exosome's labeling technique used. The studies aiming at tumor targeting have shown that after a systemic administration of exosomes (in absence of targeting ligands) are able to reach the tumor tissue and accumulate in it [14,40,50]. For example, in a melanoma murine model, it has been described that after 24 h of systemically injected HEK293T DIR-labelled exosomes, ~3% of the total tissue fluorescence was detected in the tumor without affecting the classical biodistribution pattern of exosomes [14]. Interestingly, in the study performed by Watson et al., a ~3-fold enhanced accumulation of labeled exosomes reached a breast cancer cell tumor after the blockade of monocyte/macrophage uptake, which confirms the relevance of the innate immune system for exosome clearance [40]. In addition, it has been demonstrated that exosomes may cross the blood–brain barrier. Zhuang et al. [51], showed brain accumulation after intranasal administration of exosomes encapsulated drug, which shows a peak of accumulation at 3 h and remains traceable up to 24 h at the olfactory bulb. In this study, the authors stated that particle size is a critical factor for translocation from the nasal region to the rest of the brain. To reach specifically the brain tissue by systemic administration it requires certain exosomal membrane factors, as shown by Hoshino et al. [15]. In this work, the authors report that exosomes with an ITGß3 abundance in their surface reach a 4-fold increase accumulation at the brain compared to those exosomes lacking ITGß3. Rabies virus glycoprotein (RVG) fused on the exosomes membrane has also been used to target the brain [14,52] and achieve a significant increase in brain accumulation measured by fluorescence [14]. Also, Yang et al. demonstrated the capability of modified exosomes to cross the blood–brain barrier and to target specific tumor tissue through the modification of parental cell with a construct that enabled fusing a glioblastoma targeting peptide to the N-terminus of CD47 [11].

Factors influencing the biodistribution pattern

Routes of systemic administration

Systemic administration of exosomes can be injected mainly for three different routes: subcutaneous (SC), intraperitoneal (IP), or intravenous (IV). Each systemic administration route possesses a specific biodistribution pattern, which must be considered in the design of preclinical studies in order to enhance the exosomes' arrival to the target tissue. Wiklander et al. [14] performed a comparative analysis injecting the same amount of HEK293T exosomes in C57BL/6 mice. As expected, the IV-administration showed a traditional pattern of biodistribution with higher accumulation in the liver and spleen in comparison to SC or IP after 24 h. In contrast, SC and IP injections resulted in lower accumulation of exosomes in liver and spleen whereas showed a higher accumulation in gastro-intestinal tract and pancreas. These data were corroborated in the study conducted by Mendt et al. [53], in which the administration of exosomes derived from MSC *via* IP or IV injections followed a similar pattern of distribution.

Exosomes' origin

Over the last years, a growing body of literature has shown that regardless of the origin, exosomes accumulate primarily in the liver, spleen, lung, and gastro-intestinal tract after intravenous injection [11,18,49,50]. However, the cell-type specific integrins arranged in the surface certainly determines how they accumulate in these organs [15]. Thus, DiR-labeled exosomes derived from C2C12 murine myoblast cells and bone marrow-derived dendritic cells have shown a highest accumulation in the spleen and liver, respectively [14]; whereas DiR-labeled exosomes secreted by B16F10 murine melanoma cells largely accumulate in the lung [14]. In a context of cancer metastasis, the exosomal integrin's profile strongly impacts in the pharmacokinetics of exosomes, determining their organotrophic behavior since there exist an intrinsic relation between integrins and the organotropism [15,54]. Hoshino et al. [15] demonstrated that exosomes secreted by lung, liver and brain tropic tumor cells fuse preferentially with resident cells at their predicted destination, namely lung fibroblasts and epithelial cells, liver Kupffer cells and brain endothelial cells.

Approaches for extending exosomes' half-life

As previously mentioned, phagocytes, the removal specialists of the immune system, represent the main physical barrier to systemic administration of exogenous exosomes for therapeutic purposes. Since macrophages are primarily responsible of exosome clearance in the circulation, modifications to allow them to escape from scavenger macrophages through, for example, activating the "don't eat me" signal seems to be a plausible strategy for their clinical translation pathway. In this section, we present different molecules that could be inserted or expressed on the surface of exosomes with the intention to provide a longer circulation time, and reducing the time necessary to reach the concentration level required to induce the desired biological effect on the target tissue / organ, while reducing significantly the off-target interactions. Several of the molecules selected and proposed here were inspired from mechanisms used by tumor cells to evade the immune system.

CD47

CD47, first known as integrin-associated protein (IAP), is a cell surface protein of the immunoglobulin superfamily which is heavily glycosylated and expressed in most cells in the body [55]. CD47 is a protein of 50 kDa that interacts with the immune inhibitory receptor SIRPα, predominantly expressed in neurons, dendritic cells and macrophages [56]. The CD47-SIRPα axis is a critical molecular interaction that inhibits the activation of macrophages and other myeloid cells, acting as a myeloid-specific immune checkpoint and activating the 'don't eat me' signal [26]; disruption of this 'don't eat me' system results in phagoptosis of viable cells [57]. The inhibition of phagocytosis by CD47-SIRPα interaction occurs after tyrosine phosphatase activation and inhibition of myosin accumulation at the submembrane assembly site of the phagocytic synapse which reduce macrophage-target cell interaction [58].

As reviewed by Brown and Neher [57], most human cancers overexpress CD47 and transduce inhibitory signals through SIRPα on myeloid cells as a survival mechanism to "defend" against the attack of the immune system. In tumor tissue, CD47 expression level correlates with tumorigenicity in mice and mortality in humans [59,60]. In the work by Majeti et al. [61], the overexpression of CD47 on human leukemia cell line contributes to pathogenesis by inhibiting phagocytosis of these cells through the CD47-SIRPα interaction, while the disruption of those interaction with a monoclonal antibody directed against CD47 induce phagocytosis of leukemic cells by macrophages. In several preclinical models, immunotherapy based on anti-CD47 to block CD47-SIRPα axis stimulates phagocytosis of cancer cells *in vitro*, which results in an anti-tumoral effect *in vivo* [26].

CD47 expression has been detected in various subtypes of unmodified exosomes, including human foreskin fibroblasts, human mesenchymal stem cells (MSC), jurkat T cells and platelets [22,62–65]. In the same way that it protects tumor cells from macrophage-mediated phagocytosis, the presence of CD47 on the surface of the exosome is shown to contribute to the suppression of their clearance in the bloodstream and enhance the efficiency of targeted content delivery [11,22]. The group led by Raghu Kalluri generated $CD47^{high}$ exosomes with Alexa Fluor 647 (AF647)-labeled short interfering RNA (siRNA) which showed an enhanced retention in the circulation and better accumulation in the liver, lung and pancreas than liposomes after IP administration [22]. Exosomes derived from CD47 knockout (k/o) cells showed less retention, corroborating that the presence of CD47 in the exosomal membrane inhibits their phagocytosis by circulating SIRPα+CD11+ monocytes. As expected, when the authors disrupt the CD47-SIRPα axis using antibody anti-CD47, they observed a significant increase of AF647+CD11b+ monocytes in the circulation. Interestingly, CD47 k/o mice showed lower levels of circulating exosomes compared to age-matched controls. In addition, when authors injected these $CD47^{high}$ exosomes a noticeable reduction in circulating AF647+CD11b+ monocytes were observed, suggesting that the presence of CD47 on exosomes allows an evasion from phagocytosis by the circulating monocytes and increases exosomes half-life in the circulation, which consequently induces a greater accumulation in target tumor tissue.

Corroborating the previous findings with the research work recently carried out by Yang et al. [11] and Belhadj et al. [66], it was also shown that the expression of the CD47 protein on the surface of exosomes endows them with the ability to evade phagocytosis and consequently to increase their circulating half-life. Yang et al. [11] reported that CD47 overexpression in exosomes extended the half-life by 3-fold in the bloodstream, with no obvious *in vivo* toxicity or immunogenicity in mice at the different dosages and time points tested. Likewise, Belhadj et al. [66] observed that the incorporation of CD47 in the surface membrane of exosomes reduce the endocytosis by macrophages, enhancing the uptake by the target tumor cells, prolonging the *in vivo* circulation time, lowering the distribution to the liver and spleen, and increasing tumor accumulation.

In another experimental approach, Rodriguez et al. [67] also demonstrated that CD47 allows a delay in the macrophage-mediated clearance of nanoparticles. Using CD47 recombinant protein coupled to nanoparticles, they observed an increase in the half-life of IgG-opsonized nanoparticles in the circulation and improved the delivery of dye and cytostatic drugs to tumors *in vivo*. Likewise, Hu et al. [68] corroborated that red blood cell membrane coating functionalizes sub-100 nm particles with native CD47 giving them less susceptibility to be phagocytosed by macrophages. CD47 has also been utilized to mitigate the immune response to clinically used biomaterials to enhance the bioavailability of therapeutic agents and enhance the longevity and efficacy of medical devices [69].

CD31/PECAM-1

CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is another candidate for the 'don't eat me' signal. CD31 is a 130 kDa transmembrane glycoprotein member of the immunoglobulin superfamily known as an endothelial marker and expressed in endothelial, hematopoietic and immune cells [70]. CD31 is mainly associated with vascular biology, participating in important processes as cell-to-cell adhesion, transmigration of monocytes and inflammation [70,71]. This molecule is composed of 3 structures, six extracellular domains, an intramembrane domain and a cytoplasmic tail that have two immunoreceptor tyrosine-based inhibition motifs (ITIMs) that have multiple ligand, like SHP-1 and -2, SH2 domain containing inositol phosphatase 2, PI3K, and phospholipase C-ɣ1 [72].

In 2002, the research group of Brown et al. [25] described for the first time that CD31 is a cell-surface molecule that prevents the phagocytosis of viable cells by transmitting detachment' signals, considered as a 'don't eat me' signal to macrophages. In a set of experiments, the authors demonstrate that, under conditions of flow, initial interaction between phagocytes and dying cells occurs through CD31, which determine whether phagocytosis of the damaged cell will be performed. In turn, in viable cells a signaling pathway activated by binding of CD31 results in the generation of a detachment signal to avoid being swallowed by macrophages. Using CD31-negative Jurkat cells, the authors demonstrated the inability of these cells to adhere to macrophages, a condition reversed with the transfection of functional CD31 with similar efficiency to parent CD31-positive Jurkat cells. Likewise, through the expression of a 'signaling disabled' CD31 mutant they observed the inhibition of the 'don't eat me' signals, suggesting that detachment requires an intracellular signaling downstream of CD31 binding.

CD31 expression has been detected in exosomes derived from endothelial cells, endothelial progenitor cells and platelets, as part of a specific characterization profile of nanovesicles isolated from this type of parent cell [73]. However, to date there have been no studies evaluating the anti-phagocytic effect of CD31 expression on the exosome or whether CD31 overexpression on the surface of the exosomal membrane can alter some parameters of the exosomes' pharmacokinetics such as the half-life time in the blood stream or their biodistribution' pattern in the body.

CD24

CD24 is a small (20 to 70 kDa), heavily glycosylated mucin-like cell surface protein expressed in a wide array of cells including B cells, dendritic cells, neural cells, epithelial cells between others [74]. Recently, CD24 has been identified as another potent 'don't eat me' signal that modulates the antitumor innate immune response [23]. Using RNA sequencing data from the TARGET and TCGA databases, Barkal et al. [23] observed CD24 upregulation in almost all tumor types analyzed relative to normal tissues, being specially overexpressed in triple-negative breast cancer and ovarian cancer. The authors demonstrated that CD24 promotes immune evasion through its interaction with the inhibitory receptor sialic-acid-binding Ig-like lectin 10 (Siglec-10) expressed by tumor-associated macrophages. The role of CD24–Siglec-10 interactions in regulating macrophage-mediated antitumor immune responses was studied in a co-culture model coupled to a flow-cytometry-based phagocytosis assay. Genetic ablation of CD24 (ΔCD24) increased the phagocytosis of breast cancer cells by Siglec-10 + M2-like macrophages; whereas Siglec-10 knockout macrophages exacerbated their phagocytic capability towards breast cancer cells. In addition, genetic ablation and therapeutic blockade of CD24 using monoclonal antibody resulted in a macrophage-dependent reduction of tumor growth *in vivo* and an increase in survival time, which reveal that CD24 – as an anti-phagocytic signal – have a therapeutic potential in cancer immunotherapy.

CD24-mediated phagocytosis inhibition occurs when binding to Siglec-10 activates an inhibitory signaling cascade mediated by SHP-1 and/or SHP-2, protein tyrosine phosphatases associated with the two ITIMs in the cytoplasmic tail of Siglec-10, which blocks Toll-like-receptor-mediated inflammation and the cytoskeletal rearrangement required for cellular engulfment by macrophages [75–77].

CD24 expression has been detected in exosomes isolated from urine and amniotic fluid of normal individuals, and it is considered together with CD9, CD63 and CD81 as universal markers of blood exosomes in healthy individuals and patients with different cancers [78–82]. However, their role in the context of exosomes clearance has not been studied yet, which opens a window of opportunities to develop a research work in which it uses as 'don't eat mé signal to generate invisible exosomes to the immune system. It is important to note that the use of this molecule in exosomes must consider that circulating monocytes show a lower expression of Siglec-10 compared to tumor-associated macrophages (TAMs) [23]. Nonetheless, this receptor could be induced with macrophage type 2 (M2) cytokines (TGF-β1, IL-10 or IL-4) *in vitro* and could be overexpress in diseases with M2-like circulation cytokines profile [23].

β2-microglobulin (β2M), component of the MHC class I

Beta-2-microglobulin (β2M) subunit of the major histocompatibility class 1 (MHC class I) complex is also another protein that uses cancer cells to protect themselves from immune cells. Recently discovered in the laboratory of Irving L. Weissman, the expression of MHC class I component β2M in cancer cells act as a critical regulator of the effector function of macrophages within the tumor microenvironment [83]. MHC class I component β2M interacts with the inhibitory receptor called leukocyte immunoglobulin-like receptor B1 (LILRB1), whose expression is upregulated on the surface of macrophages, including TAMs. The inhibition of macrophage phagocytosis activated by the MHC class I–LILRB1 signaling axis is due to the cytoplasmatic domain of LILRB1, composed of a long tail of four ITIMs. Once the tyrosine phosphorylation occurs, LILRB1 recruits SHP-1 which leads to the cascade of inhibitory signal of phagocytosis [84–86]. Disruption of either MHC class I or LILRB1 potentiate phagocytosis of tumor cells, which defines the MHC class I–LILRB1 signaling axis as an important regulator of the effector function of innate immune cells [83]. MHC class I–LILRB1 signaling has been studied in the myeloid lineage for its inhibitory role in monocyte activation [87]. As has also been mentioned in the other molecules, until today it is unknown whether the expression of MHC class I component β2M in exosomes plays a role in their clearance of the bloodstream. However, β2M subunit of the MHC class I complex seems to be a strong candidate or a complement in avoiding the

phagocytosis mediated by macrophages to improve the efficiency of targeted content delivery.

PD-L1

Cancer cells frequently overexpress the programmed cell death ligand 1 (PD-L1), which interacts with the immune checkpoint receptor called programmed cell death protein 1 (PD-1), to escape from the immune system [88,89]. First discovered in T cell, PD-1 is expressed by a variety of hematopoietic cells like B, NK, dendritic cells and macrophages [24,88,90], and is considered an immune checkpoint molecule that plays an important role in downregulating the immune system proinflammatory activity [90]. PD-L1 is expressed on hematopoietic cells including T cells, B cells, macrophages, dendritic cells, and mast cells, but also in several non-hematopoietic healthy cells including vascular endothelial cells, keratinocytes, pancreatic islet cells, astrocytes, placenta syncytiotrophoblast cells, and corneal epithelial and endothelial cells [91]. PD-1 and PD-L1 are type I transmembrane proteins from the immunoglobulin (Ig) superfamily, that have 3 domains: extracellular, transmembrane and cytoplasmatic. The difference between them are the extracellular and the cytoplasmatic domain, being an Ig-V like for PD-1 and an Ig-V and Ig-C-like for PD-L1 on the extracellular domain and for the cytoplasmatic one of PD-1 consist in two tyrosine-based signaling motifs, and for PD-L1 is a shot tail with no signaling motif [88,89]. Despite of the disruption of PD-1/PD-L1 axis using monoclonal antibodies have shown remarkable clinical efficacy in patients with a variety of cancers mainly mediated by the activation of T cells, it was not until 2017 that PD-1/PD-L1 therapy was shown that also function through a direct effect on macrophages. Gordon et al., authors of this discovery, reported that both mouse and human TAMs express PD-1, which negatively correlates with phagocytic potency against tumor cells. Likewise, the blockade of PD-1/PD-L1 increases macrophage phagocytosis, which results in a reduction of tumor growth, and lengthens survival in mouse models of cancer in a macrophage-dependent fashion [24].

As mentioned by Daassi et al., exosomes expressing PD-L1 may be produced by tumor cells, immune cells, MSC or other cells in the tumor microenvironment or outside of the tumor [92]. PD-L1-expressing exosomes have shown the property to inhibit the anti-tumor immune responses since they have immunosuppressive capabilities on T-cell activation [93]; however, the effect of exosomal PD-L1 on other immune cells as macrophages or even natural killer cells, dendritic cells, B-cells, regulatory T-cells and effector T-cells are still unknown. From the above it is inferred that the anti-phagocytic effect that could be induce by exosomes expressing PD-L1 on circulating monocytes/macrophages is not known, so PD-L1 is an interesting candidate molecule to test in its possible ability to make exosomes invisible for the immune system.

CD44

CD44 is another candidate to generate exosomes that remain in the bloodstream for longer since the use of monoclonal antibodies to block CD44 inhibit phagocytosis mediated by macrophages [94]. CD44 is a transmembrane glycoprotein, also referred to as P-glycoprotein, encoded by a single gene, ubiquitously expressed throughout the body, and with a molecular weight of 85–200 kDa [95]. CD44 is known for controlling inflammatory sites by stimulation of leukocyte extravasation and thus promoting infiltration; likewise, CD44 has been shown to have a role in the activation of cytotoxic T cells by binding to the C3bi component of complement [96]. In addition to its function in inflammatory and immune responses, CD44 also contributes to the ingestion and clearance of particles and apoptotic cells [97]. In 2005, CD44 was identified as a competent phagocytic receptor that efficiently mediates internalization of large particles [97]. The mechanism of CD44-mediated phagocytosis involves inside-out signals transmitted to complement receptor-3 (CR3, Mac1, CD11b/CD18) through the GTPase Rap1 [98]. Also, CD44 has been implicated in the host response to bacterial infection [99–101].

CD44 expression has been found in several types of cancer cell-derived exosomes, whose expression has been associated with several pro-tumorigenic properties such as invasiveness and chemoresistance [102–105]. However, it has not yet been investigated whether the expression of CD44 on the surface of tumor exosomes or those derived from other cell lines contributes or not to the residence time of them in the blood circulation.

Dehydroxymethylepoxyquinomicin (DHMEQ)

This molecule was developed by Matsumoto et al. [106] and it is a derivative from epoxyquinomicin C, a molecule previously isolated from Amycolatopsis sp. and was intended to be used as antibiotic and anti-inflammatory agent.

Matsumoto and his group demonstrated the inhibitory effect DHMEQ on NF-κB [106]. NF-κB is a transcription factor that mediates the expression of a variety of cellular genes regulating the inflammatory response and can be activated by a large spectrum of chemically diverse agents and cellular stress conditions including bacterial lipopolysaccharides (LPS), microbial and viral pathogens, cytokines and growth factors [107]. In the studies of Suzuki et al. [108], they establish a relation between macrophages and DHMEQ showing that DHMEQ inhibited LPS-induced NF-κB activation, iNOS expression, and inflammatory cytokine secretion by active macrophages due to the inhibition of NF-κB. Furthermore, DHMEQ also inhibited the phagocytosis of E. coli by RAW264.7 murine macrophage-like cells treated with LPS or IL-1β, thus being evidence for the involvement of NF-κB in the regulation of phagocytosis by use of this inhibitor [108]. There is also evidence of the use of DHMEQ in transplant to prevent early damage, this being possible by the inhibition of the high mobility group complex-1 (HMGB1), macrophages and proinflammatory cytokine secretion, resulting in engraftment of transplanted islets even with fewer islet grafts [109]. The possible clinical approach to use DMHEQ could be as a pre-treatment to systemic administration of exosomes therapeutics to downregulate the phagocytosis mediated by macrophages with the intention of inducing a longer circulating half-life of them.

App1: Proteins in fungi

The *cryptococcus*-specific protein antiphagocytic protein 1 (App1) regulates *Cryptococcus neoformans* virulence by controlling macrophage-driven fungal phagocytosis [110]. App1 is a small protein of 20 kDa produced and secreted extracellularly by the fungus, *Cryptococcus neoformans*, an environmental human pathogen causing a life-threatening meningoencephalitis [111], and found in the serum of infected patients [112]. The inhibition of the macrophage-mediated fungal phagocytosis effect occurs in a dose-dependent and complement-mediated manner [112]. As reported by Luberto et al. [112], the use of recombinant App1 inhibits attachment and ingestion of yeast cells by alveolar macrophages, while ΔApp1 mutant is readily swallowed by them. Despite App1 being considered as an anti-phagocytic factor, there is no evidence of this molecule being useful in therapeutic settings.

Modification strategies of exosome surface

Currently, there are several protocols available that allow modifying both the cargo or the surface membrane of exosomes to

functionalize them, for example for improve their therapeutic function, targeted drug delivery, half-life in circulation or their tracking system *in vitro* and/or *in vivo* to facilitate their use for nanomedicine purposes. Modifications of exosome structures to create an 'invisible cloak' on their surface can be engineered at the cellular level or alternatively can be modified post isolation. Endogenous exosomes modifications generally rely on molecular biology approaches to manipulate exosomes components at cellular level [113]. Although effective, these modification methods prior to exosomes isolation are often time-consuming and challenging especially when using primary cells like MSC or dendritic cells. Post-isolation modifications typically consist in chemical reactions and physical modifications to attach biomacromolecules or fluorophores directly to the exosome. Although these exogenous modification strategies are less time-consuming, it depends on the nature and size of the molecule to attach, and of an extensive knowledge of the structure of exosomes to know how to interact with them in a nanoscale [113]. Fig. 2 and Table 2 summarize the different existing methodologies for inserting different molecules on the surface of exosomes.

Pre-isolation modification strategies

Pre-isolation methods usually involve the transfection of the parental cell with a construct to express a membrane protein inexistent in the cell of origin, or the modification of naturally expressed one to express an interest peptide or fluorophore [11,114]. In general terms, this strategy involve exosomes-producing cells are loaded with expression vectors, as plasmid or virus, with a chimeric gene or protein which contains genes or proteins that will be part of exosomes as CD63, CD9, CD81 or LAMP2b fused with a specific protein [115]. Since these chimeric genes or proteins are expressed in parental cells, they also are expressed in exosomes due to the presence of exosomes' related protein [115]. These methods are mainly used to track exosomes *in vivo* [18,116], reach specific tissues [52] or functionalize exosomes for nanomedicine purposes.

The research work carried out by Rivoltini et al., uses parental cell transfection as a method to express on the surface of the exosome membrane a protein that does not exist in native exosomes [117]. In this work, K562 cells genetically modified for TRAIL expression efficiently release homogeneous exosomes cargoing active TRAIL capable of induce apoptosis in tumor cells *in vitro* and control cancer progression *in vivo*, without affecting the classic biodistribution pattern of exosomes administered systemically. Taking advantage of pre-existing proteins in the exosomes' surface, another widely used method makes chimeric constructs displaying fluorophores or peptides with specific purpose. An additional benefit of the chimeric exosome methods is that it could later be loaded with an exogenous molecular cargo as drugs or siRNAs. This strategy was used by Bellavia et al. for the treatment of Chronic Myeloid Leukemia (CML), disease characterized by the overexpression of the IL-3 receptor in its CML-blasts. In this work, the researchers could generate exosomes that express IL-3 by creating a fusion protein with LAMP2b, a well-characterized exosomal membrane protein. Exosomes with a fragment of IL-3-LAMP2b in their surface were subsequently loaded with Imatinib – a selective inhibitor of Bcr-Abl oncoprotein – to act as a vehicle for drug delivery to specifically target CML cells *in vivo* [118]. The fusion with LAMP2b has also been carried out to target the brain using a neuron-specific RVG peptide to deliver a specific siRNA against BACE1, a therapeutic target in Alzheimer's disease [52] or with an αv integrin-specific iRGD peptide, to target αv integrin-positive breast cancer cells [114]. However, it is important to point out that some peptides fused to the N terminus of LAMP2b might not display effectively on the exosomal surfaces [119].

Previous research has reported the fusion of fluorescent reporters such as GFP or RFP to the exosomal membrane protein CD63 on their extra and/or intracellular domains, without alter the

Table 2
Engineering methods to modify the exosomes surface.

Type of molecule to attach	Methodology suggested	Example	References
Protein (>30 kDa)	Pre-isolation PEGylation Liposome fusion	PD-L1, CD47, CD24 and CD31	[22,132,135]
Protein or peptide (<30 kDa)	Click chemistry PEGylation	β2M and App1	[126,127,134]
Organic molecule	Click chemistry	DHMEQ	[125,137]

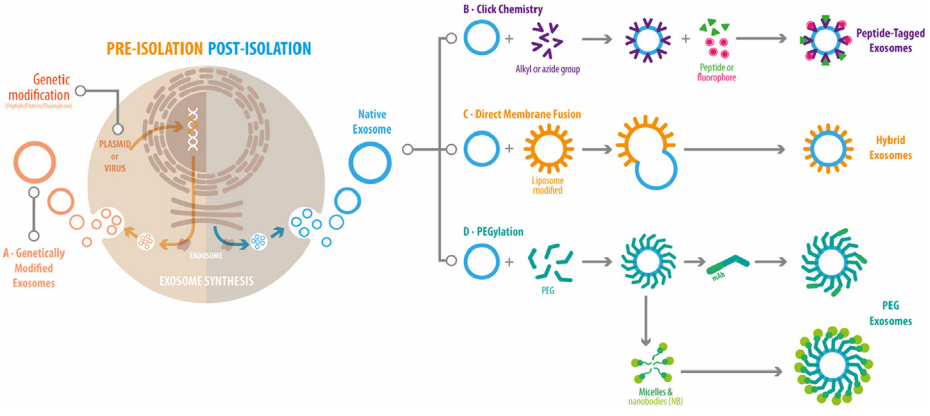


Fig. 2. Schematic diagram of the different strategies that exist to add modifications to the surface membrane of exosomes. Functionalization strategies of exosomes surface can be classified into two major approaches: pre- and post-isolation techniques. A) The pre-isolation approach usually involves genetic engineering cells to express a specific peptide, protein or fluorophore in their secreted exosomes for a specific use in nanomedicine. B-D) The post-isolation approach generally includes chemical reactions or physical modifications to add proteins or molecules directly to unmodified or native exosomes. Click chemistry method is an azide-alkyne cycloaddition that allows the incorporation through covalent bonds of different biomolecules directly to the exosomes' surface to endow them with a specific functionality (B). Direct membrane fusion between exosomes and synthetic liposomes is a method that allows modifying the properties of the exosome membrane *via* artificial functionalization of liposomes. Using freeze–thaw method or PEG, the specific modification into exosomes is directly integrated using liposomes embedded with specific peptides, proteins or antibodies (C). PEGylation is a method that use the PEG as a link to conjugate different molecules to the exosomes' surface. To functionalize these nanoparticles, exosomes can be embedded directly in PEG as a surface tagging technique to later attach the desired molecule to its surface. Also, it is possible to prepare a mix of protein- or nanobody-PEG-micelles which is subsequently incubated with the exosomes to adhere the desired molecule to them (D).

physical and biochemical characteristics typical of exosomes, in order to generate engineered exosomes for imaging or tracking [116,120]. In addition, chemiluminescence-based detection using luciferase or the radiolabeled compound iodine-125 have also been fused to the membrane-associated protein Lactadherin on exosomes as a method to obtain exosomes for molecular imaging techniques [18,49].

Altogether, pre-isolation modification methods are mainly based on different techniques of the molecular biology to introduce endogenous modifications on exosome surface; these strategies are effective for displaying genetically engineered proteins on the surface of exosomes with the limitation that cannot be applied to molecules other than the genetically encodable peptides and proteins. Although this strategy can be used for the generation of 'invisible' exosomes using some of the molecules mentioned above (such as CD47, CD44, CD31 among others), and importantly preserving the integrity and classic functionality of exosomes, this method certainly has the challenge of being able to separate during the exosomes' purification process those that were modified from the native ones.

Post-isolation modification strategies

Click chemistry

Click chemistry is a methodology that can be used to directly attach molecules to the exosome surface through covalent bonds [113], allowing the modification of the original structure of the exosomal surface through the incorporation of different agents, such as fluorescent dyes or imaging and targeting agents, that can be covalently linked in the surface of exosomes to provide them with a desired functionality [115]. Click chemistry is an azide-alkyne cycloaddition that can be done with or without copper as a catalyst, and is useful to functionalize the surface of exosomes mainly as a tool to label them for tracking and linking fluorophores, but also to attach different macromolecules like biotin or peptides without changing exosome size, internalization pattern or their properties like the capacity to cross the blood–brain barrier (BBB) [121–124]. For this methodology, the surface of the exosomes must be modified adding an alkyl or azide group to generate potential chemical active sites. The addition of terminal alkyls could be made in the amines found on exosomal proteins or in the head of the membrane lipid phosphatidylethanolamine [121], while the addition of azide are commonly performed in the culture of the parental cell adding an azide-bearing amino acid analogue to methionine (L-azidohomoalanine) or azidosugars (N-azidoacetyl-D-mannosamine) to introduce unnatural azides [122,125]. Another option is the use of a strain-promoted alkyne azide cycloaddition like dibenzylcyclooctyne (DBCO), to react with an azide linker without the use of copper as a catalyst [125]. Click chemistry is a quick and efficient method compared with traditional cross-linking reactions such as maleimide–thiol coupling and provides better control over the conjugation site [113].

Using the click chemistry technology, glioma-targeting exosomes were generated by the group led by Qiusha Tang [126]. In this study, exosomes derived from Raw274.6 murine macrophage-like cells were firstly loaded with superparamagnetic iron oxide nanoparticles and curcumin as anti-tumor agents by electroporation; subsequently, by click chemistry method exosome membrane were conjugated with neuropilin-1-targeted peptide (RGE-peptide) by a cycloaddition reaction of sulfonyl azide to obtain glioma-targeting exosomes with imaging and therapeutic functions. These engineered exosomes proved to be able to cross the BBB and efficiently reach the tumor site, besides to exert significant antitumoral effects. Importantly, the authors of this study also reported that the click chemistry method does not change the exosomes properties and allow stable modification to remain the surface of the exosome up to 4 weeks at −80 °C storage [126]. Other studies that also used the click chemistry methodology to insert modifications to the exosomes surface but with the copper-free approach using DBCO have also been shown to be effective in functionalizing exosomes [127,128]. Using this method, exosomes derived from MSC linked to a peptide with high affinity to integrin αvβ3, a protein expressed in reactive endothelial cells after ischemia, were injected systemically in a transient middle cerebral artery occlusion (MCAO) mice model [127]. The engineered exosomes successfully cross the BBB, target the lesion region of the ischemic brain and accumulate in it in a greater proportion than unmodified exosomes. In addition, when these modified exosomes are loaded with curcumin they exert a strong suppression of the inflammatory response and cellular apoptosis in the lesion region [127], and increased the animal survive and angiogenesis in the ischemic zone when loaded with miR-201 after multiples injections [128].

Altogether, click chemistry is a technique that can be used to functionalize the surface of exosomes with small molecules, large biomacromolecules, and polymers to supply them with specific skills without alter exosome size and function [121]. This methodology can be used to modify some pharmacokinetic parameters, for example, exosomes' surface may be conjugated with targeting ligands, such as antibodies and peptides, to allow specific interactions of exosomes with target cells or alternatively to evade non-target cells interaction, such as those that occur with phagocytic cells after systemic administration. In this area, this technique can be used to incorporate some of the different molecules associated with the "don't eat me" signal into the exosomal membrane (as CD24, β2M, App1), with the aim of increasing the circulating half-life of exosomes for therapeutic purposes. Click chemistry can be also used to affect exosomes biodistribution and to label exosomes with fluorescent, radioactive, and MRI agents for precise *in vivo* tracking of injected exosomes.

Direct membrane fusion

The direct membrane fusion between exosomes and synthetic liposomes is a useful methodology to modify exosomes membrane surface [129,130]. Using freeze–thaw method or polyethylene glycol (PEG), direct membrane fusion between both nanoparticles allow modification in exosomes through the artificial functionalization of liposomes modifying their type and proportions of phospholipids or protein content. Although this approach is efficient, chemical-free and does not modify the morphology of the exosomes, the type of lipid composition in the liposome can modify the cellular uptake efficiency of exosomes [129]. This technique has been reported as an easy and useful tool to load therapeutic agents or encapsulate large plasmids like CRISPR–Cas9 expression vectors in exosomes [130,131]. Introduction of exogenous membrane proteins also could be made by this membrane-engineering method. Moritani et al. reported a cell-free protein synthesis method for the mammalian membrane protein connexin-43 and its direct integration with a uniform orientation as a functional oligomer into liposome membranes [132]. In conclusion, using the membrane fusion approach it is possible to easily modify the properties of the exosome surface using liposomes embedded with peptides or antibodies as targeting moieties or PEG. Although there is no evidence of using this methodology to modify the exosome surface protein content, the fusion of proteoliposomes and exosomes membranes could facilitate the incorporation of external protein into the exosomes making it an option to attach 'don't eat me' molecules as CD47, CD31, CD24 among others.

Pegylation

PEGylation is the coating of nanoparticles with PEG. This methodology shields the coated surface from aggregation,

opsonization and phagocytosis, prolonging their half-time in circulation [133]. PEG can be used as a link to conjugate different molecules to the surface that needs to be modified. Using this approach, Kooijmans et al. [134] manage to 'decorate' the exosomes' surface with a specific targeting ligand conjugated to PEG. In this work, authors used nanobodies specific for the epidermal growth factor receptor (EGFR) conjugated to phospholipid (DMPE)-PEG derivatives to prepare nanobody-PEG-micelles, which were mixed with EVs at 40 °C to incorporate those nanobodies (15 kDa) to the surface of EVs. After making this 'decoration' on the surface of the exosomes, the authors observed no changes in their morphology, size distribution or protein composition. Exosomes modified using this method successfully targeted EGFR + tumor cells *in vitro*, and importantly, without affecting their classical biodistribution pattern. In addition, modified exosomes managed to enhance in 6-fold their circulation time in bloodstream in comparison with unmodified exosomes. In a similar approach, Si et al. [135] developed an efficient surface tagging technique to generate monoclonal antibody (mAb)-exosomes. In this work, mAb against somatostatin receptor 2 (SSTR2) were attached to the surface of HEK293 derived exosomes through PEGylation to target SSTR2-overexpressing neuroendocrine cancer cells. Both exosome and mAb were modified to display an 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG (DSPE- PEG) 'arm', successfully linking them and maintains not only the integrity of the exosomes but also their biomarkers and size distribution which confirm that this technique does not alter the physical and biochemical characteristics typical of exosomes [135]. To validate the targeted drug delivery efficiency, mAb-exosomes were loaded with the histone deacetylase inhibitor romidepsin, which efficiently induced cytotoxicity in cancer cells *in vitro* and *in vivo*.

Conclusions and perspectives

Despite the many advantages that exosomes possess as therapeutic tools, today their systemic application is technically challenging because the mononuclear phagocytic system drastically reduces the amount of exosome particles available to reach the therapeutic concentration in the target tissue. This implies the need to generate a higher dose of exosomes to meet clinical requirements, which is technically already a challenge due to the limited number of cellular sources that exist capable of secreting sufficient amounts of exosomes to generate a dose of clinical-grade. Performing modifications on the surface of exosomes to bypass the immune system is a plausible strategy to ensure a longer half-life in the bloodstream and thus modify the pharmacokinetic parameters of therapeutic exosomes without introducing adverse effects. Consequently, this strategy would provide the advantage of reaching the pharmacological concentration of exosome-based therapy in the tissue of interest in a shorter time, with the additional benefit that these engineered exosomes can also be artificially loaded with a specific therapeutic molecule. Although designing modifications in exosomes to target a specific site is a good option to improve 'targeting specificity', this strategy does not imply an improvement in the residence time of exosomes in plasma. In fact, this strategy can also be strongly affected by the barrier that phagocytic cells impose to the number of circulating exosomal particles available to target and accumulate in the target tissue, contributing to false negative results. Indeed, today it is very likely that there are innumerable pre-clinical studies with results that demonstrate low therapeutic efficiency or even no therapeutic effect that saw their options to reach the clinic truncated because of the rapid clearance suffered by exosomes systemically. If these studies incorporated some of the modifications proposed in this work, they could potentially improve their therapeutic efficiency or desired biological effect and continue their way to the clinic.

Finally, it is important to note that all structural modifications made on the surface of therapeutic exosomes must be evaluated for their safety and efficacy profile in *in vivo* models. Ideally, in experimental settings that closely resemble human immune conditions, such as in humanized murine models. Thus, the preclinical results obtained will be able to predict with greater precision not only the behavior of exogenous exosomes *in vivo*, but also the safety and efficacy of the therapy designed for specific pathological conditions.

Declaration of Competing Interest

F. A-M received stipends from Cells for Cells. The other authors indicated no potential conflicts of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Acknowledgments

The authors specially thank Dr. Maroun Khoury and Dr. Jimena Cuenca (Medicine Faculty, Universidad de los Andes, Santiago, Chile) for their helpful scientific discussions and Mr. Jacob Buckles Price for the English revision of the manuscript.

This work was supported by National Agency for Investigation and Development-ANID, FONDECYT Regular #1190411 to F.A-M.

References

[1] Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol 2002;2:569–79. doi: https://doi.org/10.1038/nri855.
[2] Baglio SR, Rooijers K, Koppers-Lalic D, Verweij FJ, Pérez Lanzón M, Zini N, et al. Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes enriched in distinctive miRNA and tRNA species. Stem Cell Res Ther 2015;6. doi: https://doi.org/10.1186/s13287-015-0116-z.
[3] Alcayaga-Miranda F, Varas-Godoy M, Khoury M. Harnessing the angiogenic potential of stem cell-derived exosomes for vascular regeneration. Stem Cells Int 2016;2016. doi: https://doi.org/10.1155/2016/3409169.
[4] Alcayaga-Miranda F, González PL, Lopez-Verrilli A, Varas-Godoy M, Aguila-Díaz C, Contreras L, et al. Prostate tumor-induced angiogenesis is blocked by exosomes derived from menstrual stem cells through the inhibition of reactive oxygen species. Oncotarget 2016;7:44462–77. doi: https://doi.org/10.18632/oncotarget.9852.
[5] Rosenberger L, Ezquer M, Lillo-Vera F, Pedraza PL, Ortúzar MI, González PL, et al. Stem cell exosomes inhibit angiogenesis and tumor growth of oral squamous cell carcinoma. Sci Rep 2019;9. doi: https://doi.org/10.1038/s41598-018-36855-6.
[6] Sun Y, Mao Q, Shen C, Wang C, Jia W. Exosomes from β-cells alleviated hyperglycemia and enhanced angiogenesis in islets of streptozotocin-induced diabetic mice. Diabetes, Metab Syndr Obes Targets Ther 2019;12:2053–64. doi: https://doi.org/10.2147/DMSO.S213400.
[7] Li T, Yan Y, Wang B, Qian H, Zhang X, Shen L, et al. Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. Stem Cells Dev 2013;22:845–54. doi: https://doi.org/10.1089/scd.2012.0395.
[8] Ha D, Yang N, Nadithe V. Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. Acta Pharm Sin B 2016;6:287–96. doi: https://doi.org/10.1016/j.apsb.2016.02.001.
[9] Yang Y, Hong Y, Cho E, Kim GB, Kim IS. Extracellular vesicles as a platform for membrane-associated therapeutic protein delivery. J Extracell Vesicles 2018;7. doi: https://doi.org/10.1080/20013078.2018.1440131.
[10] Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, et al. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-*versus*-host disease. Leukemia 2014;28:970–3. doi: https://doi.org/10.1038/leu.2014.41.
[11] Yang Z, Shi J, Xie J, Wang Y, Sun J, Liu T, et al. Large-scale generation of functional mRNA-encapsulating exosomes via cellular nanoporation. Nat Biomed Eng 2020;4:69–83. doi: https://doi.org/10.1038/s41551-019-0485-1.
[12] Nassar W, El-Ansary M, Sabry D, Mostafa MA, Fayad T, Kotb E, et al. Umbilical cord mesenchymal stem cells derived extracellular vesicles can safely ameliorate the progression of chronic kidney diseases. Biomater Res 2016;20. doi: https://doi.org/10.1186/s40824-016-0068-0.

[13] Turturici G, Tinnirello R, Sconzo G, Geraci F. Extracellular membrane vesicles as a mechanism of cell-to-cell communication: advantages and disadvantages. Am J Physiol – Cell Physiol 2014;306. doi: https://doi.org/10.1152/ajpcell.00228.2013.
[14] Wiklander OPB, Nordin JZ, O'Loughlin A, Gustafsson Y, Corso G, Mäger I, et al. Extracellular vesicle *in vivo* biodistribution is determined by cell source, route of administration and targeting. J Extracell Vesicles 2015;4:1–13. doi: https://doi.org/10.3402/jev.v4.26316.
[15] Hoshino A, Costa-Silva B, Shen T-L, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. Nature 2015;527:329–35. doi: https://doi.org/10.1038/nature15756.
[16] Mathieu M, Martin-Jaular L, Lavieu G, Théry C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat Cell Biol 2019;21:9–17. doi: https://doi.org/10.1038/s41556-018-0250-9.
[17] Smyth T, Kullberg M, Malik N, Smith-Jones P, Graner MW, Anchordoquy TJ. Biodistribution and delivery efficiency of unmodified tumor-derived exosomes. J Control Release 2015;199:145–55. doi: https://doi.org/10.1016/j.jconrel.2014.12.013.
[18] Takahashi Y, Nishikawa M, Shinotsuka H, Matsui Y, Ohara S, Imai T, et al. Visualization and *in vivo* tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection. J Biotechnol 2013;165:77–84. doi: https://doi.org/10.1016/j.jbiotec.2013.03.013.
[19] Charoenviriyakul C, Takahashi Y, Morishita M, Matsumoto A, Nishikawa M, Takakura Y. Cell type-specific and common characteristics of exosomes derived from mouse cell lines: yield, physicochemical properties, and pharmacokinetics. Eur J Pharm Sci 2017;96:316–22. doi: https://doi.org/10.1016/j.ejps.2016.10.009.
[20] Yamashita T, Takahashi Y, Nishikawa M, Takakura Y. Effect of exosome isolation methods on physicochemical properties of exosomes and clearance of exosomes from the blood circulation. Eur J Pharm Biopharm 2016;98:1–8. doi: https://doi.org/10.1016/j.ejpb.2015.10.017.
[21] Matsumoto A, Takahashi Y, Chang HY, Wu YW, Yamamoto A, Ishihama Y, et al. Blood concentrations of small extracellular vesicles are determined by a balance between abundant secretion and rapid clearance. J Extracell Vesicles 2020;9. doi: https://doi.org/10.1080/20013078.2019.1696517.
[22] Kamerkar S, Lebleu VS, Sugimoto H, Yang S, Ruivo CF, Melo SA, et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. Nature 2017;546:498–503. doi: https://doi.org/10.1038/nature22341.
[23] Barkal AA, Brewer RE, Markovic M, Kowarsky M, Barkal SA, Zaro BW, et al. CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy. Nature 2019;572:392–6. doi: https://doi.org/10.1038/s41586-019-1456-0.
[24] Gordon SR, Maute RL, Dulken BW, Hutter G, George BM, McCracken MN, et al. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. Nature 2017;545:495–9. doi: https://doi.org/10.1038/nature22396.
[25] Brown S, Heinisch I, Ross E, Shaw K, Buckley CO, Savill J. Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. Nature 2002;418:200–3. doi: https://doi.org/10.1038/nature00811.
[26] Weiskopf K. Cancer immunotherapy targeting the CD47/SIRPα axis. Eur J Cancer 2017;76:100–9. doi: https://doi.org/10.1016/j.ejca.2017.02.013.
[27] Barrès C, Blanc L, Bette-Bobillo P, André S, Mamoun R, Gabius HJ, et al. Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages. Blood 2010;115:696–705. doi: https://doi.org/10.1182/blood-2009-07-231449.
[28] Matsumoto A, Takahashi Y, Nishikawa M, Sano K, Morishita M, Charoenviriyakul C, et al. Role of phosphatidylserine-derived negative surface charges in the recognition and uptake of intravenously injected B16BL6-derived exosomes by macrophages. J Pharm Sci 2017;106:168–75. doi: https://doi.org/10.1016/j.xphs.2016.07.022.
[29] Feng D, Zhao WL, Ye YY, Bai XC, Liu RQ, Chang LF, et al. Cellular internalization of exosomes occurs through phagocytosis. Traffic 2010;11:675–87. doi: https://doi.org/10.1111/j.1600-0854.2010.01041.x.
[30] Czernek L, Chworos A, Duechler M. The uptake of extracellular vesicles is affected by the differentiation status of myeloid cells. Scand J Immunol 2015;82:506–14. doi: https://doi.org/10.1111/sji.12371.
[31] Imai T, Takahashi Y, Nishikawa M, Kato K, Morishita M, Yamashita T, et al. Macrophage-dependent clearance of systemically administered B16BL6-derived exosomes from the blood circulation in mice. J Extracell Vesicles 2015;4:1–8. doi: https://doi.org/10.3402/jev.v4.26238.
[32] Chow A, Zhou W, Liu L, Fong MY, Champer J, Van Haute D, et al. Macrophage immunomodulation by breast cancer-derived exosomes requires Toll-like receptor 2-mediated activation of NF-κB. Sci Rep 2014;4. doi: https://doi.org/10.1038/srep05750.
[33] Kim SH, Bianco NR, Shufesky WJ, Morelli AE, Robbins PD. Effective treatment of inflammatory disease models with exosomes derived from dendritic cells genetically modified to express IL-4. J Immunol 2007;179:2242–9. doi: https://doi.org/10.4049/jimmunol.179.4.2242.
[34] Van Rooijen N, Sanders A, Van Den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. J Immunol Methods 1996;193:93–9. doi: https://doi.org/10.1016/0022-1759(96)00056-7.
[35] Subra C, Laulagnier K, Perret B, Record M. Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. Biochimie 2007;89:205–12. doi: https://doi.org/10.1016/j.biochi.2006.10.014.
[36] Green DR, Beere HM. Gone but not forgotten. Nature 2000;405:28–9. doi: https://doi.org/10.1038/35011175.
[37] Rak J. Extracellular vesicles – biomarkers and effectors of the cellular interactome in cancer. Front Pharmacol 2013;4. doi: https://doi.org/10.3389/fphar.2013.00021.
[38] Wei H, Malcor JDM, Harper MT. Lipid rafts are essential for release of phosphatidylserine-exposing extracellular vesicles from platelets. Sci Rep 2018;8. doi: https://doi.org/10.1038/s41598-018-28363-4.
[39] Zani I, Stephen S, Mughal N, Russell D, Homer-Vanniasinkam S, Wheatcroft S, et al. Scavenger receptor structure and function in health and disease. Cells 2015;4:178–201. doi: https://doi.org/10.3390/cells4020178.
[40] Watson DC, Bayik D, Srivatsan A, Bergamaschi C, Valentin A, Niu G, et al. Efficient production and enhanced tumor delivery of engineered extracellular vesicles. Biomaterials 2016;105:195–205. doi: https://doi.org/10.1016/j.biomaterials.2016.07.003.
[41] Hao S, Bai O, Li F, Yuan J, Laferte S, Xiang J. Mature dendritic cells pulsed with exosomes stimulate efficient cytotoxic T-lymphocyte responses and antitumour immunity. Immunology 2007;120:90–102. doi: https://doi.org/10.1111/j.1365-2567.2006.02483.x.
[42] West GB. Plenary lecture 'Mast Cells Revisited'. Agents Actions 1986;18:5–18. doi: https://doi.org/10.1007/BF01987971.
[43] Saunderson SC, Dunn AC, Crocker PR, McLellan AD. CD169 mediates the capture of exosomes in spleen and lymph node. Blood 2014;123:208–16. doi: https://doi.org/10.1182/blood-2013-03-489732.
[44] Chonn A, Cullis PR, Devine DV. The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. J Immunol 1991;146:4234–41.
[45] Roerdink F, Wassef NM, Richardson EC, Alving CR. Effects of negatively charged lipids on phagocytosis of liposomes opsonized by complement. BBA - Biomembr 1983;734:33–9. doi: https://doi.org/10.1016/0005-2736(83)90071-8.
[46] Moein Moghimi S, Hamad I, Andresen TL, Jørgensen K, Szebeni J, Moein Moghimi S, et al. Methylation of the phosphate oxygen moiety of phospholipid-methoxy(polyethylene glycol) conjugate prevents PEGylated liposome-mediated complement activation and anaphylatoxin production. FASEB J 2006;20:2591–3. doi: https://doi.org/10.1096/fj.06-6186fje.
[47] Aung T, Chapuy B, Vogel D, Wenzel D, Oppermann M, Lahmann M, et al. Exosomal evasion of humoral immunotherapy in aggressive B-cell lymphoma modulated by ATP-binding cassette transporter A3. Proc Natl Acad Sci USA 2011;108:15336–41. doi: https://doi.org/10.1073/pnas.1102855108.
[48] Wang AL, Lukas TJ, Yuan M, Du N, Tso MO, Neufeld AH. Autophagy and exomoses in the aged retinal pigment epithelium: possible relevance to drusen formation and age-related macular degeneration. PLoS ONE 2009;4. doi: https://doi.org/10.1371/journal.pone.0004160.
[49] Morishita M, Takahashi Y, Nishikawa M, Sano K, Kato K, Yamashita T, et al. Quantitative analysis of tissue distribution of the B16BL6-derived exosomes using a streptavidin-lactadherin fusion protein and Iodine-125-labeled biotin derivative after intravenous injection in mice. J Pharm Sci 2015;104:705–13. doi: https://doi.org/10.1002/jps.24251.
[50] Lai CP, Mardini O, Ericsson M, Prabhakar S, Maguire CA, Chen JW, et al. Dynamic biodistribution of extracellular vesicles *in vivo* using a multimodal imaging reporter. ACS Nano 2014;8:483–94. doi: https://doi.org/10.1021/nn404945r.
[51] Zhuang X, Xiang X, Grizzle W, Sun D, Zhang S, Axtell RC, et al. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. Mol Ther 2011;19:1769–79. doi: https://doi.org/10.1038/mt.2011.164.
[52] Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJA. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol 2011;29:341–5. doi: https://doi.org/10.1038/nbt.1807.
[53] Mendt M, Kamerkar S, Sugimoto H, McAndrews KM, Wu CC, Gagea M, et al. Generation and testing of clinical-grade exosomes for pancreatic cancer. JCI Insight 2018;3. doi: https://doi.org/10.1172/jci.insight.99263.
[54] Xu R, Rai A, Chen M, Suwakulsiri W, Greening DW, Simpson RJ. Extracellular vesicles in cancer—implications for future improvements in cancer care. Nat Rev Clin Oncol 2018;15:617–38. doi: https://doi.org/10.1038/s41571-018-0036-9.
[55] Oldenborg P-A. CD47: A cell surface glycoprotein which regulates multiple functions of hematopoietic cells in health and disease. ISRN Hematol 2013;2013:1–19. doi: https://doi.org/10.1155/2013/614619.
[56] Matozaki T, Murata Y, Okazawa H, Ohnishi H. Functions and molecular mechanisms of the CD47-SIRPα signalling pathway. Trends Cell Biol 2009;19:72–80. doi: https://doi.org/10.1016/j.tcb.2008.12.001.
[57] Brown GC, Neher JJ. Eaten alive! Cell death by primary phagocytosis: "Phagoptosis". Trends Biochem Sci 2012;37:325–32. doi: https://doi.org/10.1016/j.tibs.2012.05.002.
[58] Tsai RK, Discher DE. Inhibition of "self" engulfment through deactivation of myosin-II at the phagocytic synapse between human cells. J Cell Biol 2008;180:989–1003. doi: https://doi.org/10.1083/jcb.200708043.
[59] Wang Y-C, Feng L, Yin C-Y, Ma L-N, Wei Y-W, Wang C-M, et al. Effectiveness of anti-CD47 antibody and Ara-C combination targeting therapy NOD/SCID mouse of myeloid leukemia. Zhongguo Dang Dai Er Ke Za Zhi 2013;15:577–82.

[60] Jaiswal S, Jamieson CHM, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell 2009;138:271–85. doi: https://doi.org/10.1016/j.cell.2009.05.046.

[61] Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD, et al. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell 2009;138:286–99. doi: https://doi.org/10.1016/j.cell.2009.05.045.

[62] Sadallah S, Eken C, Martin PJ, Schifferli JA. Microparticles (ectosomes) shed by stored human platelets downregulate macrophages and modify the development of dendritic cells. J Immunol 2011;186:6543–52. doi: https://doi.org/10.4049/jimmunol.1002788.

[63] Kim HS, Choi DY, Yun SJ, Choi SM, Kang JW, Jung JW, et al. Proteomic analysis of microvesicles derived from human mesenchymal stem cells. J Proteome Res 2012;11:839–49. doi: https://doi.org/10.1021/pr200682z.

[64] Kaur S, Chang T, Singh SP, Lim L, Mannan P, Garfield SH, et al. CD47 signaling regulates the immunosuppressive activity of VEGF in T cells. J Immunol 2014;193:3914–24. doi: https://doi.org/10.4049/jimmunol.1303116.

[65] Kaur S, Elkahloun AG, Arakelyan A, Young L, Myers TG, Otaizo-Carrasquero F, et al. CD63, MHC class I, and CD47 identify subsets of extracellular vesicles containing distinct populations of noncoding RNAs. Sci Rep 2018;8. doi: https://doi.org/10.1038/s41598-018-20936-7.

[66] Belhadj Z, He B, Deng H, Song S, Zhang H, Wang X, et al. A combined "eat me/don't eat me" strategy based on extracellular vesicles for anticancer nanomedicine. J Extracell Vesicles 2020;9. doi: https://doi.org/10.1080/20013078.2020.1806444.

[67] Rodriguez PL, Harada T, Christian DA, Pantano DA, Tsai RK, Discher DE. Minimal "self" peptides that inhibit phagocytic clearance and enhance delivery of nanoparticles. Science 2013;339(80-):971–5. doi: https://doi.org/10.1126/science.1229568.

[68] Hu CMJ, Fang RH, Luk BT, Chen KNH, Carpenter C, Gao W, et al. "Marker-of-self" functionalization of nanoscale particles through a top-down cellular membrane coating approach. Nanoscale 2013. doi: https://doi.org/10.1039/c3nr00015j.

[69] Tengood JE, Levy RJ, Stachelek SJ. The use of CD47-modified biomaterials to mitigate the immune response. Exp Biol Med 2016;241:1033–41. doi: https://doi.org/10.1177/1535370216647130.

[70] Woodfin A, Voisin MB, Nourshargh S. PECAM-1: A multi-functional molecule in inflammation and vascular biology. Arterioscler Thromb Vasc Biol 2007;27:2514–23. doi: https://doi.org/10.1161/ATVBAHA.107.151456.

[71] Feng YM, Chen XH, Zhang X. Roles of PECAM-1 in cell function and disease progression. Eur Rev Med Pharmacol Sci 2016;20:4082–8.

[72] Merchand-Reyes G, Robledo-Avila FH, Buteyn NJ, Gautam S, Santhanam R, Fatehchand K, et al. CD31 acts as a checkpoint molecule and is modulated by FcγR-mediated signaling in monocytes. J Immunol 2019;203:3216–24. doi: https://doi.org/10.4049/jimmunol.1900059.

[73] Hromada C, Mühleder S, Grillari J, Redl H, Holnthoner W. Endothelial extracellular vesicles-promises and challenges. Front Physiol 2017;8. doi: https://doi.org/10.3389/fphys.2017.00275.

[74] Fang X, Zheng P, Tang J, Liu Y. CD24: from A to Z. Cell Mol Immunol 2010;7:100–3. doi: https://doi.org/10.1038/cmi.2009.119.

[75] Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. Nat Rev Immunol 2007;7:255–66. doi: https://doi.org/10.1038/nri2056.

[76] Abram CL, Lowell CA. Shp1 function in myeloid cells. J Leukoc Biol 2017;102:657–75. doi: https://doi.org/10.1189/jlb.2mr0317-105r.

[77] Dietrich J, Cella M, Colonna M. Ig-like transcript 2 (ILT2)/leukocyte Ig-like receptor 1 (LIR1) inhibits TCR signaling and actin cytoskeleton reorganization. J Immunol 2001;166:2514–21. doi: https://doi.org/10.4049/jimmunol.166.4.2514.

[78] Keller S, Rupp C, Stoeck A, Runz S, Fogel M, Lugert S, et al. CD24 is a marker of exosomes secreted into urine and amniotic fluid. Kidney Int 2007;72:1095–102. doi: https://doi.org/10.1038/sj.ki.5002486.

[79] Runz S, Keller S, Rupp C, Stoeck A, Issa Y, Koensgen D, et al. Malignant ascites-derived exosomes of ovarian carcinoma patients contain CD24 and EpCAM. Gynecol Oncol 2007;107:563–71. doi: https://doi.org/10.1016/j.ygyno.2007.08.064.

[80] Rupp A-K, Rupp C, Keller S, Brase JC, Ehehalt R, Fogel M, et al. Loss of EpCAM expression in breast cancer derived serum exosomes: role of proteolytic cleavage. Gynecol Oncol 2011;122:437–46. doi: https://doi.org/10.1016/j.ygyno.2011.04.035.

[81] Tutanov O, Orlova E, Proskura K, Grigor'eva A, Yunusova N, Tsentalovich Y, et al. Proteomic analysis of blood exosomes from healthy females and breast cancer patients reveals an association between different exosomal bioactivity on non-tumorigenic epithelial cell and breast cancer cell migration *in vitro*. Biomolecules 2020;10. doi: https://doi.org/10.3390/biom10040495.

[82] Oksvold MP, Kullmann A, Forfang L, Kierulf B, Li M, Brech A, et al. Expression of B-cell surface antigens in subpopulations of exosomes released from B-cell lymphoma cells. Clin Ther 2014;36. doi: https://doi.org/10.1016/j.clinthera.2014.05.010.

[83] Barkal AA, Weiskopf K, Kao KS, Gordon SR, Rosental B, Yiu YY, et al. Engagement of MHC class I by the inhibitory receptor LILRB1 suppresses macrophages and is a target of cancer immunotherapy article. Nat Immunol 2018;19:76–84. doi: https://doi.org/10.1038/s41590-017-0004-z.

[84] Cosman D, Fanger N, Borges L, Kubin M, Chin W, Peterson L, et al. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. Immunity 1997;7:273–82. doi: https://doi.org/10.1016/S1074-7613(00)80529-4.

[85] Baía D, Pou J, Jones D, Mandelboim O, Trowsdale J, Muntasell A, et al. Interaction of the LILRB1 inhibitory receptor with HLA class Ia dimers. Eur J Immunol 2016;46:1681–90. doi: https://doi.org/10.1002/eji.201546149.

[86] Cheng H, Mohammed F, Nam G, Chen Y, Qi J, Garner LI, et al. Crystal structure of leukocyte Ig-like receptor LILRB4 (ILT3/LIR-5/CD85k): a myeloid inhibitory receptor involved in immune tolerance. J Biol Chem 2011;286:18013–25. doi: https://doi.org/10.1074/jbc.M111.221028.

[87] Fanger NA, Cosman D, Peterson L, Braddy SC, Maliszewski CR, Borges L. The MHC class I binding proteins LIR-1 and LIR-2 inhibit Fe receptor-mediated signaling in monocytes. Eur J Immunol 1998;28:3423–34. doi: https://doi.org/10.1002/(SICI)1521-4141(199811)28:11<3423::AID-IMMU3423>3.0.CO;2-2.

[88] Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008;26:677–704. doi: https://doi.org/10.1146/annurev.immunol.26.021607.090331.

[89] Okazaki T, Honjo T. The PD-1-PD-L1 pathway in immunological tolerance. Trends Immunol 2006;27:195–201. doi: https://doi.org/10.1016/j.it.2006.02.001.

[90] Hsu J, Hodgins JJ, Marathe M, Nicolai CJ, Bourgeois-Daigneault MC, Trevino TN, et al. Contribution of NK cells to immunotherapy mediated by PD-1/PD-L1 blockade. J Clin Invest 2018;128:4654–68. doi: https://doi.org/10.1172/JCI99317.

[91] Sun C, Mezzadra R, Schumacher TN. Regulation and function of the PD-L1 checkpoint. Immunity 2018;48:434–52. doi: https://doi.org/10.1016/j.immuni.2018.03.014.

[92] Daassi D, Mahoney KM, Freeman GJ. The importance of exosomal PDL1 in tumour immune evasion. Nat Rev Immunol 2020;20:209–15. doi: https://doi.org/10.1038/s41577-019-0264-y.

[93] Cordonnier M, Nardin C, Chanteloup G, Derangere V, Algros MP, Arnould L, et al. Tracking the evolution of circulating exosomal-PD-L1 to monitor melanoma patients. J Extracell Vesicles 2020;9.. doi: https://doi.org/10.1080/20013078.2019.1710899.

[94] Amash A, Wang L, Wang Y, Bhakta V, Fairn GD, Hou M, et al. CD44 antibody inhibition of macrophage phagocytosis targets Fcγ receptor– and complement receptor 3–dependent mechanisms. J Immunol 2016;196:3331–40. doi: https://doi.org/10.4049/jimmunol.1502198.

[95] Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc Natl Acad Sci U S A 1992;89:12160–4. doi: https://doi.org/10.1073/pnas.89.24.12160.

[96] Rosenblum MD, Way SS, Abbas AK. Regulatory T cell memory. Nat Rev Immunol 2016;16:90–101. doi: https://doi.org/10.1038/nri.2015.1.

[97] Vachon E, Martin R, Plumb J, Kwok V, Vandivier RW, Glogauer M, et al. CD44 is a phagocytic receptor. Blood 2006;107:4149–58. doi: https://doi.org/10.1182/blood-2005-09-3808.

[98] Vachon E, Martin R, Kwok V, Cherepanov V, Chow CW, Doerschuk CM, et al. CD44-mediated phagocytosis induces inside-out activation of complement receptor-3 in murine macrophages. Blood 2007;110:4492–502. doi: https://doi.org/10.1182/blood-2007-02-076539.

[99] Kipnis A, Basaraba RJ, Turner J, Orme IM. Increased neutrophil influx but no impairment of protective immunity to tuberculosis in mice lacking the CD44 molecule. J Leukoc Biol 2003;74:992–7. doi: https://doi.org/10.1189/jlb.0603301.

[100] Cywes C, Stamenkovic I, Wessels MR. CD44 as a receptor for colonization of the pharynx by group A *Streptococcus*. J Clin Invest 2000;106:995–1002. doi: https://doi.org/10.1172/JCI10195.

[101] Moffat FL, Han T, Li ZM, Peck MD, Falk RE, Spalding PB, et al. Involvement of CD44 and the cytoskeletal linker protein ankyrin in human neutrophil bacterial phagocytosis. J Cell Physiol 1996;168:638–47. doi: https://doi.org/10.1002/(SICI)1097-4652(199609)168:3<638::AID-JCP16>3.0.CO;2-V.

[102] Gao T, Wen T, Ge Y, Liu J, Yang L, Jiang Y, et al. Disruption of Core 1-mediated O-glycosylation oppositely regulates CD44 expression in human colon cancer cells and tumor-derived exosomes. Biochem Biophys Res Commun 2020;521:514–20. doi: https://doi.org/10.1016/j.bbrc.2019.10.149.

[103] Wang Z, von Au A, Schnölzer M, Hackert T, Zöller M. CD44v6-competent tumor exosomes promote motility, invasion and cancer-initiating cell marker expression in pancreatic and colorectal cancer cells. Oncotarget 2016;7:55409–36. doi: https://doi.org/10.18632/oncotarget.10580.

[104] Wang X, Cheng K, Zhang G, Jia Z, Yu Y, Guo J, et al. Enrichment of CD44 in exosomes from breast cancer cells treated with doxorubicin promotes chemoresistance. Front Oncol 2020;10. doi: https://doi.org/10.3389/fonc.2020.00960.

[105] Nakamura K, Sawada K, Kinose Y, Yoshimura A, Toda A, Nakatsuka E, et al. Exosomes promote ovarian cancer cell invasion through transfer of CD44 to peritoneal mesothelial cells. Mol Cancer Res 2017;15:78–92. doi: https://doi.org/10.1158/1541-7786.MCR-16-0191.

[106] Matsumoto N, Ariga A, To-E S, Nakamura H, Agata N, Hirano SI, et al. Synthesis of NF-κB activation inhibitors derived from epoxyquinomicin C. Bioorganic Med Chem Lett 2000;10:865–9. doi: https://doi.org/10.1016/S0960-894X(00)00114-1.

[107] Hatada EN, Krappmann D, Scheidereit C. NF-κB and the innate immune response. Curr Opin Immunol 2000;12:52–8. doi: https://doi.org/10.1016/S0952-7915(99)00050-3.

[108] Suzuki E, Umezawa K. Inhibition of macrophage activation and phagocytosis by a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin. Biomed Pharmacother 2006;60:578–86. doi: https://doi.org/10.1016/j.biopha.2006.07.089.
[109] Kuraya D, Watanabe M, Koshizuka Y, Ogura M, Yoshida T, Asahi Y, et al. Efficacy of DHMEQ, a NF-κB inhibitor, in islet transplantation: I. HMGB1 suppression by DHMEQ prevents early islet graft damage. Transplantation 2013;96:445–453.. doi: https://doi.org/10.1097/TP.0b013e31829b0744.
[110] Williams V, del Poeta M. Role of glucose in the expression of *Cryptococcus neoformans* antiphagocytic protein 1, App1. Eukaryot Cell 2011;10:293–301. doi: https://doi.org/10.1128/EC.00252-10.
[111] Stano P, Williams V, Villani M, Cymbalyuk ES, Qureshi A, Huang Y, et al. App1: An antiphagocytic protein that binds to complement receptors 3 and 2. J Immunol 2009;182:84–91. doi: https://doi.org/10.4049/jimmunol.182.1.84.
[112] Luberto C, Martinez-Mariño B, Taraskiewicz D, Bolaños B, Chitano P, Toffaletti DL, et al. Identification of App1 as a regulator of phagocytosis and virulence of *Cryptococcus neoformans*. J Clin Invest 2003;112:1080–94. doi: https://doi.org/10.1172/JCI18309.
[113] Hood JL. Post isolation modification of exosomes for nanomedicine applications. Nanomedicine 2016;11:1745–56. doi: https://doi.org/10.2217/nnm-2016-0102.
[114] Tian Y, Li S, Song J, Ji T, Zhu M, Anderson GJ, et al. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. Biomaterials 2014;35:2383–90. doi: https://doi.org/10.1016/j.biomaterials.2013.11.083.
[115] Rayamajhi S, Aryal S. Surface functionalization strategies of extracellular vesicles. J Mater Chem B 2020;8:4552–69. doi: https://doi.org/10.1039/d0tb00744g.
[116] Suetsugu A, Honma K, Saji S, Moriwaki H, Ochiya T, Hoffman RM. Imaging exosome transfer from breast cancer cells to stroma at metastatic sites in orthotopic nude-mouse models. Adv Drug Deliv Rev 2013;65:383–90. doi: https://doi.org/10.1016/j.addr.2012.08.007.
[117] Rivoltini L, Chiodoni C, Squarcina P, Tortoreto M, Villa A, Vergani B, et al. TNF-related apoptosis-inducing ligand (trail)-armed exosomes deliver proapoptotic signals to tumor site. Clin Cancer Res 2016;22:3499–512. doi: https://doi.org/10.1158/1078-0432.CCR-15-2170.
[118] Bellavia D, Raimondo S, Calabrese G, Forte S, Cristaldi M, Patinella A, et al. Interleukin 3- receptor targeted exosomes inhibit *in vitro* and *in vivo* chronic myelogenous leukemia cell growth. Theranostics 2017;7:1333–45. doi: https://doi.org/10.7150/thno.17092.
[119] Richardson JJ, Ejima H. Surface engineering of extracellular vesicles through chemical and biological strategies. Chem Mater 2019;31:2191–201. doi: https://doi.org/10.1021/acs.chemmater.9b00050.
[120] Stickney Z, Losacco J, McDevitt S, Zhang Z, Lu B. Development of exosome surface display technology in living human cells. Biochem Biophys Res Commun 2016;472:53–9. doi: https://doi.org/10.1016/j.bbrc.2016.02.058.
[121] Smyth T, Petrova K, Payton NM, Persaud I, Redzic JS, Graner MW, et al. Surface functionalization of exosomes using click chemistry. Bioconjug Chem 2014;25:1777–84. doi: https://doi.org/10.1021/bc500291r.
[122] Wang J, Li W, Lu Z, Zhang L, Hu Y, Li Q, et al. The use of RGD-engineered exosomes for enhanced targeting ability and synergistic therapy toward angiogenesis. Nanoscale 2017;9:15598–605. doi: https://doi.org/10.1039/c7nr04425a.
[123] Song S, Shim MK, Lim S, Moon Y, Yang S, Kim J, et al. *In situ* one-step fluorescence labeling strategy of exosomes *via* bioorthogonal click chemistry for real-time exosome tracking *in vitro* and *in vivo*. Bioconjug Chem 2020. doi: https://doi.org/10.1021/acs.bioconjchem.0c00216.
[124] Lee TS, Kim Y, Zhang W, Song IH, Tung CH. Facile metabolic glycan labeling strategy for exosome tracking. Biochim Biophys Acta – Gen Subj 2018;1862:1091–100. doi: https://doi.org/10.1016/j.bbagen.2018.02.001.
[125] Wang M, Altinoglu S, Takeda YS, Xu Q. Integrating protein engineering and bioorthogonal click conjugation for extracellular vesicle modulation and intracellular delivery. PLoS ONE 2015;10:1–12. doi: https://doi.org/10.1371/journal.pone.0141860.
[126] Jia G, Han Y, An Y, Ding Y, He C, Wang X, et al. NRP-1 targeted and cargo-loaded exosomes facilitate simultaneous imaging and therapy of glioma *in vitro* and *in vivo*. Biomaterials 2018;178:302–16. doi: https://doi.org/10.1016/j.biomaterials.2018.06.029.
[127] Tian T, Zhang HX, He CP, Fan S, Zhu YL, Qi C, et al. Surface functionalized exosomes as targeted drug delivery vehicles for cerebral ischemia therapy. Biomaterials 2018;150:137–49. doi: https://doi.org/10.1016/j.biomaterials.2017.10.012.
[128] Zhang H, Wu J, Wu J, Fan Q, Zhou J, Wu J, et al. Exosome-mediated targeted delivery of miR-210 for angiogenic therapy after cerebral ischemia in mice. J Nanobiotechnol 2019;17:1–13. doi: https://doi.org/10.1186/s12951-019-0461-7.
[129] Sato YT, Umezaki K, Sawada S, Mukai SA, Sasaki Y, Harada N, et al. Engineering hybrid exosomes by membrane fusion with liposomes. Sci Rep 2016;6:1–11. doi: https://doi.org/10.1038/srep21933.
[130] Piffoux M, Silva AKA, Wilhelm C, Gazeau F, Tareste D. Modification of extracellular vesicles by fusion with liposomes for the design of personalized biogenic drug delivery systems. ACS Nano 2018;12:6830–42. doi: https://doi.org/10.1021/acsnano.8b02053.
[131] Lin Y, Wu J, Gu W, Huang Y, Tong Z, Huang L, et al. Exosome–liposome hybrid nanoparticles deliver CRISPR/Cas9 system in MSCs. Adv Sci 2018;5:1–9. doi: https://doi.org/10.1002/advs.201700611.
[132] Moritani Y, Nomura SIM, Morita I, Akiyoshi K. Direct integration of cell-free-synthesized connexin-43 into liposomes and hemichannel formation. FEBS J 2010;277:3343–52. doi: https://doi.org/10.1111/j.1742-4658.2010.07736.x.
[133] Suk JS, Xu Q, Kim N, Hanes J, Ensign LM. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. Adv Drug Deliv Rev 2016. doi: https://doi.org/10.1016/j.addr.2015.09.012.
[134] Kooijmans SAA, Fliervoet LAL, Van Der Meel R, Fens MHAM, Heijnen HFG, Van Bergen En Henegouwen PMP, et al. PEGylated and targeted extracellular vesicles display enhanced cell specificity and circulation time. J Control Release 2016;224:77–85. doi: https://doi.org/10.1016/j.jconrel.2016.01.009.
[135] Si Y, Kim S, Zhang E, Tang Y, Jaskula-Sztul R, Markert JM, et al. Targeted exosomes for drug delivery: biomanufacturing, surface tagging, and validation. Biotechnol J 2020;15:1–12. doi: https://doi.org/10.1002/biot.201900163.
[136] Hikita T, Miyata M, Watanabe R, Oneyama C. Sensitive and rapid quantification of exosomes by fusing luciferase to exosome marker proteins. Sci Rep 2018. doi: https://doi.org/10.1038/s41598-018-32535-7.
[137] Bak M, Jølck RI, Eliasen R, Andresen TL. Affinity induced surface functionalization of liposomes using Cu-free click chemistry. Bioconjug Chem 2016;27:1673–80. doi: https://doi.org/10.1021/acs.bioconjchem.6b00221.

Nicol Parada obtained her degree in Biochemistry from the University of Concepción-Chile. In 2017, she obtained her MSc degree in Clinical Biochemistry and Immunology from the same university, focusing her work in Endothelial Progenitor Cells and their possible use in the field of cardiovascular therapies. In 2018, she joined the Nanoregenerative Medicine Laboratory of the Universidad de los Andes-Chile as Assistant Researcher, focusing her interest in the development of therapies based on stem cells and their extracellular derivatives. Nicol has participated in several poster and oral presentations at national and international meetings, and published in internationally recognized peer-reviewed journals.

Alfonso Romero-Trujillo obtained his bachelor's degree in Biotechnology Engineering from the Universidad Tecnológica Metropolitana-Chile. In his professional career, he has focused on studying and developing different therapeutic tools for cancer, such as the design and development of an antibody to treat gastric cancer. In 2019, he joined to the Laboratory of Nano-Regenerative Medicine of Medicine Faculty in the Universidad de los Andes (Chile) to study the anti-angiogenic role of miRNA's contained in stem cells exosomes to treat cancer. He has participated in various poster and oral presentations at national meetings and published one research manuscript in internationally recognized peer-reviewed journal.

Nicolás Georges is an undergraduate student of Biotechnology Engineer from Universidad Tecnológica Metropolitana of Chile. Nicolas Georges has 1,5 years of experience extracting and modifying alkaloids with potential anticancerogenic effect. Currently, Nicolás is doing his undergraduate thesis in the Laboratory of Nano-Regenerative Medicine of the Faculty of Medicine in the Universidad de los Andes of Chile in the field of small extracellular vesicles derived from stem cells, specifically in the use of exosomes as anti-angiogenic agents for cancer treatment.

Francisca Alcayaga-Miranda obtained her Veterinary degree from Universidad Mayor-Chile and her PhD from Universidad Autónoma de Barcelona-Spain. In 2011, she joined to the Laboratory of Nano-Regenerative Medicine in Universidad de los Andes (Chile). Currently, she is focused on the development of Exosomes-based Therapies to treat cancer and degenerative diseases. Author of more than 15 original scientific and reviews in peer-reviewed journals and 2 books chapters in the oncology field, she is also co-inventor of 2 patents. Currently, Dra. Alcayaga-Miranda is Senior Researcher in Cells for Cells & Consorcio Regenero, and Associate Professor in Medicine Faculty of Universidad de los Andes.