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# Extracellular vesicles and their current role in cancer immunotherapy


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Contents

# 1. Introduction

Extracellular vesicles (EVs) are lipid bilayer-delimited particles released into the extracellular environment by different types of cells under both physiological and pathological conditions. Based on their size and the mechanism of biogenesis, they can be classified into three groups: microvesicles, apoptotic bodies and exosomes. The extracellular vesicles are characterized by a very heterogeneous molecular composition; therefore, they are involved in numerous biological processes like coagulation, angiogenesis, modulation of the immune response, and inflammation. Most of all they play a key role in the intercellular communication. Indeed, they act as natural carriers in the transfer of biomolecules such as DNA, RNA, proteins, lipids and metabolites. Thanks to the transfer capacity and the possibility of targeting specific cells, they can be used in drug and gene delivery for the treatment of cancer and other diseases. In recent years, the use of vesicles as biological carriers has been extended to cancer immunotherapy. This new technique of cancer treatment involves the use of extracellular vesicles to transport molecules capable of triggering an immune response to damage cancer cells. In particular, several studies have analysed the possibility of using extracellular vesicles in the new therapeutic cancer vaccines, which represent a particular form of immunotherapy. Currently, in the literature there are no publications that systematically collect the studies made on therapeutic anticancer vaccines based on extracellular vesicles. Therefore, the purpose of this work is to illustrate some of the clinical studies, already carried out or still in progress, and to give a systematic reorganization of what has been produced in the literature so far. The first part of this Master Thesis provides a general overview of extracellular vesicles and isolation methods aimed at obtaining extracellular vesicles capable of carrying a therapeutic load. In addition, there is a digression on exosomes that explains which are the aspects that make these vesicles preferable as new therapeutic vehicles. In particular, the intrinsic biological characteristics and the different cellular origins that influence the molecular composition as well as the biological function of the exosomes are analysed. For example, it has been observed that exosomes derived from dendritic cells are able to trigger immune responses thanks to their molecular composition derived from their cellular origin and therefore could be effective in immunotherapy. The second part focuses on what types of molecules can be loaded inside the extracellular vesicles and provides the description of the loading methods. This analysis is then integrated with the illustration of some of the studies found in the literature on gene and drug delivery. An overview of the main surface functionalization techniques aimed at introducing specific surface markers for cell targeting, including post isolation methods and engineering of parental cells, concludes this part. The last part of the Thesis describes the different existing forms of immunotherapy, then focusing on therapeutic cancer vaccines and on the use of extracellular vesicles in immunotherapy. The section dedicated to cancer vaccines shows their constituent elements and analyses some clinical trials currently active for the study of vaccines based on dendritic cells. The section dedicated to the role of extracellular vesicles in immunotherapy focuses on cancer vaccines based on extracellular vesicles. Finally, the clinical and pre-clinical studies reported prove the significant therapeutic potential of extracellular vesicles also in the treatment of advanced cancer patients. Therefore, this new methodology in the treatment of cancer opens up new perspectives in the fight against cancer.

# 2. Extracellular vesicles

According to the latest literature reports, extracellular vesicles (EVs) are spherical particles enclosed by a phospholipid bilayer released into the extracellular environment by several cell types during both physiological and pathological conditions [1]. EVs have been isolated from many biological fluids, including blood, milk, saliva, amniotic fluid, cerebrospinal fluid, synovial fluid and urine [2]. Although initially thought to be part of a disposal mechanism through which the cell ejected its waste [3], EVs have gained increasing attention in the last years. Since the discovery that they work as vehicles for communication and transfer of cellular material between different tissues and cells, many research and literature reviews have emerged so far. In particular, EVs mediate intercellular communication, enabling the transfer of DNA, RNA, proteins, lipids and metabolites derived from their cellular origin [4]. Though the presence of proteins in EVs was reported alongside the discovery of EVs, the existence of RNA in EVs was only demonstrated during the past decade. Furthermore, EVs are also involved in processes such as coagulation, angiogenesis, cell survival, waste management, modulation of the immune response, and inflammation [5]. The International Society for Extracellular Vesicles (ISEV) defines EVs as lipid bilayer-delimited particles released from cells and unable to replicate. The presence of the lipid bilayer protects the EV cargo from degradation while the EVs move from donor to recipient cells [3]. Packaging also allows to store cargo in a more efficient manner and to deliver it at dedicated target cells by modifying the vesicles with cell type-specific adhesion receptors. Consequently, there is a growing interest in the clinical applications of vesicles which can potentially be used for therapy and biomarkers for health and disease [6]. Because of their heterogeneity, agreement has not yet been reached on the specific markers for defining EVs subtypes, but it is possible to classify them, depending on their size and biogenesis' mechanism, into three groups: microvesicles, apoptotic bodies and exosomes [7].

## *2.1. Microvesicles*

### 2.1.1. Biogenesis and cargo profiles

Microvesicles are heterogeneous cell-derived membrane vesicles that are shed from the surface of cells into the extracellular environment in a highly regulated process [8]. They are large vesicles ranging from 100–1000 nm in diameter, detected in blood, urine, synovial fluid, and many other body fluids under both physiological and disease conditions. Moreover, elevated MV concentrations have been also observed in atherosclerotic plaques and tumour tissues [9]. In general, microvesicles are formed by outward protrusion or budding of the plasma membrane through ARF6-mediated (ADP-ribosylation factor 6) rearrangement of the actin cytoskeleton as shown in Figure 2.1. This process can be triggered by an increase in intracellular cytosolic calcium that activates calpain, a calcium-sensitive protease that detaches membrane proteins from the intracellular cytoskeleton, and gelsolin, which is bound to actin filaments. This leads to remodelling of the cytoskeleton, by cleaving the actin protein network, enabling blebbing to occur. The mechanisms of formation and release of MVs remain only partially understood. In fact, it has been observed that calcium is not the only initiator involved: in various cell types, specific receptors or proteins are effective in releasing MVs. For example, in dendritic cells, macrophages and microglia, the activation of the purinergic receptor-channel promotes the release of MVs [10]. Microvesicles biogenesis also involves vertical trafficking of molecular cargo to the plasma membrane and a redistribution of membrane lipids. The specific function of microvesicles is determined by the composition of their cargo, which is in turn dependent

upon the cell type from which they are shed, the microenvironment and the triggers preceding their release. Generally, microvesicles carry membrane-derived receptors, proteins, including cytokines, chemokines, proteins involved in cellular signalling, lipids, carbohydrates [2], and nucleic acids (DNA and RNA) including mRNAs, microRNAs (miRNAs), small-interfering RNAs (siRNAs) [9]. Microvesicles are fundamental in altering the extracellular environment and intercellular signalling, as well as in facilitating cell invasion through cell-independent matrix proteolysis. When released into the extracellular environment, microvesicles can release their cargo, which modifies the extracellular milieu, or they can interact with recipient cells via endocytosis, fusion, or activation of signalling pathways through receptors [8]. For example, microvesicles from platelets exposing P-selectin were shown to bind to P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of leukocytes, leading to leukocyte accumulation and aggregation. Microvesicles can also transfer functional receptors to target cells, allowing cell signalling in cells that originally lacked the receptor. In the end microvesicles, while taking part in cellular communication, affect processes such as coagulation, thrombosis, angiogenesis, immune modulation and inflammation [2].

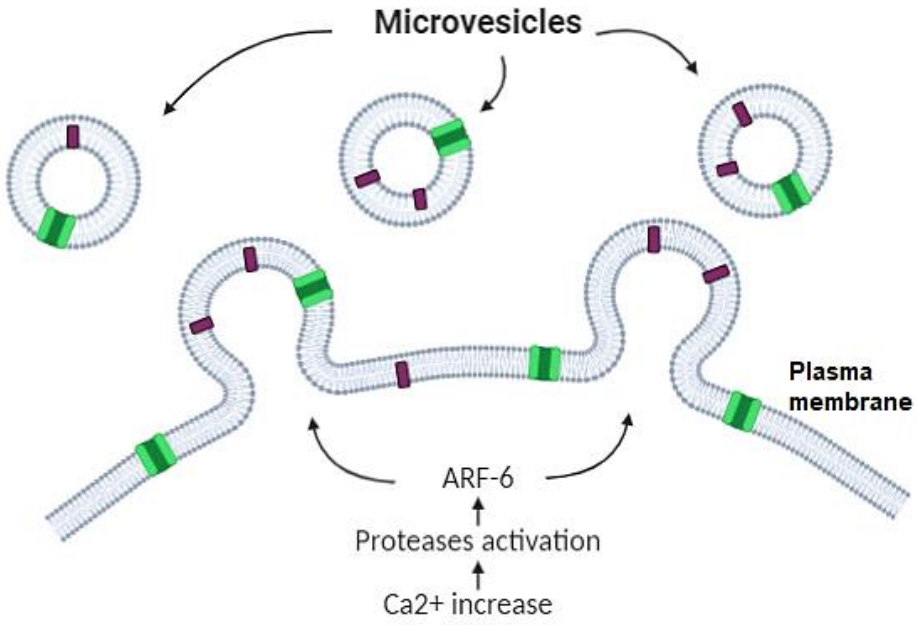


**Figure 2.1**: Calcium-dependent microvesicles biogenesis. Created with Biorender.com.

### 2.1.2. Microvesicles for therapeutic and drug delivery

Thanks to new technologies enabling for isolating microvesicles and because of their ability to transfer biomolecules such as nucleic acids, microvesicles can be exploited for targeted drug and therapeutic delivery. Drug delivery may also be improved using engineered microvesicles. The first report of delivery of a therapeutic mRNA/protein via MVs for treatment of cancer has shown that genetically engineered vesicles are viable delivery vehicles. In particular they showed the ability to deliver suicide genes to cancerous schwannoma recipient cells [8]. MVs were harvested from cells which stably expressed the suicide gene mRNA and protein–cytosine deaminase (CD) fused to uracil phosphoribosyltransferase (UPRT), a potent prodrug-activating combination. MVs were isolated from these cells and used to treat schwannoma tumour in an orthotopic mouse model. It was demonstrated that MV-mediated delivery of CD-UPRT

mRNA/protein by direct injection into schwannomas together with systemic treatment with the prodrug (5-fluorocytosine (5-FC)), which is converted within tumour cells to 5-fluorouracil (5-FU), an anticancer agent, led to regression of these tumours [11]. Recently, a team of researchers from Michigan State University (USA) has been able to demonstrate the effectiveness of using microvesicles as a delivery agent in cancer therapeutics using a breast cancer mouse model [12]. They had previously demonstrated that microvesicles are able to deliver plasmid DNA to cells and that the efficiency of delivery is partially determined by the plasmid size and sequence. In this study, microvesicles are loaded with engineered minicircle DNA and tested in breast cancer models in mice. The minicircle DNA encodes a thymidine kinase fusion protein that activates prodrugs (ganciclovir and CB1954) in breast cancer cells. They have found that microvesicles loaded with this minicircle DNA delivers the prodrug-enzyme coding genes to cancer cells 14-times more effectively than microvesicles loaded with regular plasmids and are even more successful at killing cancerous cells. Microvesicles could not only offer effective drug delivery but would actually provide a safe alternative to chemotherapy, since researchers would be able to target the treatment to cancer cells only. In the end, the results from this study confirm that minicircular DNA delivery via microvesicles could be considered a promising approach to cancer therapy. At the moment, a Phase I clinical trial using microvesicles for cancer treatment is set to begin soon in the USA, focusing on pancreatic cancer. In the meantime, the team plans to continue improving the effectiveness and safety of the method so that this promising approach can, one day soon, become a reality.

## *2.2. Apoptotic bodies*

Apoptotic bodies are membrane-bound vesicles ranging from 50-5000 nm in diameter, released from cells undergoing apoptosis as pictured in Figure 2.2 [4]. Apoptosis is a physiologically-programmed cell death that does not induce inflammatory responses [13]. It is commonly appearing in multicellular organisms because it represents a homeostatic mechanism for maintaining cell populations in tissues and has a key role during the processes of development and aging. Apoptosis is constituted by a sequence of steps which lead to the formation of the so-called apoptotic bodies. During the first stage, the induction, several types of stimuli or conditions are able to trigger the signalling of apoptosis. Following induction, caspases and aspartic acid-specific proteases start a process of weakening of the cell cytoskeleton, that is proteolytic cascade process [14] Apoptotic caspases are subcategorised as initiator caspases and executioner caspases: once initiator caspases are activated, they produce a chain reaction, activating several other executioner caspases which in turn carry out the degradation of cellular components [15]. In the early stages of apoptosis, the cleavage of the main components of cytoskeleton by caspases results in retraction of the cell and dynamic membrane blebbing. As the cell cytoplasm pushes against unsupported areas of the plasma membrane, membrane blebs are formed. This can be due to myosin-dependent contraction of cortical bundles of actin, pushing the cytosol against the cell cortex and causing blebs in areas where the cytoskeleton has deteriorated. Other noticeable events of this process are: (a) nuclear fragmentation, resulting from the disintegration of the nuclear lamina via proteolysis by caspases and the collapse of the nuclear membrane, and (b) condensation of chromatin accompanied by the hydrolysis of nuclear DNA (from "How Did My Cells Die? Choosing the Right Apoptosis Assay in Biomol.com, 2019, url: biomol.com/resources/biomol-blog/how-did-my-cells-die-choosing-the-right-apoptosis-assay). Other cellular components also undergo fragmentation and their pieces are dispersed into plasma membrane blebs. The splitting of cellular content through the membrane blebbing determines the formation of distinct membrane-enclosed vesicles: the apoptotic bodies. These apoptotic bodies are then engulfed by phagocytes for final degradation [13]. To this end, apoptotic cells can release molecular factors known as "find-me" signals to

attract phagocytes. Apoptotic bodies can also expose “eat-me” signals like phosphatidylserine on their surface and be recognized by macrophages for removal *via* phagocytic receptors such as CD36 [16]. These signals are critical to ensure the immunologically silent characteristic of apoptosis. Therefore, in autoimmune diseases, a defect in the clearance of ApoBDs formation may contribute to the development of autoimmunity [14]. Depending on the mechanism used by a particular cell type undergoing apoptosis, they comprise a content including chromatin, glycosylated proteins, large amounts of low molecular weight RNA and intact organelles such as mitochondria and nuclear fragments. It is not yet clear why different cell types need to disassemble differently and the functional significance of such diversity. Nonetheless recent studies have demonstrated that apoptotic bodies are also involved in the progression and formation of the tumour microenvironment and metastasis because these vesicles can transfer bioactive molecules to “target” cells [17]. It has also been demonstrated that ApoBDs seem to have a greater procoagulant effect on cancer native cells. These results highlight the potential of ApoBDs to contribute to the prothrombotic state and anticancer immunity. Even if further studies are mandatory to provide scientific evidence in biology and medicine, apoptotic body formation represents a process closely involved in both cell clearance and intercellular communication [14], both of which have implications in immune regulation.

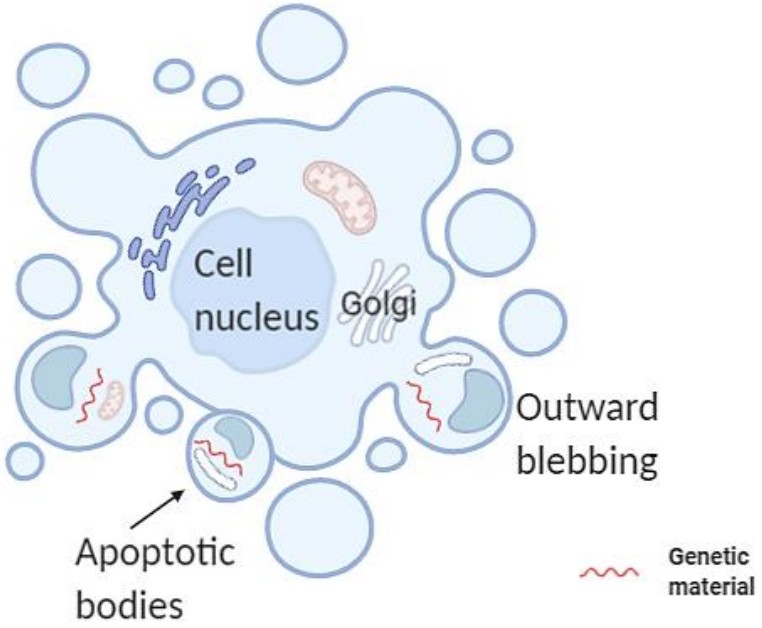


**Figure 2.2**: Cell apoptosis and apoptotic bodies formation. Created with Biorender.com.

## *2.3. Exosomes*

### 2.3.1. Biogenesis and main features of the exosomes

Exosomes are phospholipid bilayer vesicles of around 40–100 nm in size that derive from multivesicular bodies (MVBs) [18]. They are secreted by all cell types and can be found in most body fluids, including blood, saliva, and urine. An exosome is a “nanosphere” with a bilayer membrane, containing various types of lipids and proteins derived from the parent cells [19]. Exosome biogenesis can be divided into different phases. It starts with the formation of early endosome from plasma membrane, then matured into late endosomes. The limiting membrane of late endosomes undergoes inward budding and forms vesicles inside the lumen, thus forming the multivesicular bodies (MVBs). At the end, the fusion of these MVBs with the plasma

membrane leads to the release of intraluminal vesicles (ILVs), named as exosomes [20]. The processes that govern the formation of ILVs inside MVBs and the fusion with the PM are not completely understood [4]. There are two known pathways which can lead to the formation of MVBs as shown in Figure 2.3. The first pathway involves endosomal sorting complexes required for transport (ESCRT), while the second is ESCRT-independent. ESCRT is a protein machinery composed of four soluble multi-protein complexes that are ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. These complexes work together to facilitate MVB formation, vesicle budding, and protein cargo sorting [21]. ESCRT-0 is responsible for cargo sorting. Indeed, it binds to ubiquitin moieties that are attached to membrane proteins on endosomes, and thus executes the first sorting step in the MVB pathway. Thus, these proteins will be incorporated into ILVs and, later, become part of the released exosomes. ESCRT-0 also recruits ESCRT-I to the endosomal membrane. Tumour susceptibility gene 101 (TSG101), a component of ESCRT-I, forms a complex with the ubiquitinated cargo protein and helps in the activation of ESCRT-II complex, inducing bud formation. This complex then involves the sequestration of MVB proteins and the recruitment of the de-ubiquitination enzyme to remove the ubiquitin from the cargo proteins before sorting them into ILVs. In the last stage, ESCRT-III is recruited by ESCRT-II and gets disassembled by vacuolar protein sorting-associated protein 4 (VPS4) adenosine triphosphatase (ATPase), resulting in closing of the cargo-containing vesicle and pinching off of the vesicles. On the contrary, the ESCRT-independent mechanism seems to involve molecules such lipids, tetraspanins and heat shock proteins. In particular, it depends on the conversion of sphingomyelin to ceramide by sphingomyelinases. This conversion leads to ILVs formation because it has been demonstrated that lipids such as ceramides induce the inward curvature of the limiting membrane of MVBs [19] [4].

The presence of exosomes in extracellular space was identified as early as in late 1980s. Exosomes secreted from cells were initially proposed as cellular waste resulting from cell damage or as by-products of cell homeostasis [21]. Currently, these extracellular vesicles are considered functional vehicles, because they are able to deliver molecular cargoes to target cells and reprogram the recipient cells distal from their release. Generally, exosomes contain proteins, DNA, mRNA, miRNA, lipids, but their molecular composition directly depends on the donor cell from which they are derived, so this may contribute to their diverse and multiple physiological roles. It is known that exosomes play a major role in intercellular communication, but they are also involved in many biological processes, including the maturation of erythrocytes, antigen presentation in immune responses, coagulation, inflammation, and angiogenesis[19]. Exosomes also participate in cancer progression and metastasis by transferring bioactive molecules between cancer and various cells in the local and distant microenvironments [22].

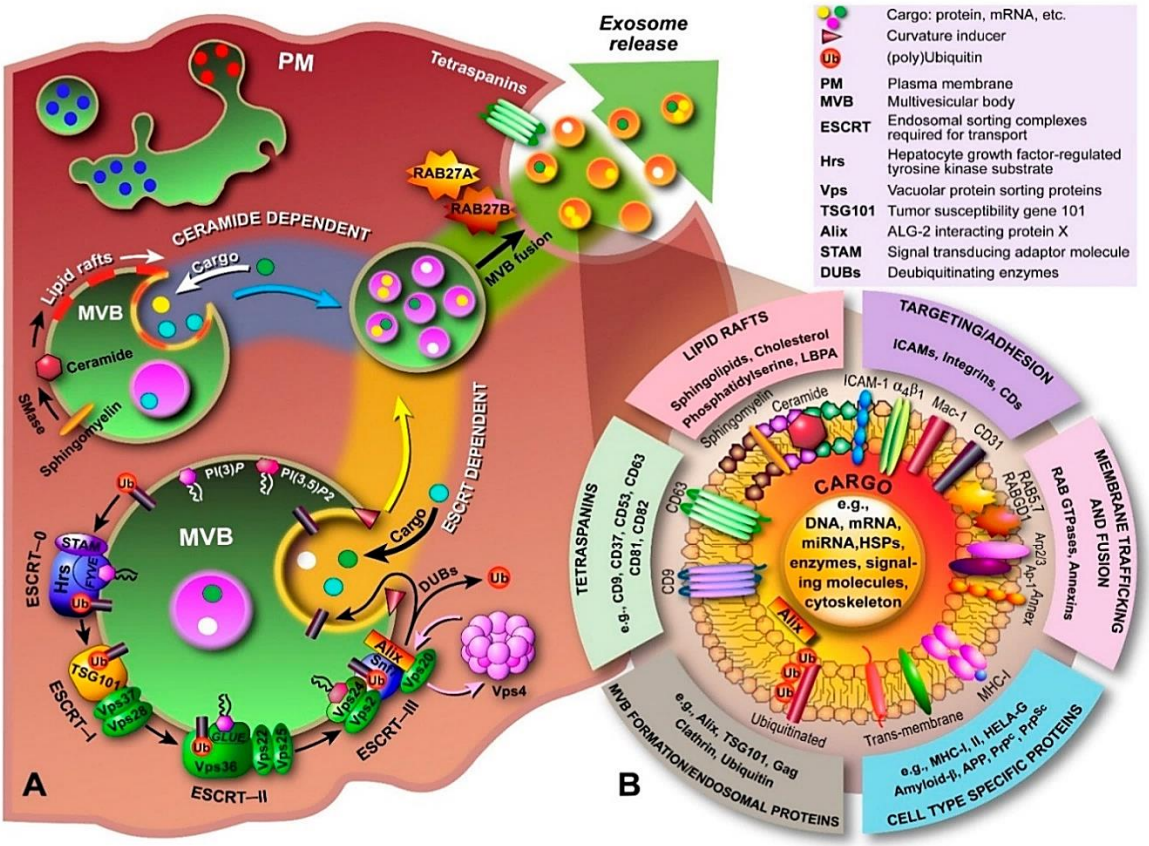


**Figure 2.3**: Exosomes biogenesis and molecular composition. Figure taken from: H. Kalra, G. P. C. Drummen, and S. Mathivanan, "Focus on extracellular vesicles: Introducing the next small big thing," *Int. J. Mol. Sci.,* 2016.

### 2.3.2. Exosomes: ideal candidates for therapeutic delivery systems

Exosomes are employed as natural drug delivery vehicles to deliver a wide range of therapeutics, including genetic material, into the cells. Their potential as therapeutic delivery vehicles is further enhanced by the low immunogenicity and toxicity enabling them to be ideal candidates for designing novel drug delivery systems. Many different cell types such as B cells, dendritic cells, T cells, platelets, tumour cells, mesenchymal stem cells, human embryonic kidney cell, various cancer cell lines are known to secrete exosomes. The great variety of substances they are able to transfer suggests that exosomes may have different biological roles depending on their cellular origin. All these exosomes share a conserved set of proteins including tetraspanins (CD81, CD63, CD9), Alix and Tsg101, highlighting their similar biological functions. At the same time, they have cell type-specific proteins that reflect their cellular source and their special biological activities. For example, exosomes released from maturing reticulocytes are rich in transferrin receptors that the reticulocytes have to dispose of. Those from epididymis are rich in proteins that are essential for the maturation of male gametes, while urinary exosomes secreted by kidney tubules transport aquaporin, a kidney specific protein, and so on for all other cell types [23] [24]. Other two important factors to completely exploit exosomes as new delivery system are the amenability to modifications to enhance exosomal targeting capability and the scalability of the process. Generally, it is possible to introduce modifications to the exosomal membrane proteins which are responsible for cell targeting. However, exosomes from a vast variety of cells have been also investigated to solve the problem of cell targeting in clinical therapeutic applications. For example, tumour cells actively release and utilize exosomes. In particular, tumour cell-derived exosomes carry

tumour-associated antigens specific to the tumours from which they are derived. Exosomes can deliver these antigens to dendritic cells (DCs) and induce a T-cell-mediated immune response against tumour cells[25]. Therefore, there are appealing aspects to the use of tumor-derived exosomes for the delivery of therapeutic agents and vaccines in cancer immunotherapy. Cancer immunotherapy is the artificial stimulation of the immune system to treat cancer, improving on the immune system's natural ability to fight the disease [26]. This new approach in cancer treatment exploits the fact that cancer cells often present tumour antigens, molecules on their surface that can be detected by the antibody proteins of the immune system. Then, antibodies bind to the tumour antigens marking and identifying the cancer cells for the immune system to inhibit or kill them. However, at the same time, tumour-derived exosomes could induce apoptosis of activated cytotoxic T cells, impairment of monocyte differentiation and induction of myeloid-suppressive cells, that are inhibitors of T cells [27] [28]. As a result, the release of exosomes by tumours may allow them to evade "immunosurveillance" and interfere with cancer immunotherapy. The potential risk in using tumour exosomes and the possibility in aggravating the pathological condition of the patient, instead of improving it, makes choosing the proper exosomes crucial for therapy. For the reasons mentioned above, exosomes isolated from fruits and plants have been considered as alternative options for clinical use because they come from reliable sources and are generally considered safe. In addition, agricultural products such as fruits and milk are scalable sources from which to isolate exosomes [29]. However, despite the high yield and superior safety profiles of exosomes isolated from food, these exosomes are not the best choice. In fact, unlike those derived from tumours, these are unable to stimulate the immune system for the cancer treatment. As a consequence, immune cell-derived exosomes are receiving greater attention. They have been shown to be particularly capable of evading immune phagocytosis, thus they can avoid rapid clearance, circulate longer and prolong their efficacy [1]. Dendritic cells (DCs), in particular, have been investigated as sources of exosomes for drug delivery and therapeutic vaccine applications [30] [31]. DCs are antigen-presenting cells so their main function is to capture and process antigen material and then present it on the cell surface to the T cells of the immune system as shown in Figure 4.1. They act as messengers between the innate and the adaptive immune systems [32]. Consequently, DC-derived exosomes contain antigen presenting molecules, adhesion molecules and costimulatory molecules, that are the necessary equipment required for generating powerful immune responses and thus for exosome-based vaccines. This new technique for the therapeutic administration of the vaccines is based on the production of exosomes, engineered with the vaccine antigen of interest, to induce a powerful cytotoxic T cell (CTL) mediated immune response against a large number of tumour and viral antigens. In the end, many cell types can secrete exosomes, however, the only cell type known to have scalable capacity to produce exosomes is the mesenchymal stem cell (MSC). Mesenchymal stem cells (MSCs), also referred to as mesenchymal stromal cells, are adult stem cells capable of self-renewal and multilineage differentiation [33]. They were originally found in the bone marrow, but they were later identified in other tissues including adipose tissue, muscle, peripheral blood, placenta and umbilical cord. MSCs have several cellular characteristics that are highly desired qualities of drug delivery vehicles and these make them ideal candidates as producers of exosomes for drug delivery [23]. Some of these features are the ease of isolation from ethically non-controversial human materials, the large ex vivo expansion capacity and the immunosuppressive properties. [34]. Preliminary data suggest that some of the immunomodulatory properties of MSC were transferred to their exosomes, which suggests that transplanting MSC exosomes would unlikely lead to adverse effects. However, as MSCs are not infinitely expansible, a large-scale production of these exosomes would require replenishment of MSCs from human embryonic stem cells (hESCs). R. W. Y. Yeo et al. investigated the immortalization of hESC-MSC with *MYC* gene to avoid this limitation without

compromising the production of therapeutically efficacious exosomes [35]. Oncogenes such as MYC gene or viral vectors encoding oncoproteins can transform a cell into an immortalised state by silencing the cell cycle checkpoint pathways and cell cycle regulators [36]. Therefore, immortalized cells are a population of cells, which can escape normal cellular senescence and keep undergoing division due to mutation. In addition, the mutations required for immortality can be intentionally induced for experimental purposes. As a result, this kind of cells can grow *in vitro* for even long periods.

## *2.4. EVs isolation methods*

The choice of the EVs isolation procedure is of primary importance for obtaining EVs suitable for therapeutic purposes. First of all, it is necessary to determine a proper extraction method to obtain a homogeneous EVs preparation with specific characteristics and biological functions [37]. Then, the process needs to be standardized with regard to the reproducibility, purity, impurities, and maintenance of EVs' functional properties. In particular, for each specific application, purity constraints must be considered. Clearly, if EVs are to be used as a source of diagnostic material, it is necessary to recover the highest possible amount of vesicles, while preservation of their structure and high purity of preparations are not necessary. In the case of EVs designed to be used as drug delivery vehicles, it is necessary to use the methods that preserve their structure and select the source that allows tissue or organ targeting [38]. Finally, the procedures should be evaluated according to their scalability within limited processing times and to the costs [39].

Several extraction methods have been developed, but the most frequently used is the differential ultracentrifugation (UC). The EV-producing medium is subjected to a series of centrifugations with different speeds. After each centrifugation step, the supernatant is transferred into a new test tube while the generated pellets are being discarded [40]. In this way, larger particles like cells, cell debris or large extracellular vesicles sediment faster and are firstly removed, while the small EVs are collected in the final pellets resulted from a higher speed centrifugation [37]. The EVs that can be obtained from a sample are exosomes, microvesicles and apoptotic bodies. Exosomes, the most extensively studied EV population, are usually isolated by centrifugation at 100,000–200,000 × g, microvesicles are isolated by centrifugation at 10,000–20,000 × g, and apoptotic bodies are obtained at a g-force of approximately 2,000 × g. This technique, however, is limited by several drawbacks, including risk of EVs aggregation, co-isolation with other non-EV structures and eventual disruption of EVs integrity due to high speed. These problems not only determine low EV recovery, but also affect the preparation purity. Furthermore, ultracentrifugation is time consuming and has limited scalability. In order to effectively reduce contamination and to separate the EVs, a density gradient ultracentrifugation step can be used [41]. The EVs are separated on the base of size and mass density (top-down gradient) or mass density only (bottom-up gradient), allowing them to float into a density gradient of viscous solution of sucrose or iodixanol. Upon centrifugation, EVs migrate to their equilibrium density with a migration speed or rate of flotation that depends on the size, shape, and density of the EV [42]. As in ultracentrifugation, the density gradient also leads to loss of functionality due to centrifugal forces that damage the EVs. Taking into account the problems encountered with these techniques, more effective alternatives to these methods need to be explored by researchers.

Precipitation with hydrophilic polymers is the second most used method after ultracentrifugation, indeed it is used by the majority of the isolation kits. This method utilizes a decrease in the solubility of compounds in the solutions of superhydrophilic polymers. [38]. The water-excluding polymers such as polyethylene glycol (PEG) can bind water molecules

and force less soluble components out of solution. Then, EVs can be isolated via centrifugation at low centrifugal forces. The main advantages of EV precipitation with PEG are simplicity and speed as well as the possibility of working in physiological pH range and weak dependence on the ion concentration. However, also the polymer-based precipitation method suffers from low purity and moderate yield. In particular, the low-purity problem is the result of co-precipitation of proteins in a sample because PEG decreases the solubilities of both EVs and proteins.

Size exclusion chromatography (SEC) has emerged as an alternative to differential centrifugation and gradient density ultracentrifugation because it does not seem to impact EV integrity so that it preserves EV functionality [42]. However, a pre-treatment and concentration of EV samples by ultracentrifugation or ultrafiltration are necessary in order to obtain the EV preparations free of proteins and other impurities. In addition, biologically active and pure samples with high yield often require multiple chromatographic steps. SEC is performed using a column containing small porous polymer beads that are the stationary phase. As the sample solution travels through the stationary phase, small particles are able to enter the porous beads. As a result, larger particles travel through the column more quickly than small particles and elute at an earlier time point than small particles. SEC overcomes the problems like vesicles disruption, aggregation and co-purification of non-EV material. More importantly, TEM analysis of EVs by UC and by SEC showed no major morphological differences and Western blot analyses revealed that both UC-EVs and SEC-EVs were enriched for ALIX and CD63, exosomes marker proteins. It is an expensive method but has high purity and yield [24].

EVs can be also fractionated by size with good accuracy using flow field-flow fractionation (FFFF), a one-phase chromatography technique which separates particles based on differences in their diffusion properties inside a flow channel. FFFF uses a liquid flow perpendicular to the channel flow as separation force. Thanks to dynamic diffusion, the particles are separated according to their size or molecular weight. FFFF combined with multiple detectors is able to rapidly characterize and separate EVs and facilitates understanding of EV function by subtype [39]. Beyond this, it is possible to isolate on large-scale clinical EVs for the purpose of developing future EV-based diagnostics and therapeutics.

To discriminate between the different EVs subtypes and overcome the hurdle of overlapping dimensional range, the purification methods by immunorecognition are introduced. These methods can be used for EV isolation from either cell culture media or body fluids. They are based on immunoaffinity so they employ immobilized antibodies to selectively capture EVs [24]. Notably, the antibodies can be immobilized on different supports, like magnetic beads, chromatographic matrix, plates and chosen to capture specific EVs subpopulation [19]. After mixing the EV sample with the antibody-coated beads, a magnetic force is applied and retains the EV-covered microbeads, while the rest of the sample is discarded. Next, the microbeads with attached EVs are eluted using appropriate buffers. [40]The advantage of this isolation method is its high ability to select a specific EV population based on a marker expression regardless of its size. Moreover, it allows to avoid further ultracentrifugation steps. However, the high specificity of immunoaffinity methods is compromised by the heterogeneous expression of EV markers on different EV subpopulations which results in reducing purity of the extracted particles [41]. Also, it is necessary to highlight that the beads, due to their limited physical surface, can only bind a certain number of EVs. This limitation, in turn, can lead to a partial loss of the vesicle population during the purification process, making this method valid only for small sample volumes.

# 3. Cargo loading methods and functionalization

## *3.1. Classification of cargo into EVs and cargo loading methods*

As a new delivery system, EVs have various advantages such low immunogenicity and toxicity and targeting ability. Also, the use of EVs overcome some limitations encountered in conventional nanoparticulate systems such as liposomes, which are vesicular structures prepared from lipids in the laboratory and widely used as drug carriers. Unlike liposomes, exosomes-based delivery systems, can cross some biological barriers such as the blood brain barrier (BBB) [43] [44], evade the lysosomal degradation and deliver cargos directly into the cytoplasm [24]. Most of all, the unique structure of EVs, constituted by a hydrophobic lipid membrane and a hydrophilic core, allows a great variety of molecules to be loaded into the EVs. Hydrophobic and hydrophilic molecules can be loaded into EVs, including anticancer drugs, miRNA, siRNA, DNA and proteins [29]. Recently, EVs have been shown to be able to carry nanoparticles (NPs) as well [45] [46] [47]. By loading NPs into EVs, it is possible to overcome problems such as particle aggregation, degradation and rapid clearance, which often occur in the use of nanoparticles. Therapeutic cargos are incorporated into exosomes by following two main loading approaches: the passive and the active cargo loading. Passive loading refers to a simple method wherein cargo is passively loaded into EVs without any external interventions. Instead, active loading requires the EVs are forced to load the cargo, using many different techniques.

### 3.1.1 Passive cargo-loading methods

Such methods typically refer to a simple co-incubation of the EVs with the therapeutic cargo at room temperature (RT) or at 37 °C for a certain period of time [48] [49]. Usually, upon incubation hydrophobic cargo diffuses into the EVs and crosses the membrane by following the diffusion gradient. Actually, the mechanism depends upon the lipophilic nature of loaded molecules. For example, some hydrophobic molecules, like curcumin, are loaded through a lipid rearrangement of the membrane that facilitates the entry of the molecule. Curcumin is a natural polyphenol with anti-inflammatory, antineoplastic, antioxidant, and chemo-preventive properties. Indeed, one of the first reports concerning drug delivery involves the use of exosomes to deliver curcumin to activate myeloid cells and treat an inflammatory disease [48]. Monocyte-derived myeloid cells represent potential effector cells during inflammatory processes because they act as scavengers and have a high capacity for taking up exosomes circulating in the peripheral blood, through their phagocytic function. Curcumin was co-incubated with a murine tumour cell line (EL-4) -derived exosomes at 22 °C for 5 minutes, and then subjected to sucrose gradient centrifugation. After the separation, exosomal curcumin was collected to make various considerations regarding of the incorporation of curcumin into the exosome. The morphology and the size of exosomal curcumin resulted similar to the original exosomes. Indeed, one of the main advantages of co-incubation is the possibility of preserving EVs morphology. Moreover, solubility, stability and bioavailability of curcumin came out increased too. In the end, the therapeutic relevance of exosomal curcumin was validated using a lipopolysaccharide (LPS)-induced septic shock mouse model. The anti-inflammatory activity *in vivo* was assessed through intraperitoneal injection, resulting in a strong decrease of mouse mortality compared to mice treated with free curcumin.

Other molecules, such as glucose, are internalized into EVs by energy-dependent mechanisms named as glucose channels, [37], while loading nanoparticles can be accomplished by modifying many parameters, such as the temperature to have a enough fluid phospholipidic

membrane of the EVs to envelop solid NPs. For example, Dumontel et al. [45] analysed the possibility to combine non-immunogenic and naturally-stable cell-derived EVs with synthetic zinc oxide nanocrystals (ZnO NCs) with the final aim to obtain a biomimetic platform for intracellular delivery of ZnO NCs. ZnO NCs have intrinsic cytotoxic properties above certain concentrations, probably due to the intracellular dissolution and release of $Zn^{2+}$ ions. These ions cause mitochondrial damage and disruption of cellular zinc homeostasis then affecting several cellular processes. These EV-mimicking NPs were called Trojan nano-horses (TNHs), to bring out the concept of biomimicry of ZnO NCs protected by the EV membrane. The EVs were extracted from KB cells by differential ultracentrifugation. ZnO NCs were functionalized with amino-propyl groups to provide a positively charged surface on the NCs, in order to improve the colloidal stability of the NCs in solution and to induce an electrostatic interaction with the negatively charged EVs surface, useful to enhance the coupling's efficiency. The coupling between EVs and ZnO NCs was carried out using two solutions containing EVs and ZnO NCs respectively. After a co-incubation at 37°C for 90 min, the sample was centrifuged at a low centrifugation acceleration, resulting in a pellet containing the first run of TNHs and a supernatant containing some uncoupled EVs which were co-incubated again with ZnO NCs. Finally, both TNHs from the first and the second run were combined to treat KB cells. This study demonstrated that the inclusion of NCs into the EVs allows colloidal stabilization of NCs in biological media and that the cytotoxic ZnO NCs can be delivered to cancer cells in a reproducible and efficient way. It is important to note that the cargo incorporation can be achieved also through the co-incubation of donor cells with the therapeutic cargo [50] [51]. This approach is named also as indirect loading, while the direct loading refers to the co-incubation of already purified EVs with the therapeutic cargo.

### 3.1.2. Active cargo-loading methods

In electroporation an electrical field is applied in a conductive solution where the EVs and cargo are dispersed [52]. The electrical field creates temporary pores in the EV membrane that allow the infiltration of the cargo in the EVs. The integrity of the EV membrane is then recovered after the cargo loading process. Tian et al. tested the feasibility of delivering the chemotherapeutic drug doxorubicin (Dox) to tumour tissue in a mouse model using engineered exosomes [53]. To reduce immunogenicity and toxicity, exosomes were isolated by ultracentrifugation from mouse immature dendritic cells (imDCs). Then, they were loaded with Dox: 100mg of engineered exosomes and 50 mg of Dox were mixed in 200 mL of electroporation buffer at 4°C. After electroporation at 350 V and 150 mF, the mixture was incubated at 37 °C for 30 min to ensure the membrane of the exosomes entirely recovered. After noting the possibility of loading the exosomes with the chemotherapeutic agent by electroporation, they demonstrated also their high antitumor activity both *in vitro* and *in vivo*. This technique can be especially used for loading large and hydrophilic molecules like siRNA and miRNA, as they cannot diffuse spontaneously through the membrane. Indeed, Alvarez-Erviti et al. first demonstrated the effective delivery of siRNA to the brain via systemic injection of exosomes in mice [43]. However, electroporation may cause RNA aggregation and exosome instability [29].

During the extrusion, the EVs and therapeutic cargo are loaded together into a syringe-based lipid extruder and extruded through membranes with 100–400 nm porous size, at controlled temperature [54]. As a result, the EV membrane is disrupted and the drug is loaded into the EVs. Deun et al. demonstrated the feasibility of mechanical extrusion to coat nanoparticles with extracellular vesicle membranes, with the ultimate aim of demonstrating that the coating of gold nanoparticles with extracellular vesicles allows to confer on the nanoparticles potential

immune-evading capacities [55]. EVs were isolated from 4T1 mouse mammary cancer cell line by size exclusion chromatography, using a discontinuous iodixanol gradient. A water suspension of EV was extruded 5 times through a 100 nm polycarbonate porous membrane using an extruder. After this first extrusion, the sample was mixed with AuNPs before being extruded 15 more time. The study, aimed to evaluate feasibility of the extrusion approach, indicated that extrusion did not alter 4T1 EVs membrane protein composition or orientation, and could be convenient as a method for functionalizing nanoparticles.

In the sonication method, EVs and therapeutic cargo are mixed together and sonicated by using a homogenizer probe [29]. As in the extrusion, the membrane integrity of EVs is compromised due to the mechanical shear stress and the cargo is allowed to diffuse into EVs. There is evidence that the integrity of the membrane can be restored [56], however irreversible damages to EVs and possible aggregation can occur [37].

In the freeze-thaw cycling approach extracellular vesicles are mixed with the cargos and then are subjected to few cycles of freezing at -80° C in liquid nitrogen and thawing at room temperature [52]. The formation of ice crystals temporarily disrupts the EV membrane allowing the encapsulation of small molecules and proteins without affecting their biological activity [57]. Nevertheless, similarly to the other techniques, freeze-thaw cycles may affect EV size and cause aggregates [37].

Saponin is used as a membrane permeabilizer to support cargo loading; it is a surface-active agent that selectively removes membrane cholesterol, thus opening pores in lipid membranes. The pores allow molecules to enter EVs. A disadvantage of saponin is the potential cytotoxic effects if residues are not fully removed prior to EV use [57].

It is very interesting to notice that in some works different loading methods are compared to find the best approach for the EV preparations [58]. Haney et al. investigated different EV-loading techniques for the development of a new exosomal-based technology for catalase delivery to central nervous system (CNS) in the treatment of Parkinson's disease (PD) [59]. Catalase is a potent antioxidant protein that is susceptible to deactivation and rapid degradation and, like other therapeutic proteins, is unable to cross the BBB following systemic administration. Therefore, it was loaded into exosomes *ex vivo* using different methods: incubation at room temperature with or without saponin permeabilization, freeze-thaw cycles, sonication, or extrusion. Exosomes were isolated from a mouse macrophage cell line using differential centrifugation. The different methods were evaluated as follows. For incubation, the exosomes from Raw 264.7 macrophages were diluted in PBS then catalase solution was added to incubate them at RT for 18 h. For saponin treatment, a mixture of catalase and exosomes was supplemented with 0.2% saponin and placed on shaker for 20 min at RT. For freeze and thaw method, the catalase and exosomes were incubated for 30 min, then rapidly freezed at−80 °C, and thawed at RT, for three times. The exosomes and catalase subjected to sonication were sonicated, cooled down on ice for 2 min, and then sonicated again. In the end, to test the extrusion technique, catalase and exosomes mixture was extruded for 10 times through an extruder with 200 nm pores diameter. Haney et al. evaluated how the loading efficiency and stability of the catalase-loaded exosomes (exoCAT) are affected by the preparation method. They observed that sonication and extrusion enabled catalase diffusion across the lipid bilayers and resulted in high loading efficiency, probably due to the extreme deformation of the exosomal membrane. The same occurred with saponin permeabilization, probably because saponin may selectively remove membrane-bound cholesterol of exosomes so creating pores in the exosomal lipid bilayers and therefore promoting catalase incorporation. Furthermore, the exoCAT obtained by sonication, extrusion and saponin treatment showed the preservation of the enzymatic activity of the catalase, so avoiding degradation of proteases, and

a prolonged and sustained release. Regarding the delivery of therapeutics to the target cells, this study demonstrated the extraordinary ability of exosomes to target cells and deliver their load to neurons. Compared to traditional nanocarriers for PD therapy such as liposomes [60] or PGLA nanoparticles [61], exosomes accumulated in very higher levels in PC12 cells, that are a common model for *in vitro* evaluation of drug neuroprotective effects. Interesting, accumulation levels varied based on the technique used for loading. In particular, sonicated exosomes showed the greatest uptake in neurons compared to exosomes obtained by incubation at RT or by freeze/thaw cycles. Hypothetically, the deformation in exosomal membranes due to sonication determined an alteration of surface proteins, thus obtaining better interactions with target cells. In accordance with the loading efficiency, exoCAT obtained by sonication and extrusion provided the most potent neuroprotection against oxidative stress *in vitro*. This could be due to the mechanism of intracellular trafficking of the different exosomal formulations in cell targeting, but they are still under investigation. Finally, specific exoCAT formulations significantly decreased brain inflammation and increased neuronal survival in a PD mouse model. The mechanism was not completely understood, but they hypothesized the encapsulation of catalase into exosomes may preserve catalase enzymatic activity, prolong the circulation time, reduce immunogenicity, and improve its interaction with epithelial cells, thus improving also therapeutic effects in PD. Indeed, two exosomal formulations obtained by saponin treatment and sonication were selected as the most efficient ones that could provide high loading, sustained drug release and potent neuroprotection in 6-OHDA mouse model. Therefore, this study showed that the exosome-mediated delivery of catalase could lead to a viable therapy for patients with PD.

## *3.2. Surface functionalization: an overview*

The functionalization of the EVs surface is carried out to improve targeting abilities, biodistribution and therapeutic applications of EVs. However, it is necessary to pay attention to the reaction conditions: excessive temperatures, pressures, or solvent exposure can cause membrane disruption and surface protein denaturation; the exposure to low or high salt concentrations can lead to osmotic stress. Furthermore, modifications or inadequate reaction conditions can induce vesicle aggregation [62].

### 3.2.1. Post-isolation methods

Several methods are used to modify the surface of EVs after their isolation and can be divided in covalent and non-covalent chemical modifications [1]. The non-covalent approaches gently provide membrane modifications based on mild reactions, such as electrostatic interactions, receptor-ligand bindings, and hydrophobic insertions [62]. For this purpose, Nakase et al. used Lipofectamine, a commercial transfection reagent containing cationic lipids, which adsorbs on the exosomal surface [63]. Lipofectamine adsorption increases the exosomal surface charge and so it helps the interaction between EVs and negatively charged target cells surface without any cytotoxicity. The functionalization with cationic lipids was also employed to cause the accumulation of a negative charged pH-sensitive fusogenic peptide, GALA. The presence of this peptide leads to the fusion of endosomal and exosomal membranes in cells and the subsequent cytosolic release [64]. Therefore, as it is evident, the functionalization based on electrostatic interaction combined with the application of the GALA peptide improve cellular uptake and also enhance cytosolic release of exosomal contents.

The most used covalent method is the click chemistry, also known as azide alkyne cycloaddition, because an alkyne moiety usually reacts with an azide group to form a stable

triazole linkage. This method can be easily performed as it can take place both in organic and aqueous media. It typically does not require temperature elevation but can be performed over a wide range of temperatures (0-160°C) and over a wide range of pH values (5 through 12) [65]. Furthermore, a study demonstrated that click chemistry did not alter exosome size and functionality [66]. Another common approach of surface functionalization of EVs and based on covalent bonding is the PEGylation. This method allows for reducing the immunogenicity of nanoformulated drugs by shielding the EVs with a polyethylene glycol (PEG) corona. A study by Kooijmans et al. showed that this surface modification significantly increases the EV circulation half-life in mice because it reduces recognition by the mononuclear phagocyte system (MPS) thus avoiding plasma protein opsonization [67]. Opsonization is an immune process which uses opsonins to tag foreign pathogens for elimination by phagocytes, while half-life describes the time it takes for the concentration of a substance to halve its steady-state when circulating in an organism. However, the presence of PEG corona reduces the EV-cell interaction and the cellular uptake of the EVs. This drawback can be overcome by functionalizing the distal end of the PEG chain with a targeting ligand [68] [67]. For example, Kim et al. have loaded exosomes (exo) with paclitaxel (PTX), an anti-cancer agent widely used against lung cancer, to be delivered to pulmonary metastases [69]. It has been shown that a variety of cancer types, including lung cancer, overexpresses the sigma receptor, a membrane-bound protein [70]. Aminoethylanisamide (AA) is a ligand with high affinity for sigma receptor and has been utilized to target lung cancer cells following the PEGylation of the exosomes. The authors used exosomes released by primary bone-marrow derived macrophages for *in vivo* experiments. They developed a specific procedure based on sonication and incubation to add PEG, and a similar procedure to incorporate a vector moiety with AA [58]. Experimental results showed that the obtained AA-PEG-exoPTX formulation showed an extraordinary ability to accumulate in target cancer cells. Also, the engineered exosomes possessed a high loading capacity (~33%) along with cancer targeting, potent inhibition of pulmonary metastases growth and prolonged survival compared to non-targeted exosomes.

### 3.2.2. Genetic engineering of parental cells for surface functionalization

Surface ligands can be added to EVs not only through EV post-isolation methods like click chemistry, but also through genetic engineering, wherein the cells that will produce the EVs are induced to express the protein or peptide of interest. This approach was employed by Alvarez-Erviti et al. for targeted delivery of siRNA-loaded exosomes to mouse brain [43]. More and more evidences demonstrate that small interfering RNAs (siRNAs) can be utilized as therapeutic agents. They can be employed in cancer and viral infections, but also for the treatment of a range of diseases through the silencing of genes, whose abnormal expressions contribute to the pathophysiology of the disease. For example, Alvarez-Erviti et al. used this approach to achieve knockdown of β-site APP-cleaving enzyme 1 (BACE 1), a therapeutic target for Alzheimer's disease [71]. They have utilized murine self-derived dendritic exosomes targeted with lysosome-associated membrane glycoprotein 2B (Lamp2b) to deliver GAPDH siRNA across the blood-brain barrier (BBB) in mice. They used self-derived dendritic cells for exosome production to reduce immunogenicity. Then, to ensure that systemically-injected exosomes targeted the brain *in vivo*, dendritic cells were transfected with a plasmid encoding Lamp2b [41]. This protein was attached with a targeting peptide derived from the Rabies Virus Glycoprotein (RVG), as this peptide is known to bind nicotinic acetyl choline receptor (AchR) present on neurons and the vascular endothelium of the BBB [18]. At the end exosomes were purified and loaded with GAPDH siRNA by electroporation. Intravenously injected RVG-targeted exosomes delivered GAPDH siRNA specifically to neurons, microglia, and oligodendrocytes in the brain. Moreover, researchers observed a strong knockdown of BACE

1 and a significant decrease in the levels of a major component of the amyloid plaques that are associated with Alzheimer's pathology. In a recent study, Yang et al. have found a similar approach to systematically deliver nerve growth factor (NGF) into ischemic cortex for the treatment of stroke, in a photothrombotic ischemia model [72]. NGF has a primary role in the growth, as well as the maintenance, proliferation, and survival of nerve cells [73]. Thus, NGF mRNA was loaded into RVG-modified exosomes, delivered into ischemic cortex, and translated into bioactive NGF protein in recipient cells. In particular, they engineered exosomes with RVG peptide on the surface for neuron targeting and loaded NGF into exosomes, with the resultant exosomes named as NGF@ExoRVG. By systemic administration of NGF@ExoRVG, NGF was efficiently delivered into ischemic cortex, resulting in the release of encapsulated NGF protein and de novo NGF protein translated from the delivered mRNA. Moreover, NGF@ExoRVG was found to be highly stable for preservation and functionality for a long time *in vivo*. In addition, the study revealed that the delivered NGF reduced inflammation by M2 microglia activation: a process which mediates the immune response in the CNS. In the end NGF promoted cell survival and increased doublecortin-positive cells, a cell population which expresses a marker of neuronal precursor cells. All these results suggest the therapeutic potential of engineered exosomes as vehicles for targeted therapy.

# 4. Cancer immunotherapy

Immune system is a defensive apparatus that the human body uses to fight illness. It is constituted by a complex "surveillance network" made up of several highly specialized organs and cells, shared by the lymphatic vessels, and located in various parts of the body. All of them cooperate, each with a specific role, to defend the organism and keep it healthy. The immune system keeps track of all of the substances normally found in the body. Any new substance that the immune system doesn't recognize triggers an alarm, causing the immune system to attack it. Unfortunately, cancer can commonly escape the immune system's natural defences, allowing cancer cells to continue to grow. Cancer immunotherapy is a growing field of cancer research dedicated to the development of novel cancer therapies by understanding and making use of immune pathways. Immunotherapy is a type of cancer treatment that stimulates the natural immune system to fight cancer by finding and destroying cancer cells. There are many types of immunotherapy including monoclonal antibodies, oncolytic virus therapy, T-cell therapy and cancer vaccines.

Monoclonal antibodies are highly specific molecules produced in laboratory from identical immune cells and engineered to work as substitute antibodies that target only a single site (epitope) on a single antigen and that enhance or mimic the immune system's attack on cancer cells [74]. They are designed to recognize specific protein receptors present on the surface of some cancer cells, in order to destroy certain types of cancer cells while minimizing the damage to healthy cells. When the monoclonal antibody recognizes the presence of the receptor on the surface of the tumour cell, it binds to it. In this way, it stimulates the body's immune system to attack cancer cells. It can also induce cancer cells to self-destruct or can block the receptor preventing it from binding to a different protein that stimulates the cancer growth. Some examples of commercialized monoclonal antibodies for cancer treatment or maintenance cancer therapy are trastuzumab and pertuzumab for breast cancer and rituximab for non-Hodgkins lymphoma.

Oncolytic viruses (OVs) can replicate in cancer cells but not in normal cells, leading to lysis of the tumour mass. OVs can also stimulate the immune system in order to facilitate a strong and durable response against the tumour itself. However, viruses are recognized by the immune system as pathogens and the consequent anti-viral response could represent a big hurdle for OVs. Finding a balance between anti-tumour and anti-viral immunity is a priority for researchers in the field [75].

T cell therapy requires genetic modification of patient's autologous T cells in the laboratory so that they express a receptor, called chimeric antigen receptor (CAR) specific for a tumour antigen. T cells are taken from a patient's blood, then the gene for CAR expression is added to the T cells in the laboratory. After *ex vivo* cell expansion, CAR T cells are given to the patient by infusion, then they bind the antigen on the cancer cells and kill them [76].

Lastly, cancer vaccines enable the patient's immune system to recognize and fight cancer cells more effectively [77]. This discussion will investigate cancer vaccines more in detail and in particular the role of extracellular vesicles as delivery vehicles to enhance and amplify the effect of the immune response against tumour cells.

## *4.1. What is a cancer vaccine?*

Vaccines were originally developed as prophylactic agents, administered to healthy individuals to induce long-term immunity against a pathogen and to prevent the outbreak of viral diseases.

First of all, cancer vaccines are different from the vaccines that work against viruses. Actually, cancer vaccines are classified as i) prophylactic and ii) therapeutic. In the field of immunotherapy, prophylactic vaccines are used for cancer prevention [78]. Their main purpose is to reduce the incidence and mortality of cancer; hence they are administered to healthy individuals. Furthermore, prophylactic vaccines only apply to the few virally induced malignancies, such as the vaccine against hepatitis B virus that can cause liver cancer, or the human papilloma virus (HPV) vaccination, which aims to prevent genital cancers induced by high-risk HPV strains. In contrast to prophylactic vaccines, instead of preventing disease, therapeutic vaccines are designed to boost the immune system to attack a disease that already exists. There are two aspects to take into account. Often, when the cancer is diagnosed, it is already well established and has largely evaded the control of the immune system. Moreover, the vast majority of tumours that are classically treated with surgery, chemotherapy or radiotherapy definitively disappears. In some patients, however, the tumour may relapse and become increasingly resistant to treatment [79] [80] [81]. This happens because in some cases a certain number of cancer cells can escape treatment and remain in the body, even if the anticancer therapy was initially very effective. These cells are able to reproduce the tumour even years after its first appearance, and consequently the disease becomes more difficult to eradicate with classic treatments. In this context, therapeutic cancer vaccines have been developed for patients who already have a growing, established tumour. As evidence of this, recent clinical trials propose the combined or sequential use of different therapeutic strategies to create a "multimodal" therapeutic approach [82]. For example, therapeutic cancer vaccines are administered after complete surgical removal of the tumour, followed or not by chemotherapy, in lung cancer or cutaneous melanoma patients (NCT00530634, NCT04245514, NCT02211131, NCT04330430). The main goal of therapeutic vaccines is to induce strong antigen-specific T cell responses, particularly CD8+ cytotoxic T lymphocytes (CTLs) mediated responses, with the assistance of suitable adjuvants which enhance the immune response [83]. CTLs are capable of rejecting tumour cells via recognition of tumour-associated antigenic epitopes expressed by human leukocyte antigen (HLA) class I molecules on the cancer cells [84]. The choice of antigen is the most important component of cancer vaccine design. Ideally, the antigen should be expressed specifically by cancer cells and not in normal cells, it should be present on all cancer cells such that the cancer cannot escape immune attack by downregulating the antigen, and finally it should be highly immunogenic [85]. Antigens meeting all of these criteria do not exist, but they can be divided into two general classes: tumour-associated antigens (TAAs) and tumour-specific antigens (TSAs).

TAAs are self-antigens expressed in tumour cells but may be expressed in normal cells as well. As self-antigens, T cells that bind with high affinity to TAAs are typically deleted by tolerance mechanisms. Immune tolerance is an important means by which growing tumours manipulate the tumour microenvironment with the aim of preventing elimination by the host immune system. Tolerance is the result of the so called immune checkpoints. These pathways include the presence of protein receptors on the surface of immune system cells, which, if bound to specific ligands, prevent the immune system from attacking cells indiscriminately. Often, these specific ligands are expressed by tumor cells. In this way, cancer cells are able to trigger inhibitory signals that make immune cells inert or tolerant. For example, the binding between the checkpoint proteins PD-1 on the surface of T cells and the PD-L1 inhibitory receptors on tumor cells keeps T cells from killing tumour cells in the body, by also inhibiting T cells proliferation (Figure 4.2) [86] [87]. Thus, a cancer vaccine using these antigens must be potent enough to “break tolerance”. Moreover, in many vaccine clinical trials the immune response does not seem to be strong enough to achieve significant efficacy. Indeed, Hollingsworth and Jansen [85] explain that such vaccines stimulate activation and proliferation of antigen-specific

CD8 T cells to a level of < 1% of the total circulating CD8 T cells, if compared to antiviral vaccines, which typically yield > 5% antigen-specific CD8 T cells.

Conversely, TSAs, or tumour neoantigens, are truly tumour-specific. They are recognized as foreign by the immune system, and consequently, reactive T cells are not easily eliminated by immune tolerance mechanisms [88]. These immunogenic neoantigens are the result of several hotspot mutations occurring in multiple cancer patients. Like cancer mutations, the majority of neoantigens are unique to each patient and vary depending on tumour type. For this reason, generation of a cancer vaccine against a patient's individual neoantigens requires a personalized approach. First of all, the patient's tumour genome is sequenced and mutations are identified. Next, neoantigens are predicted via computerized algorithms and possibly confirmed experimentally. At the end, a vaccine expressing the predicted neoantigens is constructed and delivered to the patient [85].

Antigens to be employed in therapeutic vaccines can be delivered to patients in different formulations and making use of different vectors, including peptide restricted epitopes from TAAs, whole tumour antigens in the form of a protein, recombinant viruses, autologous or allogeneic tumour cells, heat shock proteins transporting immunogenic peptides, or DNA constructs encoding TAAs. In addition, in order to administer the vaccine, antigenic materials can be injected directly, coupled to immunostimulatory adjuvants, or used for *ex vivo* loading of antigen presenting cells (APCs), usually dendritic cells (DCs) [89] [84].

The most popular vaccine category is peptide-based vaccine consisting of immunogenic restricted epitopes, usually from tumour-specific or tumour-associated antigens. In typical peptide vaccination protocols, the antigenic material is conjugated to a carrier protein [90]. The binding with the carrier protein helps enhance immune response by increasing the half-life of the epitope. Further, the carriers are generally known to have immunogenic properties, and thus the simple covalent binding of epitopes to these species can often be sufficient to enhance the immune response. It is clear that most cancer cells can be differentiated from healthy cells by either overexpression of certain endogenous proteins or mutation of those proteins. Thus, any gene product that is expressed differentially or in a mutated form in cancer cells is a potential vaccine target, just like the HER2 gene and its modified form HER2/neu oncogene. HER2/neu is an epidermal growth factor receptor and one of the most studied oncogenes in cancer. Human epidermal growth factor (EGF) is a protein naturally produced by the human body, which when attached to another protein, such as HER2 or CerbB2, stimulates the multiplication of cancer cells. About 30% of breast cancers develop along with the amplification of the HER2/neu gene or the overexpression of its protein product [91]. Its overexpression also occurs in other cancers such as ovarian cancer, stomach cancer, colorectal cancer and in aggressive forms of uterine cancer. Tumours characterized by HER2 overexpression are known as HER2-positive and can be highly aggressive [92]. Several peptide-based vaccines derived from the HER2 receptor have been developed in the last few years. NeuVax$^{TM}$ is a 9-amino acid peptide derived from the extracellular domain of HER2 combined with granulocyte-macrophage colony-stimulating factor (GM-CSF). It stimulates specific CD8+ cytotoxic T lymphocytes (CTLs) that recognize and destroy HER2 expressing cancer cells. Initial studies found that the peptide promotes T-cells to lyse HER2-positive cancer cell lines. Also, in mouse models, T-cells stimulated with this peptide have shown to efficiently destroy HER2 expressing cells in colon carcinoma and renal cell carcinoma [93]. Human trials demonstrated that NeuVax$^{TM}$ is well tolerated in humans, while a phase III clinical trial determined that NeuVax$^{TM}$ monotherapy does not impact breast cancer recurrence as compared to placebo (NCT01479244). In addition, NeuVax$^{TM}$ may be promising in combination therapies. Indeed, two phase II clinical trials investigating NeuVax$^{TM}$ treatment in HER2-positive breast cancer combined with trastuzumab are ongoing (NCT01570036, NCT02297698). Trastuzumab works by interfering with one of the ways in

which breast cancer cells grow and divide. In particular, it attaches to the HER2 protein, thereby preventing the human growth factor in the epidermis from reaching the neoplastic cells and, consequently, preventing their division and growth. Trastuzumab also acts as a stimulator of the body's immune cells to help them destroy cancer cells. The main objectives of these two trials are disease-free survival at 24 months and 36 months and invasive disease-free survival from time of initiation of trastuzumab maintenance therapy (trastuzumab monotherapy) to time of invasive local, regional or distant recurrence, new primary, or death due to any cause. However, the results are still not available.

GP2 is another 9-amino acid peptide derived from HER2 and capable of inducing a CTL response *in vitro*. Moreover, clinical testing demonstrated that the vaccine was well tolerated and patients demonstrating increased HER2-specific CTLs. These are just two of the peptide-based vaccines designed specifically for breast cancer treatment [90]. It is also important to mention another peptide-based vaccine, HerVaxx. It consists of a fusion peptide made of three peptides derived from the extracellular domain of the HER2 conjugated to the carrier protein diphtheria toxin [94]. In the last few months a Phase 2 HerVaxx study was updated to evaluate the overall survival and the progression-free survival and to measure the efficacy, safety and immune response in 68 patients with metastatic gastric cancer overexpressing the HER-2 protein (NCT02795988).

In the examples described so far, the vaccine is directly injected to the patient. As already mentioned, however, it is possible to administer the vaccine also through the *ex vivo* loading of dendritic cells (DCs). DCs are known to be the most effective antigen presenting cells and play a central role in coordinating innate and adaptive immune responses. DCs are antigen-presenting cells (APCs) that activate T lymphocytes through major histocompatibility complex (MHC) signalling as pictured in Figure 4.1 [95]. These properties led to many attempts in the development of DC-based vaccines [96]. In particular, these vaccinations have produced encouraging clinical results in some patients with advanced cancers.

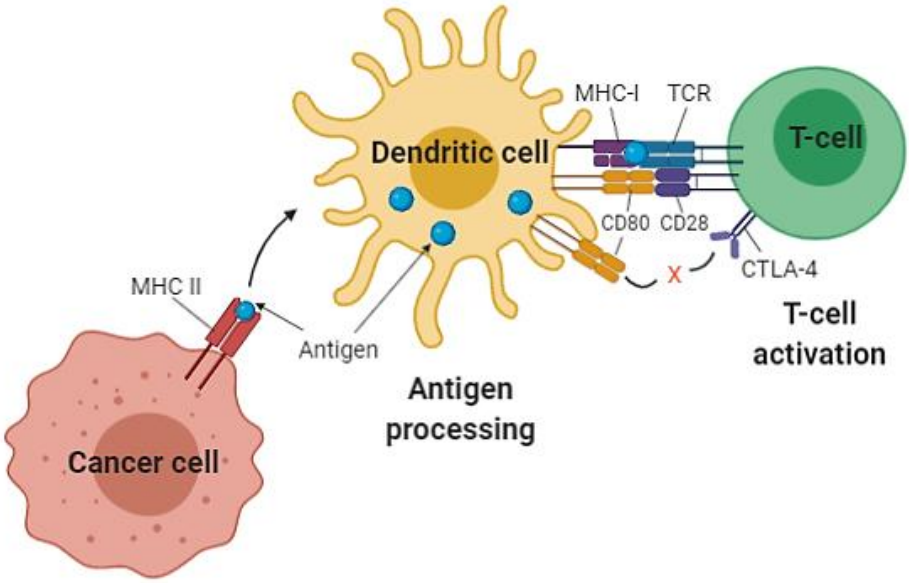


**Figure 4.1**: Initiation of the adaptive immune response to cancer by dendritic cells. The dendritic cells capture and process antigen material and then present it on the cell surface to activate naive T-cells. Once activated, T-cells begin proliferating and can attack cancer cells. Created with Biorender.com.

In 2010, the FDA approved the sipuleucel-T (PROVENGE®) for metastatic castration-resistant prostate cancer (mCRPC) [97]. Sipuleucel-T is an infusional autologous DC vaccine based on enriched blood APCs cultured with a recombinant fusion protein consisting of prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony-stimulating factor (GM-CSF), that are, respectively, an antigen expressed in prostate cancer tissue and an immune cell activator [98]. DCs are isolated from the patient's peripheral blood mononuclear cells (PBMC), loaded with tumour antigens PAP *ex vivo*, activated, and then reinfused back into the patient. In 2010, a phase III clinical trial enrolled 512 mCRPC patients to receive sipuleucel-T or placebo administered with three intravenous infusions at 2-week intervals (NCT00065442). This study demonstrated a small but significant increase in median overall survival (OS) of 25.8 versus 21.7 months and an extended 3-year survival (31.7% vs. 23.0%) in sipuleucel-T group compared with placebo group. Also, the toxicity profile was good with transient flu-like symptoms and fever being the most common side effects. However, the complexity and price of sipuleucel-T production have hindered its widespread use. Nonetheless, sipuleucel-T serves to demonstrate that autologous DC vaccines can work. Indeed, several other DC vaccines are being developed. There are different types of therapeutic candidate DC vaccines currently undergoing clinical trials for numerous types of cancer, some of them are listed in Table 4.1.

**Table 4.1**. Representative selection of the currently-active clinical trials investigating dendritic cell-based cancer vaccines

| Condition | Treatment | Clinical phase | NCT identifier |
|---|---|---|---|
| Breast cancer | HER-2 pulsed DC Vaccine | Phase I | NCT02063724 |
| Brain tumours | Autologous DCs pulsed with CSC Lysate | Phase I | NCT02010606 |
| Prostate cancer | Autologous DCs loaded with mRNA from Primary Prostate Cancer Tissue + hTERT + Survivin | Phase I/II | NCT01197625 |
| Sarcoma/Soft Tissue Sarcoma/Bone Sarcoma | DC vaccine + tumour lysate + imiquimod | Phase I | NCT01803152 |
| Brain Metastases | Personalized cellular vaccine: tumour antigen mRNA-pulsed autologous DCs | Phase I | NCT02808416 |
| Newly Diagnosed Glioblastoma | AV-GBM-1: autologous DCs loaded with autologous tumor antigens derived from self-renewing TICs | Phase II | NCT03400917 |
| Multiple Myeloma | ASCT + DC Myeloma fusion vaccine + MAb CT-011 (Pidilizumab) | Phase II | NCT01067287 |
| AML | DC AML Fusion Vaccine | Phase II | NCT01096602 |
| Advanced Breast Cancer | DCs co-cultured with CIK cells + Capecitabine monotherapy | Phase II | NCT02491697 |

Legend:
**DC= dendritic cell, CSC= cancer stem cell, hTERT= human telomerase reverse transcriptase, TIC= tumour-initiating cell, ASCT= autologous stem cell transplantation, MAb=monoclonal antibody AML= Acute myelogenous leukaemia, CIK cells= cytokine-induced killer cells.**

These studies highlight that these new strategies can become real possibilities in the fight against cancer, therefore they must be increasingly explored, as they can seriously improve and enhance the capabilities of existing cancer drugs.

## *4.2. EVs in anti-tumour immunotherapy*

The capabilities of the DCs as powerful and versatile APCs make them suitable to be the vehicles in cancer vaccines and anti-tumour immunotherapy. However, many drawbacks hinder their use in clinical treatments. DCs are a heterogeneous cellular population which comprises several subsets with different phenotypical and functional capacities. Depending on the subset and on the received stimuli, DCs can display different capacities for antigen presentation, migration, and cytokine secretion. In particular, they can induce different T cell behaviours, by polarizing them into effector or tolerogenic cells [99]. Because of this heterogeneity they can thus promote either antitumor activity or regulation of immune tolerance, which is known to be a very limiting factor in vaccine success [100]. For example, tumour cells can secret soluble immunosuppressive cytokines that could convert immature DCs into tolerogenic DCs, which may activate regulatory T ($T_{reg}$) cells. $T_{reg}$ cells generally suppress or downregulate induction and proliferation of effector T cells, by promoting tumour proliferation [101]. Another limitation in the use of DCs is also due to their difficult storage aimed at maintaining their efficacy even for long periods of time. Finally, applying such therapies across large populations is expensive and requires monitoring of well-defined quality control parameters. The use of DC-derived exosomes (Dex) has been indicated as a solution to many problems associated with DC-based immunotherapy. Dex are characterized by unique molecular composition that allows them to maintain the immunostimulatory abilities of DCs. Indeed, Dex contain MHC-I and MHC-II molecules, which can stimulate cytotoxic and helper T cells ($T_h$ cells), respectively, as well as costimulatory (CD86, CD40) and adhesion molecules (ICAMs), which can elicit strong immune responses toward cancer cells [102]. Furthermore, the lipid composition of exosomal membrane allows to storage Dex at -80°C for more than 6 months maintaining high stability. Finally, treatment with cell-free Dex may be more resistant to immunomodulatory events such as immunotolerance that occurs in tumours than other anticancer vaccines [103]. Some important preclinical studies carried out to evaluate the immunogenicity of Dex and their possibility of use in the production of new therapeutic vaccines are collected in Table 4.2.

**Table 4.2**: Preclinical studies evaluating Dex immunogenicity for cancer vaccines

| Authors | Method | Main outcomes | Refs |
|---|---|---|---|
| *Théry C. et al.* | *In vitro* | Dex can transfer functional peptide-loaded MHC class I and II complexes to DCs. | [104] |
| *André F. et al.* | *In vitro* and *in vivo* | Dex harbouring MHC class I/peptide complexes require DC for efficient priming of CTLs. | [105] |
| | *In vivo* | Dex mimic the capacity of mature DCs to initiate peptide-specific CD8+ T cell responses. | |
| *Segura E. et al.* | *In vitro* | Dex from immature DCs (imDC) and mature DCs (mDC) have different protein composition due to maturation signals. MHC class I molecules are up-regulated in mDC and reduced in mature exosomes. Molecules stimulating CD4 T cells are up-regulated in mDC and mature exosomes. | [106] |

| | | | |
|---|---|---|---|
| *Sprent J.* | *In vitro* | Peptide-pulsed Dex are immunogenic for CD8+ T cells also in the absence of APCs. | [107] |
| | *In vivo* | Peptide-loaded Dex induce high proliferative responses and CTLs induction, so priming CD8+ T cells. | |
| *Viaud S. et al.* | *In vivo* | Dex administration promotes proliferation, activation and cytotoxicity of NK cells. | [108] |
| | *In vitro* | Human Dex harbouring IL-15Rα lead to NK cell proliferation and IFNγ production | |

Exosome-based vaccines have been proven safe in multiple phase I trials in different types of cancers, so they could represent a new strategy for cancer immunotherapy. In particular, a phase I clinical study investigated the safety, feasibility and efficacy of administering autologous Dex loaded with antigenic HLA-presented peptides of melanoma-associated antigen (MAGE) to patients with advanced NSCLC [109]. MAGE gene products are of particular interest, thanks to their wide expression in many tumours and their potential to induce tumour-specific CTL responses against expressing-MAGE tumour cells, resulting in tumour cell lysis. Notably, antigen-specific CTLs induced by MAGE gene-derived peptides have proven to be highly efficacious in the prevention and treatment of various types of tumours and for this reason MAGE has been used as a target for tumours [110]. In this phase I trial, Morse et al. isolated Dex by ultracentrifugation from peripheral blood mononuclear cells and loaded them with MAGE-A3, -A4, -A10, and MAGE-3DPO4 peptides. Patients were enrolled into three cohorts that varied in the method of peptide loading and concentration. In particular, peptides were loaded either directly into Dex following purification from the DC culture or indirectly into cultured DCs. The quantity of Dex administered to the patients in each cohort was identical: $1.3 \times 10^{13}$ MHC class II molecules in a volume of 3 mL with a combination of subcutaneous (90% of the volume) and intradermal (10%) injections weekly for 4 weeks. Dex therapy administered to 9 patients resulted well-tolerated without evidence of toxicity or autoimmune reactions. By contrast, *in vitro* immunologic analysis detected an increase in T cell activity in only one of tested patients, probably due to $T_{reg}$-mediated suppression of immune cells. In 2/3 patients who had analysable samples, an increase in $T_{reg}$ was observed after the conclusion of Dex therapy. However, this was not confirmed due to the small number of samples available for the analysis. Other possible explanations were related to non-optimized or low-sensitive assays, inadequate antigen presentation, or the lack of antigen-specific T cells in the circulation. Actually, it was hypothesized that the immunologic activity of Dex might be due to activation of natural killer (NK) cells. NK cells are a type of cytotoxic lymphocyte fundamental in the innate immune system. They have the ability to recognize tumour or infected cells in the absence of antigen-specific cell surface receptors and kill them by producing cytokines [111]. Morse et al. observed an increase in NK activity following immunization in two over four of the analysed patients. They explained that cytokines released in response to Dex therapy could have caused activation of NK cells or that Dex could have directly activated NK cells. Finally, the main clinical results were a very good disease stability, which was observed in two patients who had disease progression at the start of the study, and continued disease stability over 12 months in two of four initially stable patients. A similar study was carried out in 15 patients with stage III/IV malignant melanoma. MAGE peptides were loaded either directly into autologous DC derived-exosomes or indirectly into cultured DCs and then used as cancer vaccine [50]. Patients received a 4-weeks vaccination with antigen given intradermally (1/10th) and subcutaneously (9/10th) every week. All patients underwent assessment of tumour status

at 2 weeks after the fourth exosome vaccination. Dex-based cancer vaccine was well-tolerated, and in 4 patients some extent of therapy response was observed; in particular, stable disease was observed in two patients receiving the highest dosage of directly-loaded exosomes. While one partial response and a minor response were observed in 2 patients who were then subjected to a continuation therapy allowing for stabilization. However, as in the previous study, no significant T cell response was observed.

Despite the clinical benefits of DC exosome-based vaccination in the treatment of cancer, not all patients are responding to treatment due to all the possible obstacles described above. Tumour-derived exosomes (Tex) are another type of exosomes investigated for the improvement of antitumor immune responses. Tex contain tumour-associated antigens expressed in the parental tumour cells and major histocompatibility complex (MHC) class I molecules [112]. Thus, Tex could present tumour antigens to DC and induce $CD8^+$ T cell-dependent antitumor immune responses. In addition, tumour cells release a larger amount of exosomes compared to exosomes secreted from healthy cells. As a result, they seem to be a source of tumour antigens for antitumor immunotherapy and could represent a novel type of cell-free cancer vaccine [113] [114]. However, previous studies reported that Tex can promote immune escape through different immunosuppressive mechanisms as the accumulations of $T_{reg}$ cells in the tumour microenvironment or the expression of toll-like receptors (TLR) on the tumour cell surface [115]. Indeed, generally Tex are mediators of immune cell-tumour cell communication and regulators of immune responses, so they are involved in the progression, regression and drug resistance of tumours and contribute to metastasis development [116]. Many studies verified the feasibility and functionality of Tex to stimulate immune responses against cancer in mouse models. For instance, a study by Bu et al., showed that a single vaccination with L1210 leukaemia-released exosomes not only prevented tumour formation but also elicited protection against tumour growth in syngeneic mice [117]. Treated mice and control group, constituted by unvaccinated mice, were stimulated with L1210 tumour cells 2 weeks after immunization with L1210 exosomes. Regarding tumorigenesis, all unvaccinated mice displayed tumours in less than 10 days, whereas 87.5% of the exosome-vaccinated mice were tumour free. At the same time, a significant protection against tumour growth was observed, after 60 days, at a level of 85% with 5 μg vaccination dose and at 60% when the dose was reduced to 2.5 μg. Also, exosomal vaccine produced significantly stronger anti-L1210 CTL activity than the control group. Other studies on leukaemia vaccination proposed Tex as a possible antigen source for DC-based vaccination. An important advantage of loading DC with Tex relies on the processing of the Tex tumour antigens and the presentation of tumour antigen peptides through MHC, which strongly facilitates the capture of tumour-peptide specific $CD8^+$ T cells. Moreover, the enrichment of tumour antigens in Tex and the equipment of Tex with markers that facilitate the uptake by DC highlight the possibility to enhance the activation of CTL, combining the advantages deriving from the combined use of Tex and DCs. Indeed, S. Hao et al. demonstrated that exosome-pulsed DCs induced stronger antitumor immunity than exosomes and DCs alone [118]. Gu et al. demonstrated *in vitro* that suppression of Th activation by Tex is circumvented in the presence of DC, then they proposed the vaccination with Tex-loaded DC (DC-TEX) by loading patients' DCs with Tex circulating in the peripheral blood [119]. They tested the vaccine in a murine myeloid leukaemia. They demonstrated that Tex-pulsed DCs significantly enhanced the survival of vaccinated mice and induced CTL immune responses more effectively. They also verified that, beside tumour antigens, the availability of Tex markers facilitating uptake by DC contributes to pronounced CTL activation. Other studies that have evaluated the possibility of using extracellular vesicles as a vehicle in anti-cancer vaccines are shown in Table 4.3.

**Table 4.3**: Preclinical studies investigating the use of EVs in cancer vaccines

| Therapeutic agent | Condition | Outcome | Refs |
|---|---|---|---|
| Irradiated C6 glioma cell-derived MVs (IR-MVs) | Malignant C6 glioma | *In vivo* vaccination with IR-MVs promotes antitumor immune response leading to the apoptosis of glioblastoma cells and increases Th cells and CTL infiltration into the tumour. | [120] |
| DC-derived-exosomes functionalized with costimulatory molecules, MHCs, antigenic Ovalbumin peptide and anti-CTLA-4 antibody (EXO-OVA-mAb) | B16-OVA melanoma tumour model | Exosomes are targeted to T cells *in vivo*. EXO-OVA-mAb are able to effectively prime T-cell activation and proliferation, *in vitro* and *in vivo*. The fraction of memory T cells is increased in mice treated with vaccination. The antitumor efficacy is confirmed by the infiltration of both CD4 + and CD8 + cells and the CTLs/Treg ratio within the tumour site of vaccinated mice. | [121] |
| Interferon-γ-modified prostate cancer cell-derived exosomes | RM-1 prostate cancer | Vaccine induces macrophages differentiation and the production of antibodies, reduces tumour angiogenesis and metastasis rate, inhibits tumour growth and prolongs survival time of mice with metastatic prostate cancer. | [122] |
| Interferon-γ-modified prostate cancer cell-derived exosomes + IFN-γ-modified RM-1 cell vaccine | RM-1 prostate cancer | Exosomal vaccine improves the T cell response generated by the tumour cell vaccine and downregulates in the expression of IDO1 and PD-L1 immune checkpoints. Combination therapy show the highest tumour-specific cytotoxic activities compared to vaccine monotherapies and tumour growth is significantly suppressed. | [122] |
| Mature DCs pulsed with ovalbumin protein-pulsed DC-derived exosomes (EXO-pulsed DCs) | B16-OVA melanoma tumour model | EXO-pulsed DCs stimulate CD8+ T-cell proliferation and differentiation into CTL effectors *in vitro* and *in vivo*. EXO-pulsed DCs induce stronger immunity against lung tumour metastases and can eradicate established tumours. They also induce strong long-term OVA-specific CD8+ T-cell memory | [123] |

As described in the second chapter, exosomes, and more broadly EVs, are also natural carriers of RNA and can be employed to deliver siRNA in silencing of genes for cancer treatment [124] [125]. In addition, it was observed that silencing of immunosuppressive genes through siRNA combined with immune checkpoint blockade therapy provides a promising method in cancer immunotherapy [126] [127]. As already mentioned, immune checkpoint blockade plays a key role in preventing the interruption of immune responses. Actually, immune checkpoint inhibitors, such as monoclonal antibodies against programmed death 1 (PD-1), inhibits immunosuppressive molecules and restores the ability of the CTLs to kill cancer as shown in Figure 4.2 [128].

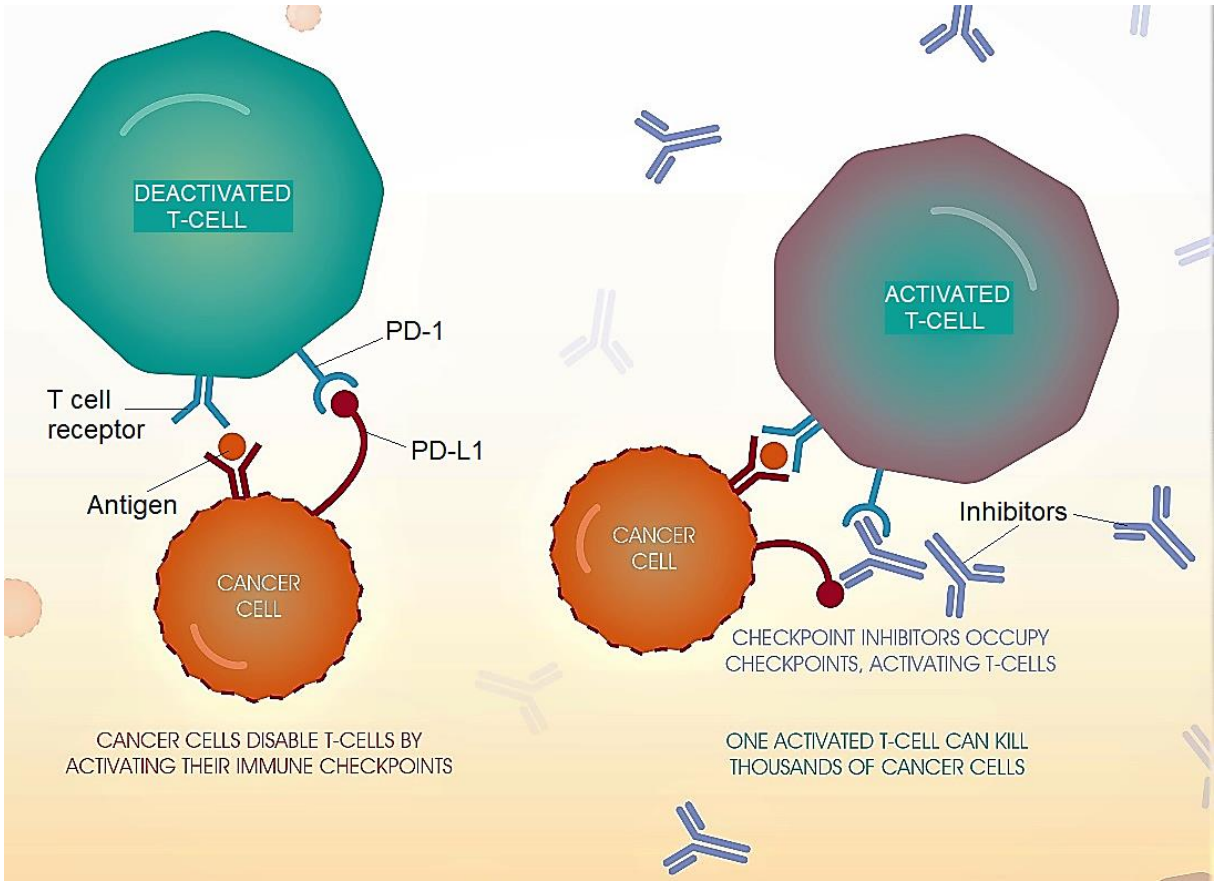


**Figure 4.2**: Checkpoint inhibitors are able to "break" the immune tolerance. (Left) Cancer cells are able to deactivate T-cells at their immune checkpoints. (Right) Checkpoint inhibitors occupy checkpoint binding sites, preventing cancer cells from switching off T-cell activity so they can attack cancer cells. Figure adapted from: "*Nobel Prize 2018 elevates awareness of immunotherapy research",* graphic by Shireen Dooling via Arizona State University website.

For all these reasons, Matsuda et al. used extracellular vesicles (EVs) for intrahepatic delivery of small interfering RNA (siRNA) to directly target β-catenin in hepatocellular carcinoma (HCC) [129]. β-catenin is involved in the signalling pathway of cell proliferation and often its alteration causes the development of carcinomas. The Wnt/β- catenin pathway, in particular, has been identified as an important oncogenic contributor to immune escape because its activation is frequently associated with poor spontaneous T cell infiltration across most human cancers [130] [131]. By taking into account that sensitivity to anti-PD-1 therapy needs the presence of tumour antigen-specific T cells within tumour tissue, a poor or even absent T-cell infiltration can result in immune deserts and weak response to immunotherapy. Moreover, mutations in gene encoding β-catenin were identified among the most frequent alterations associated to the development of HCC [132] [133]. Thus, in this study, together with the β-catenin siRNA-loaded EVs, also anti-PD-1-based therapy were systemically administered in order to reduce the tumour growth and at the same time, to enhance the therapeutic response to immune checkpoint inhibitors. A synthetic transgenic model of hepatocellular cancer was generated by inducing the co-expression of c-tyrosine-protein kinase Met (cMET) and mutant β-catenin in mouse livers through hydrodynamic injection (HDI) of DNA and plasmids [134]. The EVs were derived from bovine milk, which is a safe and scalable source of EVs [135]. First, the therapeutic effect of anti-PD-1 was evaluated. Three weeks after HDI, two groups of mice bearing HCC were respectively treated with 250 μg/mouse of anti-PD-1 and with phosphate-buffered saline (PBS) for control measurements, for 2 weeks. The transfection of

*Gaussia luciferase (g-luc)* and its expression level allowed to evaluate the efficacy of therapeutic interventions on tumour growth in terms of relative luminescence units [136]. It was observed a reduced rate of tumour growth determined as a change in g-luc expression over a 6-week period after the start of treatment compared with that observed in the control group. Also, the survival of mice receiving anti-PD-1 was extended with median survival of 119 days compared with 96 days in controls. To evaluate the efficacy of combined treatment with both therapeutic EVs (tEVs) and anti-PD-1, four groups of mice bearing HCC received different treatments three weeks after HDI for a period of 2 weeks. The first group received 250 μg of anti-PD-1 injected intraperitoneally 3 times per week (n = 11). The second group received tEVs ($2 \times 10^{12}$ particles/body) injected intravenously once every 3 days (n = 10) for five doses, and the third group both anti-PD-1 and tEVs (n = 10) in the same way. Last group was used as control and did not receive any treatment. Matsuda et al. first demonstrated the *in vitro* efficacy of β-catenin siRNA delivery via EVs, by incubating HepG2 cells with siRNA-loaded EVs. Through immunoblot analysis, they observed that the delivery of β-catenin siRNA, by means of EVs, decreased the β-catenin protein expression. Then, they evaluated the effect of siRNA delivery *in vivo* on the response to anti-PD-1 therapy. The tumour growth rate between 3 and 6 weeks was reduced with anti-PD-1, tEVs, or both. However, at 4 weeks after the end of treatment, a relapse was noted by an increase in g-luc expression in 38% of the anti-PD-1 group and 100% of the tEV group. By contrast, no relapse was observed in mice treated with both anti-PD-1 and tEVs, wherein a sustained reduction of growth rate (between 3 and 12 weeks) was noted, also greater than that with either treatment alone. Therefore, this confirmed that targeting an oncogenic factor can enhance the efficacy of anti-PD-1. Ultimately, the combination treatment resulted associated with the greatest degree of CD8+ T-cell infiltration within the tumour microenvironment. Moreover, it was observed that inhibition of β-catenin signalling in HCC may enhance activation of antitumor-specific T cells, inducing the generation of CD8+ effector T cells and their infiltration into the tumour microenvironment and preventing CD8+ T-cell exhaustion following an initial response to anti-PD-1 therapy. However further research is needed to clarify the molecular mechanism by which β-catenin can enhance tumoral CD8+ infiltration. The Table 4.4 collects currently active or completed clinical trials investigating the use of EVs-based immunotherapies.

**Table 4.4**: Currently active or completed clinical trials on EVs-based immunotherapies

| Condition | Treatment | Clinical phase | NCT identifier |
|---|---|---|---|
| Non-Small-Cell Lung Cancer | Tumour Antigen-loaded Dex | Phase II | NCT01159288 |
| Colon cancer | Curcumin conjugated with plant exosomes | Phase I | NCT01294072 |
| Metastatic pancreatic adenocarcinoma | iExosomes (MSC-derived Exosomes loaded with siRNA targeting $KRAS^{G12D}$) | Phase I | NCT03608631 |
| Malignant Pleural Effusion | MTX-ATMPs | Phase II | NCT02657460 |
| Malignant Pleural Effusion | Microparticles packaging methotrexate[1] | Not applicable | NCT04131231 |

[1]Treatment of malignant pleural effusion (MPE) in patients with advanced lung cancer or breast cancer. MSC= mesenchymal stem cells, MTX-ATMPs= methotrexate-autologous-tumour-derived microparticles.

## Conclusions

In recent years the study of extracellular vesicles has intensified considerably, leading to the achievement of important results in the application of extracellular vesicles for gene and drug delivery. In particular, exosomes have proved to be the ideal candidates to be used as therapeutic delivery vehicles. Based on this, the use of extracellular vesicles has also been extended to the field of cancer immunotherapy. Immunotherapy is a type of cancer treatment that stimulates the immune system to attack and destroy cancer cells. A particular form of immunotherapy is represented by therapeutic anticancer vaccines. Among the different types of cancer vaccines investigated, the most promising are the cancer vaccines based on dendritic cells, as these cells facilitate the triggering of the immune response. Several clinical trials are currently active to evaluate the efficacy of therapeutic cancer vaccines against various forms of cancer. As shown by numerous pre-clinical tests, dendritic cell-derived exosomes can also be used in the production of cancer vaccines. Clinical trials have confirmed the safety and feasibility of exosome-based cancer vaccines; however, some studies have not been fully satisfactory. In fact, vaccines are generally well tolerated by patients undergoing treatment but often no significant immune response is observed. This indicates that significant progress has been made in building safe delivery vehicles but, at the same time, the clinical efficacy of extracellular vesicles based-cancer vaccines remains to be determined. The exosomes derived from tumour cells have been proposed as an alternative to the previous ones, as they are able to improve the antitumor immune responses. Nevertheless, some studies have shown that exosomes derived from tumour cells could be involved in processes that promote tumour proliferation, therefore, they are not preferable. To overcome these drawbacks, the possibility of producing cancer vaccines based on dendritic cells loaded with exosomes derived from tumour cells has recently emerged, but they are still under investigation. The use of extracellular vesicles in immunotherapy therefore seems to be hindered only by technological problems and not by qualitative ones. Therefore, an ever-increasing effort in this direction could lead to tangible results and above all to a new and innovative way to fight cancer.

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