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Federico Coccozza

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Unravelling the potential of subtypes of extracellular vesicles and particles as protein carriers for future therapeutic applications

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Dirigée par Clotilde THÉRY

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ABSTRACT

Title: Unravelling the potential of subtypes of extracellular vesicles and particles as protein carriers for future therapeutic applications

Abstract: Extracellular vesicles (EVs) and other extracellular nano-particles (ENPs) are secreted by cells to the extracellular medium and they have been identified as important mediators of communication with other cells and the surrounding extracellular matrix. EVs carry various cargoes, including proteins, that can deliver signals to induce physiological changes in recipient cells. Because of this and other properties, EVs are actively investigated for use as therapeutic tools.

In this work we aimed to compare the potential as future therapeutic agents of different EVs subtypes, non-EV extracellular particles and proteins in 2 different pathological contexts: viral infection and anti-tumor immune responses.

In the first, we evaluated the potential of bioengineered EVs exposing a recombinant protein, ACE2, as compared with the non-EV associated protein, to serve as decoy for viruses, in particular SARS-CoV-2. We demonstrated that ACE2-EVs were efficient decoys for SARS-CoV-2 S protein-containing lentivirus. Reduction of infectivity positively correlated with the level of ACE2, was much more efficient than with soluble ACE2 and further enhanced by the inclusion of TMPRSS2, a protease involved in the priming for membrane fusion of the virus.

In the second, we separated and compared the potential of tumor-derived EV subtypes, among which we unexpectedly identified viral-like particles (VLPs) from endogenous retro-viruses, and other ENPs, to transfer antigens and modify antigen-presenting cells. We evidenced that the ENPs were poorly captured and did not affect DCs. sEVs specifically induced DC death. The mixed lsEV/VLP preparation were the most efficient to induce DC maturation and antigen presentation, followed by VLPs.

Our results highlight the importance of identifying the subtypes of EVs that carry the function of interest, to optimize any future use of EVs as therapeutic devices.

Keywords: extracellular vesicles, cancer, antigen presenting cells, SARS-CoV-2, endogenous retroviruses, extracellular nano-particles.

RÉSUMÉ

Titre: Détermination du potentiel des sous-types de vésicules et de particules extracellulaires comme porteurs de protéines en vue de futures applications thérapeutiques

Résumé: Les vésicules extracellulaires (VEs) et autres nanoparticules extracellulaires (ENP) sont sécrétées par les cellules dans le milieu extracellulaire et ont été identifiées comme des médiateurs importants de la communication avec d'autres cellules et la matrice extracellulaire qui les entoure. Les VEs contiennent diverses molécules, y compris des protéines, qui peuvent délivrer des signaux pour induire des changements physiologiques dans les cellules réceptrices. Du fait de cette propriété de communication intercellulaire particulière, les VEs sont activement étudiées pour éventuellement être utilisées à des fins thérapeutiques.

Dans ce travail, nous avons cherché à comparer les fonctions de différents sous-types de VEs, des particules extracellulaires non VEs, et des protéines dans deux contextes pathologiques différents : l'infection virale et les réponses immunitaires anti-tumorales.

Premièrement, nous avons évalué les VEs exprimant à leur surface une protéine recombinante, ACE2, reconnue par un virus, le SARS-CoV-2, par rapport à la même protéine non associée aux VEs, pour servir de leurre au virus, et ainsi réduire l'infection. Nous démontrons que les ACE2-EVs sont des leurres efficaces pour les lentivirus contenant la protéine S du SARS-CoV-2. La réduction de l'infectivité est positivement corrélée avec le niveau d'ACE2, est beaucoup plus efficace qu'avec l'ACE2 soluble et est encore renforcée par l'inclusion de TMPRSS2, une protéase impliquée dans l'amorçage de la fusion membranaire du virus.

Deuxièmement, nous avons séparé et comparé les sous-types de VEs dérivées de tumeurs, parmi lesquelles nous avons identifié une nouvelle sorte: des rétoparticules virales (VLP) provenant de rétrovirus endogènes, et d'autres particules extracellulaires, pour leur capacité à transférer un antigène et à moduler l'activation des cellules présentatrices d'antigènes. Nous mettons en évidence que les ENP sont faiblement capturées et n'affectent pas les DCs. Les sEVs induisent spécifiquement la mort des DCs. La préparation mixte lsEV/VLP est la plus efficace pour induire la maturation des DCs et la présentation des antigènes, suivie par les VLP.

Nos résultats illustrent l'importance d'identifier le(s) sous-type(s) de particule(s) portant la fonction d'intérêt, avec le but d'optimiser de futures utilisations de VEs à visée thérapeutique.

Mots clés: vésicules extracellulaires, cancer, cellules présentatrices d'antigènes, SARS-CoV-2, rétrovirus endogènes, nanoparticules extracellulaire.

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ABBREVIATIONS

ACE2: angiotensin converting enzyme 2
AF4: asymmetrical flow field-flow fractionation
AFM: atomic force microscopy
ALIX: ALG-2-interacting protein X
APCs: antigen presenting cells
ApoBDs: apoptotic bodies
ARMMs: ARRDC1-mediated microvesicles
ARRDC1: arrestin domain-containing protein 1
ATGs: autophagy-related genes
ATP: adenosine triphosphate
DAMPs: damage-associated molecular patterns
DCs: dendritic cells
DNA: deoxyribonucleic acid
dUC: differential ultracentrifugation
ENPs: extracellular nano-particles
ESCRT: endosomal sorting complexes required for transport
ERVs: endogenous retro-viruses
EVs: extracellular vesicles
FASL: Fas ligand
FC: filter concentration
GM-CSF: granulocyte-macrophage colony-stimulating factor
GO: gene ontology
HIV: human immunodeficiency virus
HMGB1: high-mobility group box 1
HSP: heat shock protein
ICD: immunogenic cell death
IFN: interferon
IL: interleukin
Kif: kinesin-like protein
MCT-1: monocarboxylate transporter-1
M-CSF: macrophage colony-stimulating factor

MFG-E8: milk fat globule-EGF factor 8
MHC: major histocompatibility complex
MLV: murine leukemia virus
MVB: multi vesicular body
nano-FCM: nano flow cytometry
NK: natural killer
NKG2D: natural killer group 2D
OMVs: outer membrane vesicles
PAMPs: pathogen-associated molecular patterns
PCR: polymerase chain reaction
PQS: pseudomonas quinolone signal
PS: phosphatidylserine
RNA: ribonucleic acid
SF: sequential filtration
SMAPs: supramolecular attack particles
SPADE: spanning-tree progression analysis of density-normalized events
TEM: transmission electron microscopy
TfR: transferrin receptor
TMPRSS2: transmembrane serine protease 2
VLPs: viral-like particles

INTRODUCTION

The high complexity of multicellular organisms and their level of organization in tissues, organs and systems (immune, digestive, nervous, etc.) requires the interaction and coordination of their fundamental units: the cells. Sensing and delivery of signals between cells is known as intercellular communication and comprises a range of diverse molecular and biophysical mechanisms. This range extends from direct contact between the membranes of the cells, in which their surface proteins or glycoproteins can interact depending on the ligand-receptor specificities, and the signals can be transduced to the core of the cells inducing biological changes, to indirect contact through the secretion of factors that diffuse in the extracellular medium and can reach its own producing cell (autocrine), other cell (paracrine) and distant targets, sometimes traveling through vessels and arriving to other organs (endocrine) (Raposo & Stahl, 2019). The secreted factors specialized in intercellular communication have been studied extensively and are categorized as hormones, cytokines, chemokines, growth factors and neurotransmitters, but also other secreted proteins, lipids, nucleic acids and carbohydrates can alter the surrounding neighbor cells.

Some decades ago, in the late 60's and 70's, extracellular vesicles (EVs), initially called "platelet dust", then "vesicles", then "exosomes", were observed in the extracellular medium (Anderson, 1969) and described as ways of disposal of undesired compounds for the cell (Harding et al., 1983a; Johnstone et al., 1987). But after some years of research, besides this function, EVs were also recognized as a new type of messenger that allowed cells to communicate. Basically, EVs are a portion of the cytosol of the originating cells enclosed by a lipid bilayer coming from a membrane of the same cell (internal compartment or plasma membrane) that are released to the external medium. In particular, differently to the other secreted factors, they are a nano- to micro-size packages of information, carrying a complex selection of lipids, proteins, genetic material and metabolites.

More recently, and together with the development of more sophisticated methods of isolation and characterization of EVs, the scientists in the field got more interested in the study of EVs heterogeneity. EVs are not a single population in terms of composition and physicochemical properties, since their content and shape depend on the place where they originated: from which cell type, in which state they were and from which subcellular compartment the EV budded. Heterogeneity also arises from the mechanism of generation: several molecular mechanisms are described for the EVs biogenesis and they leave traces and make a selection of the components that the EV will carry. Also, there is not an effective and robust tool for the

complete inhibition of EV secretion, only a few drugs exist that do it partially (Catalano & O'Driscoll, 2019). So, the main way to study the function of EVs is by isolating and using them in a certain experimental set up. And as they are not the only secreted entity in the extracellular medium of the studied cells (conditioned medium or biological fluids) and the isolation methods have different rates of purity and recovery, the study of the EV heterogeneity also, more or less implicitly, implies the study of other co-isolated factors or contaminants. For example, 2 new types of extracellular non-vesicular nano-particles called exomeres and supermere were isolated and characterized from several conditioned media and body fluids in 2018 and 2021 (H. Zhang et al., 2018; Q. Zhang, Jeppesen, Higginbotham, Graves-Deal, et al., 2021) expanding our awareness of the diversity in the cells secretome.

Therefore, the study and comparison of the subtypes of EVs, ENPs and other secreted factors may deepen our knowledge on the mechanisms of intercellular communication. It would give us a better outlook and help us to find the main secreted molecule, particle or vesicle responsible for each biological function. Such studies are important broadly, because these are the mechanisms that cells use to communicate both in physiological and pathological conditions. This understanding could bring us the possibility to exploit specific candidates for therapeutic applications.

We focused our studies in the analysis of functional proteins carried on EVs and ENPs, since they are the major effector macromolecules for cellular biology and physiology, even though we are aware that EVs and ENPs are also carriers of other functional and very important macromolecules, such as nucleic acids (RNAs, DNAs), lipids, glycoproteins, metabolites, etc. We choose 2 different pathologic scenarios: viral infections and cancer biology, where we envision a possible future therapeutic application. In the first set up we tested EVs and soluble proteins as decoys of viral infection of SARS-CoV2; and in the second we assessed the capacities of tumor EVs and ENPs as antigen carriers and immune modulators of antigen presenting cells.

I will start by doing an overview of the EVs from their discovery, definition, to some of their functions and the ongoing developments of their therapeutic applications. I will then follow by describing the current knowledge in subtypes of EVs, ENPs and endogenous retroviral particles, including the criteria of subtype definition and the role of the method of isolation and characterization. And finally, I will present the EVs in the two previously mentioned contexts: EVs as decoys of viral infection and tumor EVs as immune modulators.

1. Extracellular vesicles

History and definition of the EVs

The field of EVs has evolved greatly during the last 3 decades, including changes in its comprehension and nomenclature. Therefore, doing an overview of the history of the research and the milestones reached could help to understand the complexity and challenges in this area of biology (Couch et al., 2021).

EVs are nanoparticle structures secreted to the environment by virtually all cells. They are delimited by a lipid bilayer and contain components of the cell that secreted them (**Figure 1**).

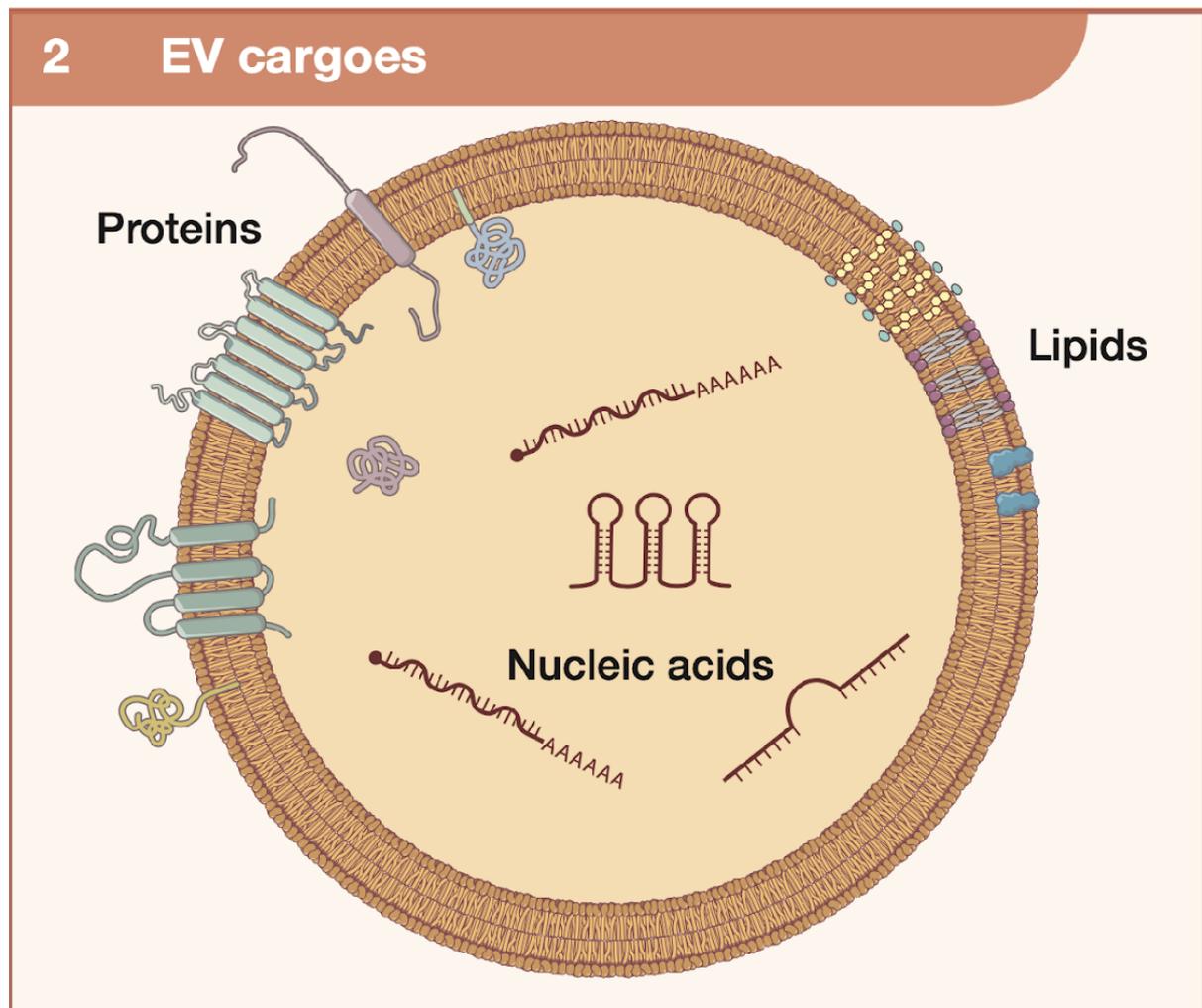


Figure 1: Scheme of an EV and its content.

Source: (Cocozza, Grisard, et al., 2020), see Annex 1.

The first records of vesicular structures found in the extracellular medium of cells are from the late 60's, produced by platelets (Wolf, 1967), cartilage (Anderson, 1969) and other body fluids (Crawford, 1971; Ronquist & Brody, 1985). They were initially named "platelet dust", then "microparticles" and "vesicles". But it was not until the 80's that a more specific function and secretion mechanism for the EVs was found, when the groups of Johnstone and Stahl published

complementary papers about the maturation of reticulocytes. In this works, the loss in the plasma membrane of the transferrin receptor (TfR) was shown to occur through a two-steps mechanism involving first endocytosis of TfR into compartments of the endocytic pathway, ultimately reaching the multi-vesicular body (MVB) and being targeted into its internal vesicles, followed by the release of these vesicles called “exosomes” by fusion of the MVB with the plasma membrane (Harding et al., 1983b; Pan & Johnstone, 1983). In conclusions of these observations, the authors attributed to exosomes the function of waste disposal of undesired proteins of the cell.

After this discovery, during the 80’s and increasingly in the 90’s, several works were published showing cellular and physiological properties and functions for EVs, including the first evidence of their capacities of altering other cells, that is to say, cell-to-cell communication. In particular, it was shown that EVs from B cells were able to present MHC-peptide complexes to T lymphocytes (Raposo et al., 1996). Then it was shown that the EVs from tumor antigen-pulsed dendritic cells were able to eradicate tumors by an antigen-specific and immune system-dependent mechanism (Zitvogel et al., 1998). These studies allowed thinking of possible therapeutic applications for the EVs and led to the first clinical trials some year later (Escudier et al., 2005).

In the decade following the year 2000, the research in EVs intensified exponentially with demonstration of their implications in cancer (Wolfers et al., 2001) and the immune system, and even more with the discovery of the capacity of EVs to transfer functional nucleic acids between eukaryotic cells (Ratajczak et al., 2006; Skog et al., 2008; Valadi et al., 2007).

During the decade of 2010, the increasing community of researchers in the EV field got more organized, starting to meet and creating in 2011 the International Society of Extracellular Vesicles (ISEV). This allowed better understanding, and initiated comparison and discussion of the great amount of information that sometimes was contradictory or confusing. This led, even, to providing specific guidelines and minimal requirements to perform research in the field (Lötvall et al., 2014; Théry et al., 2018).

Beyond all this progress in the research of EVs, there are still several unsolved biological questions and technical limitations that keep open the door to new discoveries (van Niel et al., 2022). This includes, for example, the lack of established robust markers for the different EV subtypes, the scarcity of suitable *in vivo* models for validation of the physiological relevance of EVs functions in the organisms, the limited possibility of analysis of subpopulations at single EV level, and the potential of improvement of the current techniques for EV isolation and characterization.

Biogenesis and functions

EVs are subcellular structures produced by cells of all the kingdoms of life that, from what we know, depend on a protein machinery to be formed (Woith et al., 2019). These machineries can vary from kingdom to kingdom and exist several into the same cell, but the fact that these proteins exist implies that they are subjected to natural selection and the fact of having a set of proteins able to enclose a portion of membrane and release it to the extracellular medium seems to have been evolutionary selected many times and for different reasons depending on the organism. This could be either because these proteins have an important function for the organism's survival and fitness and the EVs formation are a consequence of their functioning, or that the EVs itself have important functions.

In fact, several sorting machineries can intervene alternatively or altogether, depending on the cell, its state and the subcellular compartment, at different stages of the vesicle formation. In general, the process consists in the clustering of lipids and proteins in membrane microdomains, then some change in the physicochemical properties of the membrane induces the budding and finally the fission occurs (Colombo et al., 2014; van Niel & Raposo, 2018). Some of these mechanisms involve: the endosomal sorting complexes required for transport (ESCRT) that acts both in endosomes/MVB and in the plasma membrane; the generation of ceramide by the sphingomyelinase; the flopping of certain lipids, remarkably phosphatidylserine (PS) from one side to the other of the plasma membrane; the ESCRT-independent mechanism, through syntenin and ALIX for clustering and budding in the endosome/MVB; the tetraspanins that induce a clustering and form cone-like structures (CD63, CD81, CD82 and CD9); arrestin domain-containing protein 1 (ARRDC1) that recruits Tsg-101 and promotes the formation of ARRDC1-mediated microvesicles (ARMMs) at the plasma membrane. The EVs generation can also be driven by mechanical forces: induced by contraction of the cytoskeleton (McConnell et al., 2009), or under certain circumstances: cell division that produces secreted midbodies (Rai et al., 2021), migration that generates migrasomes, a subtype of EV (Ma et al., 2014), and apoptosis that induces the blebbing of apoptotic bodies (Sebbagh et al., 2001).

The ESCRT is a highly evolutionarily-conserved group of proteins required for the MVB formation, cytokinesis, nuclear envelope reformation, repair of damaged cellular membranes, enveloped retrovirus release and extracellular vesicles generation (Gatta & Carlton, 2019). The MVB formation is important for the cell membrane protein turn over, since it allows their degradation by endocytosis from the plasma membrane. It starts by the recruitment of the ESCRT to the early endosome that sorts in ubiquitinated proteins and lipids and forms intra-

luminal vesicles (ILVs) and thus creating the MVB, and finally the MVB fuse with the lysosome (Schmidt & Teis, 2012). But in some cases, the MVB also can fuse with the plasma membrane by another molecular mechanism involving Rab27a and Rab27b GTPases (Ostrowski et al., 2010) allowing the release of its content to the extracellular medium, and the ILVs become exosomes. The existence of this alternative pathway suggests also a function for the exosomes in the extracellular medium.

Once outside, EVs can target other cells depending on the specificities of their surface molecules (integrins, cadherins, PS, proteoglycans, lectins, etc.) and the ones of the acceptor cell. Also, they can interact by receptor-ligand contacts activating signaling pathways, or they can be specifically or unspecifically phagocytosed and digested (mainly by macrophages and antigen presenting cells) leading to the antigen presentation and nutrient transfer. Another fate of EVs, although probably in a poorly efficient way when EVs do not contain viral components (Albanese et al., 2021), they can fuse their membrane with the membranes of the acceptor cell, leading to the cytoplasmic release of their content. Alternatively or before something of this happens, the EVs interact with the extracellular matrix and eventually then can diffuse to reach a vessel and enter into the circulation, being able to arrive to different organs (**Figure 2**).

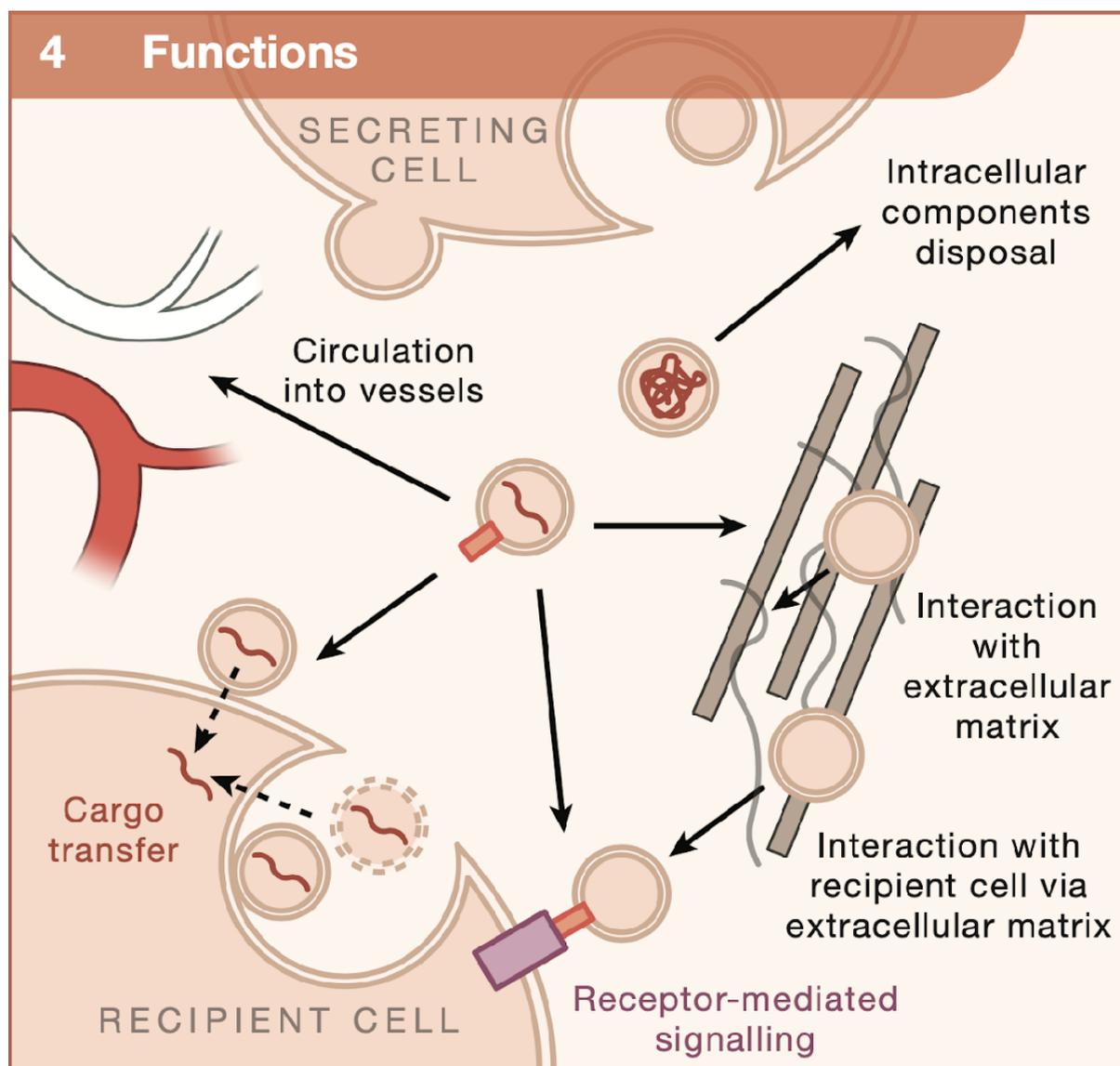


Figure 2: Functions of EVs in the extracellular space.

Source: (Cocoza, Grisard, et al., 2020), see Annex 1.

A vast review about the biological functions found in EVs was previously published (Yáñez-Mó et al., 2015). This includes the functions from mammals-derived EVs seen in body fluids: urine, saliva, synovial fluid, bile, cerebrospinal fluid, bronchoalveolar fluid, nasal fluid, uterine fluid, amniotic fluid, breast milk, blood, faeces and seminal plasma. It also includes functions in all physiological systems: vascular biology, immune system, pregnancy, embryonic development, tissue repair, bone calcification, liver homeostasis, nervous system.

However, something relevant to consider is that most of the functional capacities that have been attributed to EVs in general were studied with preparations that contained a mixture of EVs subtypes, ENPs and other components collectively considered as contaminants (soluble proteins, lipoproteins, etc.) and consequently, the specific properties of each subtype of EV and ENP or contaminants has been incompletely investigated.

Extracellular vesicles versus contaminants

Historically, researchers did not know about the heterogeneity of EVs, and they were thus studying complex mixtures of EVs, which were often called "exosomes" even when no attempt were made at separating exosomes from other subtypes of EVs, or even at separating EVs from co-isolated contaminants. Nowadays, many studies are aware of the heterogeneity of EVs, but still analyzing them as bulk packages of EVs. So, from this perspective, a strategy to discover their properties consist in their isolation from other components ("contaminants" in this context) present in the cell conditioned medium or biofluid used. Depending on the type of biofluid, the medium, complements used, and the cell type, these contaminants can vary: soluble protein, cells debris, lipoproteins (mostly in serum or plasma), protein complexes, fibers, other protein aggregates, nucleic acids (DNA, RNA) and small metabolites are examples. Some are easy to separate because of their great difference in biochemical properties, such as soluble proteins or metabolites that are several orders of magnitude smaller than EVs, but can be present in the final preparations because of their high concentrations; and others can also be co-purified because of their similar biochemical properties, such as lipoproteins (**Figure 3**). For example, by using density gradients, EVs copurify with HDL (Yuana et al., 2014).

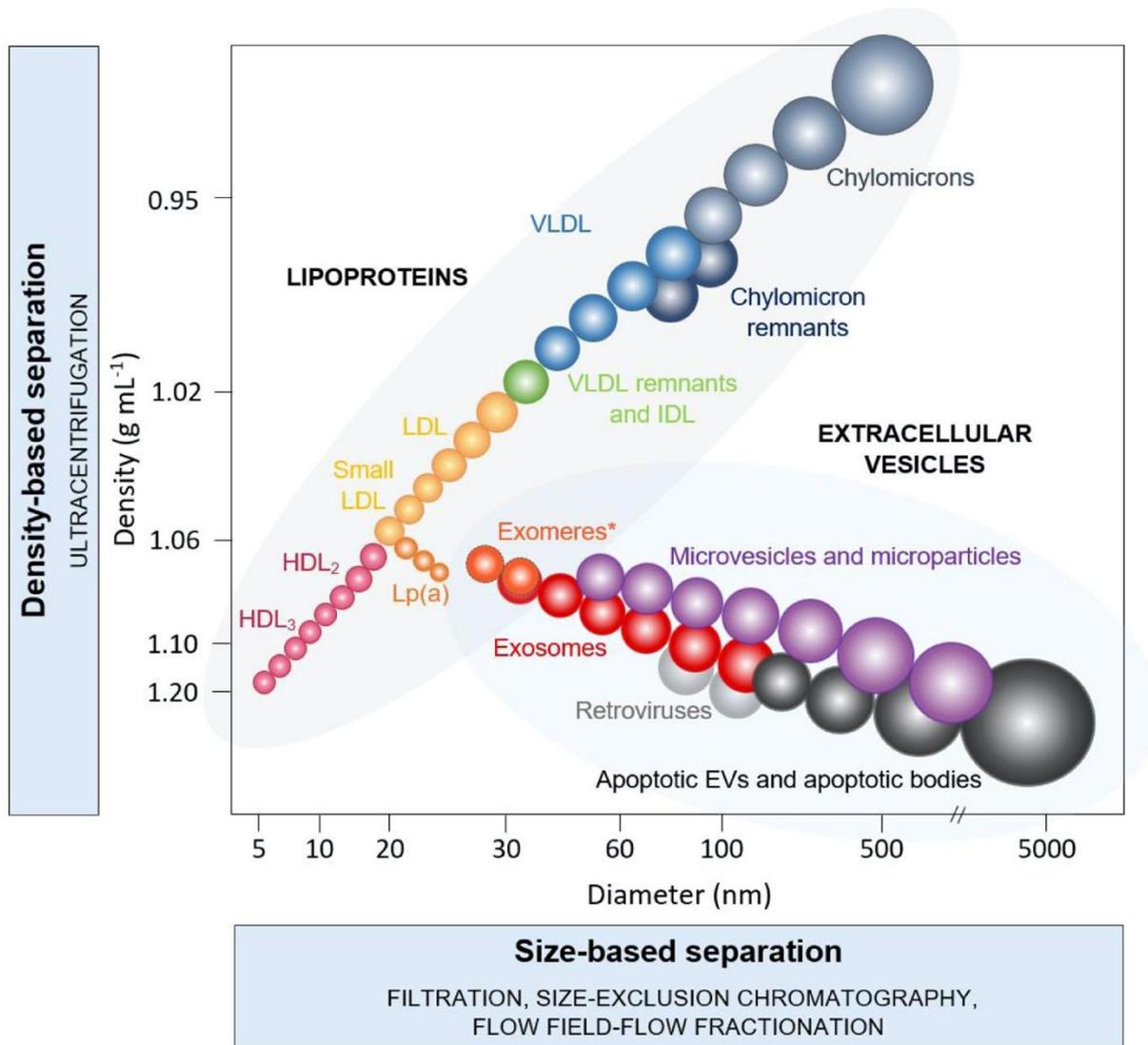


Figure 3: EVs and lipoprotein contaminant comparison in terms of size and density.
Source: (Liangsupree et al., 2021)

Over the years, with the increase on the knowledge and refinement of the techniques, the accumulating evidence points at the heterogeneity of the EVs that a single cell type could secrete at steady-state but also in different states, but also that different cell types could contribute to EVs in biofluids, and that there are also close relatives of EVs in the extracellular medium, such as other nanoparticles (ENPs), lipoproteins, viral particles, etc. This opened a new avenue for the research on intercellular communication. The next chapter will revise our knowledge about EVs and ENPs heterogeneity and the challenges that its study implies, taking in account the importance of the isolation and characterization methods.

2. Subtypes of extracellular vesicles and particles

Definition of subtypes of extracellular vesicles and particles

A population is a group of elements that share a common characteristic, and the definition of different populations can be based on qualitative differences between the populations or quantitative differences in the same parameter that distributes as distinct Gaussian curves. Subpopulations can be also defined inside a population that shares a characteristic but differs in another one. For example, EVs are a population that have in common the fact of being an enclosed, non-replicative, lipid bilayer that originates from cells, and then subpopulations could be defined based on qualitative differences (e.g., some have viral capsids inside and others not) or by quantitative differences (e.g., different sizes). It is important to point out that when a characterization or functional attribution has to be given to a certain subpopulation, either a separation or an inhibition of its secretion/formation or the identification through a marker is required. These methods normally use the physicochemical properties of the particles to separate/inhibit/track. So, they should be well tuned to separate to the best possible based of intrinsic measurable properties of the subpopulations and not on arbitrary settings decided by the researcher, otherwise mixtures of subpopulations will be isolated and the conclusions taken can be only partial.

The efforts of scientists in the development of novel isolation and detection/characterization technologies of EVs during the last years have increased the knowledge on EVs/ENPs heterogeneity (Liangsupree et al., 2021; Tkach et al., 2018; Willms et al., 2018), and highlighted the existence of novel subtypes of non-vesicular ENPs such as exomeres (H. Zhang et al., 2018), supermeres (Q. Zhang, Jeppesen, Higginbotham, Graves-Deal, et al., 2021), and supramolecular attack particles (Bálint et al., 2020) that can be isolated and studied. Some of the most common EVs and ENPs subtypes are shown in **Figure 4**.

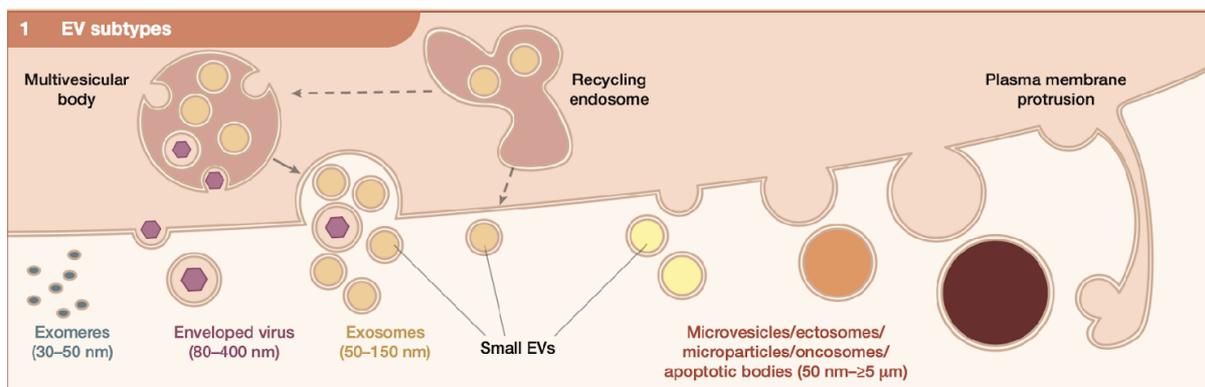


Figure 4: Subtypes of EVs and some ENPs secreted by cells.

Source: (Cocozza, Grisard, et al., 2020), see Annex 1.

Still, during the lifetime of the EV field, different criteria have been applied to define or classify EV and other ENP subtypes, making the whole picture ever more complex (Lässer et al., 2018). The main classification parameters and subtypes of EVs/ENPs are summarized here.

Subtypes of extracellular vesicles

First, I will start by the subdivisions made inside the EVs family, grouped by the parameter used to distinguish them. It is important to notice that often more than one parameter was applied in a certain work, and also that 2 parameters can group in subpopulations differently the same starting population. For example, dividing by size in sEVs and lEVs or dividing by origin in MVB (exosomes) and plasma membrane (ectosomes): in the group of sEVs there will be exosomes and ectosomes, and in the group of lEVs, ectosomes; and in the group of MVB-derived vesicles there will be sEVs, whereas in the plasma membrane-derived vesicles there will be sEVs and lEVs.

By size

EVs can be subdivided in subpopulations by their size and the size ranges used can vary depending on the model (cell type, culture conditions, purification method, etc) and the machine used to measure. Typically, EVs can be classified as small EVs (sEVs) of size ranges around 40 to 80 or 150 nm, and large EVs (lEVs) of 150 nm to 10 μm , eventually a third category of medium EVs (mEVs) can be added for example between 80 to 150 nm. The distinction of the populations of these sizes can be complicated due to the limitations of detection and resolution of the machine used to measure. The nanoparticle tracking analysis (NTA) is one of the most used techniques, and it has an inferior limit of detection dependent on the amount of light scattered by the particles, in general around 50 nm for EVs but even some bigger, not so bright EVs might also not, moreover, the presence of big particles interfere with the detection of smaller once and has a bias towards small particles (Gandham et al., 2020). However, more precise technologies exist in order to well characterize EVs by sizes as transmission electron microscopy (TEM) or Cryo-EM, atomic force microscopy (AFM), nano flow cytometry (nano-FCM) and DLS coupled to AF4, though all of them with low throughput.

EVs can be separated through differential ultracentrifugation (dUC) by their ability to pellet, hence their weight in the medium (dependent on the density difference with the medium and size), which can be somehow correlated to the size but not only, and through increasing centrifugation speeds applied to the supernatant of the previous centrifugation enriching, in

general, for decreasing size vesicles at each step. Then the obtained EVs are named by the speed applied (e.g.: 2k, 10k and 100k), after a step of washing at the same speed and time (Jeppesen et al., 2014; Kowal et al., 2016; Martin-Jaular et al., 2021). But the obtained pellets normally have a mixture of EVs of different sizes, being the 2k and 10k enriched but not pure in IEVs and the 100k in sEVs.

To separate EVs and ENPs by size, sequential filtration (SF) was proposed, consisting in the use of filters of different pore sizes (Heinemann et al., 2014). The group of Breakefield and Krichevsky characterized the landscape of coding and non-coding RNA of IEVs (800-220 nm), sEVs (220-20 nm) and ENPs (<20nm) from human glioblastomas, separated with filters of 800, 220 and 20 nm. Besides showing TEM images with well separated EVs and ENPs, they did not show a measurement of the size distribution.

A more effective technique of separation was developed using the AF4 allowing to separate with a high degree of purity IEVs (90-120 nm), sEVs (60-80 nm), and a smaller non-membranous particle type, exomeres (~35 nm), discovered and characterized in the same occasion (H. Zhang et al., 2018).

By origin

Another criteria for the definition of different EV subtypes is the subcellular compartment of biogenesis. This was in the spotlight since the beginning of the research in the EV field since, presumably, the generation of an EV in a given compartment would attract components inherent to it, resulting in a distinct EV subtype. This is true and the origin of an isolated EV subtype can be extrapolated by the main subcellular location of a set of their proteins or lipids, for example. But it exists some biases, like the fact that proteins and lipids can inhabit in more than one compartment (Mathieu et al., 2021), also that the biogenesis machineries can also act with more or less avidity in more than one compartment, and a same compartment can have more than one mechanism acting on it, leading to more than one subtype of EV from the same compartment. Thus, both the compartment of budding and the biogenesis machinery are important in the determination of EV subtypes.

Mainly, EVs can be formed in the MVB or late endosome, leading to the release of exosomes, or from the plasma membrane, producing ectosomes or microvesicles or microparticles (Colombo et al., 2014). Alternatively, EVs can contain components from other organelles, such as mitochondria by transfer of components through mitochondria-derived vesicles (MDV), an intracellular type of vesicle which goes to lysosomes or peroxisomes, but which can also reach MVB and thus exosomes, or by direct budding of entire mitochondria into ectosomes (D. Liu et al., 2020; Soubannier et al., 2012). EVs can also originate from the autophagosomes when

LC3 and autophagy-related genes (ATGs: ATG12 and ATG3) interact with Alix in the MVB or when an autophagosome fuse with the MVB forming the amphisome, and eventually releases its content extracellularly (Leidal & Debnath, 2021; Murrow et al., 2015).

Even though the compartment of origin is an important factor in the definition of an EV subtype, EVs get mixed in the extracellular medium and their separation is challenging, due to the sharing of biophysical properties. A strategy to unravel the properties and functions of these EV subtypes is the immune precipitation or detection by specific antibodies against transmembrane proteins, either to positively select as for some EV isolation kits using anti-CD63, CD9 and CD81 tetraspanins antibodies, or by depletion and assessing the lack of the biological effect. In the first case, however, the addition of antibodies could induce some side effects on the system (Ig isotypes should be used as control) or mask the effect of the target protein. It is important to notice that these approaches focus only on a subpopulation of the EVs from the specific compartment as not all of them contain these markers, their success relies on the affinity of the antibodies and they require a good presetting. For the particular case of the combination of tetraspanins, these kits claim to purify exosome, but it was shown that EVs bearing CD63 at high level are enriched in exosomes, but they are not pure exosomes, and the ones bearing CD9 are enriched in ectosomes, but also not purely (Fordjour et al., 2022; Mathieu et al., 2021). In another way, exosomes can be studied by inhibiting their release by interfering with Rab27a or Rab27b (Ostrowski et al., 2010). Or drugs that inhibit a specific biogenesis pathway.

By density

The density of EVs can vary depending on their load, that is if they are densely filled with proteins or other components or if they are more aqueous, but in general they are denser than water 1 g/ml and less dense than protein and DNA aggregates ~1,4 g/ml also found in the extracellular medium. EVs were described to have a buoyant floating density of 1,08-1,22 g/mL on sucrose gradients (Raposo & Nijman, 1996), and mainly between 1,13-1,19 g/ml, that is lighter than vesicles from the endoplasmic reticulum 1,18-1,25 g/ml and heavier than vesicles from the Golgi 1,05-1,12 g/ml (Théry et al., 2006). Some discrepancy can occur in the bibliography in the exact reported density, due to the fact that the osmotic pressure of the medium used to make the density gradient can alter the properties of the EVs, being the Optiprep iso-osmotic up to 1,32 g/ml so not changing the density of the EVs, different to the sucrose (Kowal et al., 2016; X. Li & Donowitz, 2014; Neves et al., 2009). Discrepancies can also be due to the distinct nature of the EVs from the cell type used in each case, or to the

manipulation errors, as in general the fractions from the density gradients are collected by manual pipetting.

Concerning density of the EV subtypes, microvesicles from 3 cell lines (MDA-MB-231, AsPC-1 and A375) were shown to have a density of 1,03-1,08 g/ml (Ettelaie et al., 2014), whereas apoptotic bodies 1,16-1,28 g/ml and enveloped viral particles are denser than non-viral vesicles 1,15-1,28 g/ml (McNamara & Dittmer, 2019). Human primary DCs secrete EV subpopulations of 1,115 g/ml and 1,145 g/ml evidenced by separating the 10k and 100k through iodixanol gradient (Kowal et al., 2016). B16F10 melanoma cells also secrete two subpopulations of sEVs with densities of 1,12-1,19 g/ml and 1,26-1,29 g/ml (Willms et al., 2016). Also, the RNA content of human mast cells HMC-1 and the erythroleukemic cell line TF-1 revealed 2 distinct populations of extracellular carriers, including EVs (Lässer et al., 2016). Subpopulations of EVs from mesenchymal stem cells of different densities, low (1,01-1,06 g/ml), medium (1,08-1,15 g/ml), where most of the EV marker were found, and high (1,17-1,27 g/ml), had different molecular signatures and functions (Collino et al., 2017).

By specific cargo

As previously mentioned, the association of proteins of the EVs biogenesis machineries, including recruited components for the loading into the vesicles as the formation of lipids rafts that bring certain elements, and the fact that some of the proteins involved in the biogenesis remains attached to the EVs upon release, leads to the generation of subtypes of EVs with distinct protein, lipid, and nucleic acid cargos. Also, specific cell types can carry specific markers that individually or in complexes, can end up loaded in a subtype of EV.

Separating the EVs subpopulations by cargo can be obtained as described before by immunoprecipitation, or by other strategies, such as analyzing the behavior and clustering of the components of the EVs. For example, by doing dUC and proteomics of EVs of human Jurkat T cells partially separated by 10k, 30k and 100k centrifugation, the profile of distribution of proteins in these pellets was analyzed and clustered, allowing to the distinction of, for instance, 2 EV subtypes: CD81/ADAM10/ITGB1 and CD81/CD63/syntenin (Martin-Jaular et al., 2021). Also, the heterogeneity of EVs from a human glioblastoma cell line, Gli36, was assessed by biotinylation of the EVs, capture on a chip, staining with fluorescent antibodies against EV and glioblastoma tumor markers and reading through a microscope. Afterwards, individual EVs were clustered by the staining, identifying up to 14 subpopulations (Lee et al., 2018). Through the measurement of the staining with the mitochondrial dye MitoTracker, anti-CD41, CD235, annexin V, size (FSC) and inner content (SSC) in the nano-FCM and subsequent

spanning-tree progression analysis of density-normalized events (SPADE), up to 10 subpopulations of EVs from platelets and red blood cells were found (Marcoux et al., 2016).

By morphology

Cryo-EM is a technique that allows the observation of EVs in a more reliable manner, since it does not introduce fixatives, and thus, EVs can conserve their natural shape. This revealed a great heterogeneity of EVs, including the existence of vesicles inside other, or structures of different shapes and features like membrane coating, filaments and electron dense areas (Höög & Lötvall, 2015; Zabeo et al., 2017). A classification is shown in **Figure 5**.

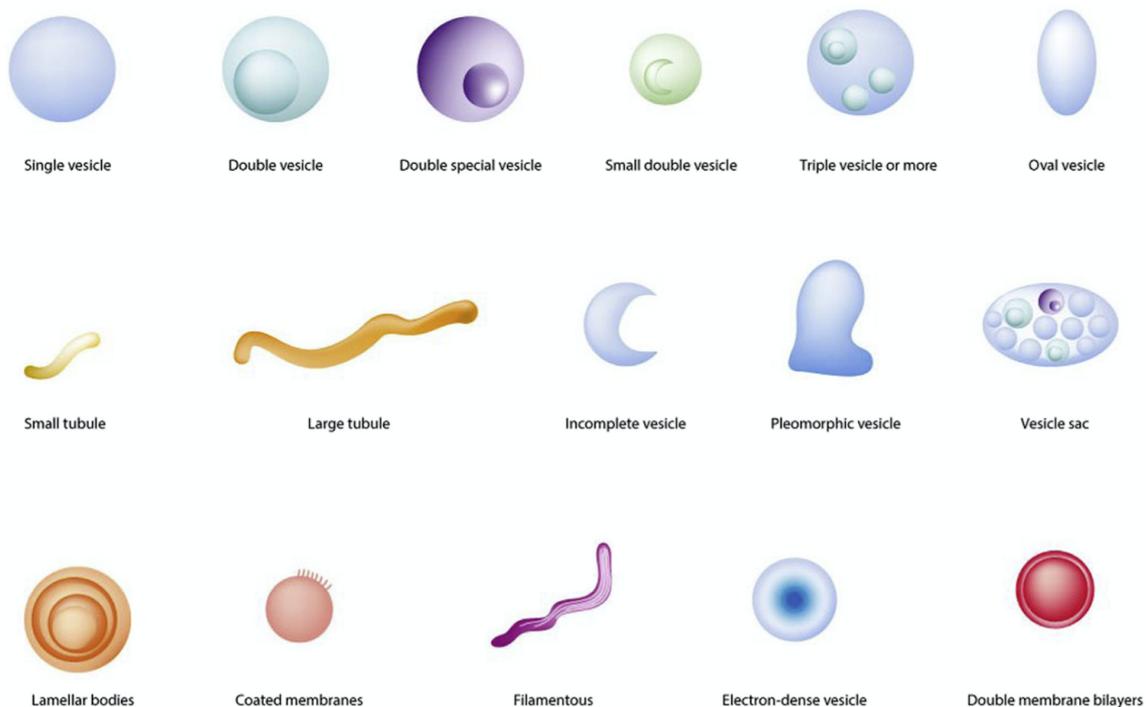


Figure 5: Classification of subtypes of EVs by their morphology.

Source: (Lässer et al., 2018).

All these morphological characteristics of EVs were also seen in unprocessed samples, proving that they are not an artefact of the preparation. However, a clear understanding of the association of each of the morphological types to the biogenesis mechanism, subcellular compartment of origin or if they are actively secreted by live cells or subproducts of cell death (cell debris) is lacking. For example, it is unclear if vesicles inside vesicles or organelles inside vesicles are products of apoptosis or they are actively secreted. The migrasomes are such a well described type of vesicle containing vesicles. It is also unknown if filaments inside vesicles, with possible contractile properties are actively secreted or if they are generated by disruption due to the manipulation.

Special EV subtypes

Apoptotic dying cells undergo a program that includes the fragmentation of the cell in a variable number of apoptotic bodies (ApoBDs) at its final stage. These apoptotic bodies are considered as a particular subtype of EV of 500 nm -2 μ m and are generated by exposure of PS in the outer leaflet of the plasma membrane and blebbing (Battistelli & Falcieri, 2020). They contain from cytosol portions to proteins, DNA, RNA, chromatin, entire organelles and any other cell component. After their release in the organism, they are quickly phagocytosed and cleared mainly by macrophages, due to the recognition of the exposed PS in the ApoBDs, with no induction of an inflammatory phenotype in normal apoptosis conditions. However, it exists several types of death for cells, and the ApoBDs can deliver information about it to the immune system. ApoBDs from infected macrophages can transfer antigens from *Mycobacterium tuberculosis* to antigen presenting cells that activate CD8 T cells (Schaible et al., 2003). The antigen-presenting cells pulsed with ApoBDs from tumor cells had antitumor effects in preclinical mouse models and human patients (Henry et al., 1999; Hus et al., 2005; Kokhaei et al., 2003; Palma et al., 2012). Something to consider is also that these studies were performed by inducing death in cells and collecting the product without a further separation, that is including all types of EVs and cellular products and not considering the EV heterogeneity. But cell death and apoptosis, even when non purposely-induced, also occur in routine cell culture and in the organisms, thus ApoBDs are present at different degrees in all types of starting materials used for EV preparations (They et al., 2001).

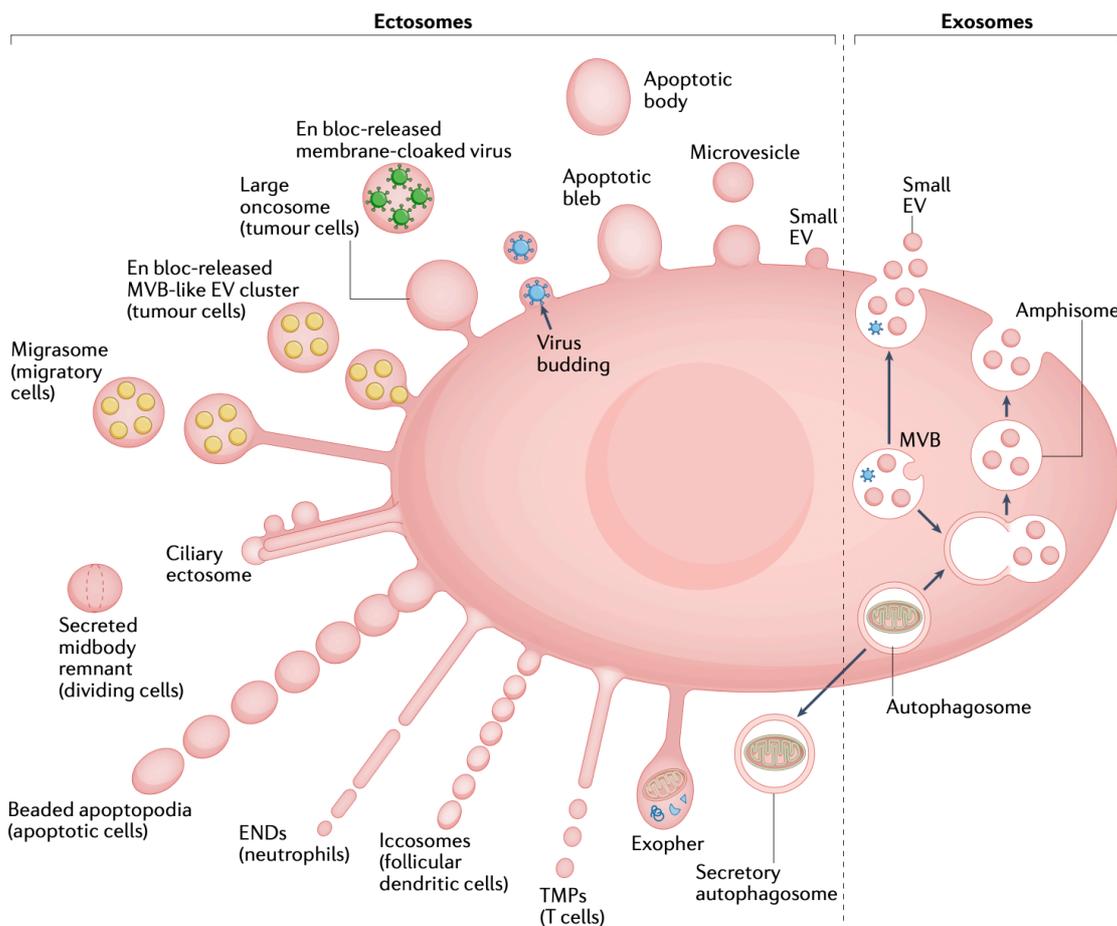


Figure 6: Heterogeneity of EVs.

Source: (Buzas, 2022)

Migrasome is an organelle that is generated when the cell migrates at the tips or intersections of retraction fibers, and can eventually brake and be released as an EV subtype (Ma et al., 2014; Yu & Yu, 2021). Migrasome consist in spherical structures containing numerous intraluminal vesicles and they originate by assembly of tetraspanin-enriched microdomains (Huang et al., 2019). Migrasomes can act as packets of information left as traces by migrating cells that can serve for chemotaxis and organ formation during development (Jiang et al., 2019). They can also be used for disposal of unwanted content such as damaged mitochondria (Jiao et al., 2021), and they can also transfer mRNA between cells (Zhu et al., 2020).

Large oncosomes are a large subpopulation of EVs (1-10 μm in diameter) secreted by tumor cells, in particular they were described in amoeboid migrating prostate cancer cells that undergo a certain cellular transformation (Di Vizio et al., 2009; Minciacchi et al., 2015).

Extracellular nanoparticles

In 2018, the group of David Lyden discovered and characterized an abundant non-membranous particle of ~35 nm that could be separated from EVs using Asymmetric Flow field-flow fractionation (AF4), which they named exomere (H. Zhang et al., 2018). This study raised awareness on the existence of subtypes of nanoparticles, other than EVs, present in the extracellular medium, later called extracellular nanoparticles (ENPs) (Jeppesen et al., 2019). A few years later, additional ENPs were discovered, for instance a smaller subtype called “supermeres” (from the supernatant of exomeres) (Q. Zhang, Jeppesen, Higginbotham, Graves-Deal, et al., 2021) and supramolecular attack particles (SMAPs) (Bálint et al., 2020). However, except for the latter which display killing activity, and the exomeres that promote hypersialylation of the surface proteins of recipient cells (Q. Zhang et al., 2019), little is known still on the biological functions of these recently discovered particles.

Retroviral particles as a subtype of extracellular vesicle

Another level of complexity of the vesicular secretome is observed upon viral infection or expression of endogenous retroviruses on cells. Viruses research started much before the discovery of EVs, and during the 20th century, viruses were defined as small infectious agents that can only multiply in living cells. That means that EVs are not in this category. But since then much more was discovered about the structure and mechanisms of viruses and EVs that make them close relatives (Nolte-'t Hoen et al., 2016). Basically, they are both nanosized structures with an external lipid membrane that contain genetic material inside and that are formed in the endosome or plasma membrane. Once outside the producing cell, both can selectively target another cell, transfer their RNA or DNA content and induce functional changes. These similarities are due to the fact that viruses depend on biological processes of the host cells to survive and to form viral particles. In particular, enveloped viruses are enclosed in a host cell-derived lipid bilayer and, in some cases, hijack the EV formation machinery like the ESCRT to be formed, as for example does the HIV (Booth et al., 2006; Gould et al., 2003). Therefore, enveloped viruses share most of their physicochemical properties with EVs and they can be considered as an EV subtype. In particular, for the case of viruses and EVs from the same infected or endogenous retroviruses expressing cell, they coexist in the same factory, making blurrier the frontier between them, as some intermediate entities, such as incomplete

non-infectious viral particles and EVs modified to carry viral proteins or other atypical host proteins, can be generated (**Figure 7**).

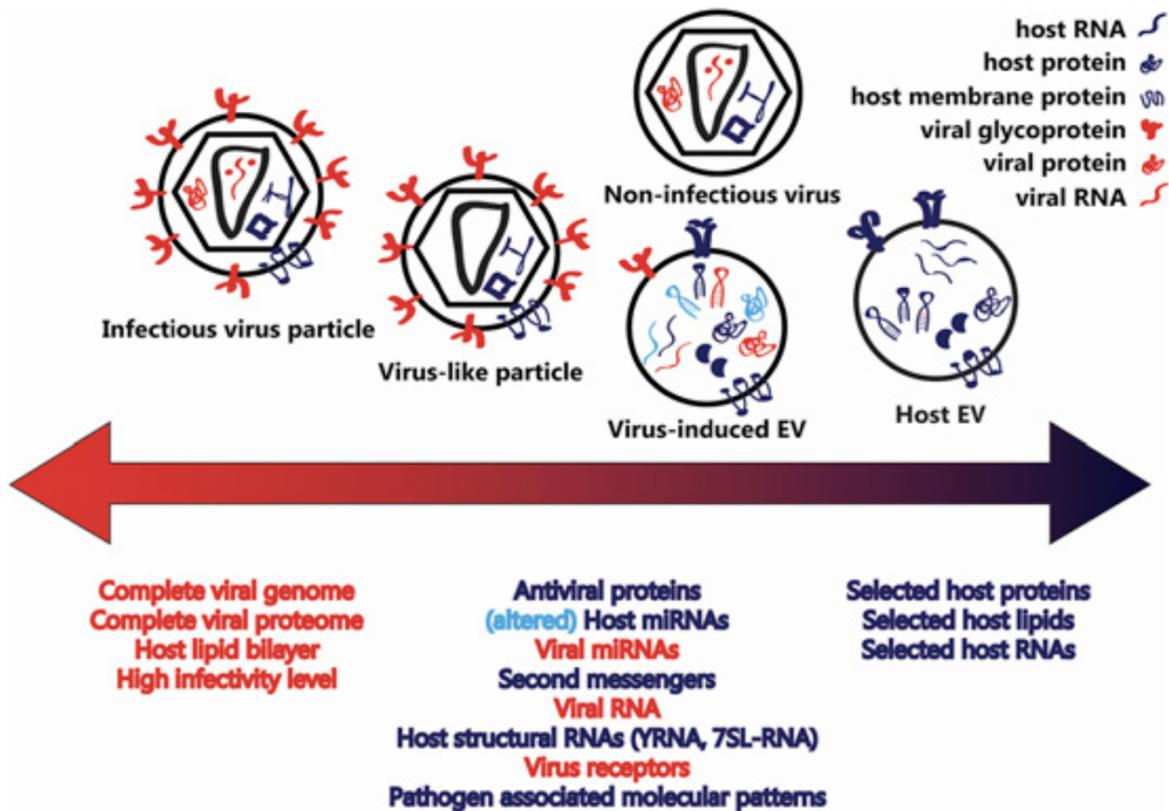


Figure 7: Gradient of particles generated in a virus infected or endogenous virus expressing cell: from complete viruses to EVs.

Source: (Nolte-'t Hoen et al., 2016).

Retroviruses contain RNA and can target cells, where their RNA genome can be reverse transcribed into dsDNA and integrate into the genomic DNA of the host cell. Eventually, with an unknown but greater than 0 probability, they can integrate in the germ line of the organism, that will give rise to an individual bearing the integrated viral genome. The retrovirus thus enters in the host organism gene pool and becomes an endogenous retrovirus (ERV) (Johnson, 2019). After this happened, its fate can be either loss or fixation in the population/species, and this will depend on genetic drift and natural selection. Once fixated in the host genome, the endogenous retrovirus genome can also increase in copy number through various mechanisms. Over the span of millions of years, the genomes of vertebrates have accumulated thousands to hundreds of thousands of ERVs loci, encompassing 8% of the human genomes. However, they are not necessarily part of a pathologic process as their ancestors, since they have coevolved with their host and are also an important source for modeling the genome and evolution. Some loci are neutral because they are repressed or mutated and others became beneficial. For

example, syncytin, an ERV-derived gene, is essential for the human development, or the gag-like protein Arc that is essential for long-lasting information storage in the mammalian brain (Ashley et al., 2018; Pastuzyn et al., 2018).

Infectious ERVs are able to cause cancer as they can insert in parts of the genome that will unleash the action of oncogenes (Kassiotis, 2014). In mice, different defectives ERVs are expressed at low levels (Shepherd et al., 2003; Stocking & Kozak, 2008), and eventually they can recombine and restore their infectivity under certain circumstances, for instance in immunodeficient models or in mouse tumor cell lines (Ottina et al., 2018; Young et al., 2012). In humans, ERVs (HERVs) are normally mutated or epigenetically silenced and thus cannot form viral particles (Kassiotis & Stoye, 2017). Nevertheless, they can get expressed at some level in physiological conditions in some tissues, specially by immune cells or in pathological conditions such as pathogenic infection or cellular transformation (Young et al., 2018). HERV particles were found in human cancer cell lines (Boller et al., 1993; Bronson et al., 1979; Mueller-Lantzsch et al., 2009). The immune system still responds to HERVs, especially when they have viral properties, so they also serve as an intrinsic warning system (Kassiotis & Stoye, 2016). Moreover, HERVs can exhibit some intrinsic antitumor effects, for instance when HERV-T is expressed, it can inhibit the tumor growth by depleting monocarboxylate transporter-1 from the membrane (MCT-1) (Blanco-Melo et al., 2017).

This means that not only infected cells, but also ERVs-expressing cells can produce retroviral particles. This is the case for example of most mouse C57Bl/6-derived cell lines, in which, eventually in a stochastic manner, a replicative defective locus (*Emv2*) that has a single mutation in the polymerase, can recombine with another MLV to regain its infectivity and successively reinfect the cell, leading to a high expression of the retroviral particles (Ottina et al., 2018). Therefore, retroviral particles, which can also be considered as an EV subtype, could constitute a component in EV preparations.

In order to investigate the specific functions of EVs and retroviral particles, they have to be separated. But to achieve a good separation is challenging, since they have similar properties such as size and relatively close densities (most EVs display densities of 1,13-1,18 g/ml, and retroviruses of 1,16-1,18 g/ml). This was achieved first by density gradient ultracentrifugation, distinguishing, at the time, viral particles from defective ones, that were actually virus-induced EVs (Eckner & Hettrick, 1977). The protocol was then optimized by using velocity gradients, for more complicated separations, such as for HIV (Cantin et al., 2008; Konadu et al., 2016). The velocity gradients differ from the density gradients by the fact that, besides consisting also in a density gradient, the equilibrium of the particles into their density is not achieved because

of shorter centrifugation times, and therefore both the size and the densities of the particles matter. More recently, other novel technologies are being developed for the separation of EVs and viral particles, such as sorting by flow cytometry (Lippé, 2018; Nolan & Duggan, 2018; van der Pol et al., 2018) or others less specific (McNamara & Dittmer, 2019).

Methods of isolation of EVs and separation of subtypes of EVs and ENPs

The current methods of isolation can at different degrees separate EVs from contaminants and/or separate EV and ENP subtypes one from each other. Also, we can compare the techniques in terms of the degree of recovery and specificity, two criteria which tend to be mutually exclusive, and a high rate in both parameters for a certain technique is so far unachieved. Some of the most commonly used methods are shown in **Figure 8**.

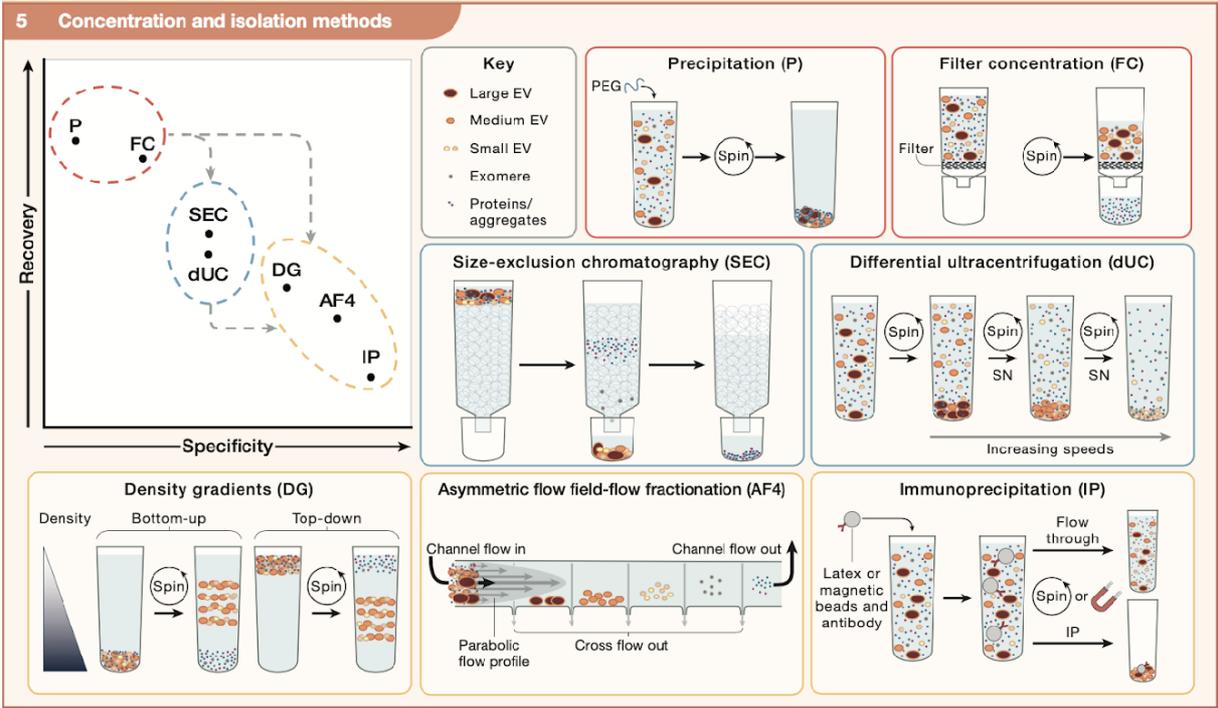


Figure 8: Isolation methods of EVs.
 Source: (Cocozza, Grisard, et al., 2020), see Annex 1.

The methods with greater recovery but less specificity are filter concentration (FC) or polymer-based precipitation (P) (e.g., with PEG, polyethylene glycol), in which EVs, ENPs, and most of the secreted products are recovered. These methods are commonly used as a first step of concentration or purification followed by another more specific.

The differential ultracentrifugation is the most used method of isolation of EVs and it allows to separate EV subtypes by their size, including exomeres (Q. Zhang et al., 2019), though as a great mixture. But it was shown to co-isolate higher amounts of soluble proteins, compared with other techniques, such as size-exclusion chromatography (SEC) (Brennan et al., 2020). This last one offers the opportunity to obtain a broad preparation of EVs of different sizes, but nevertheless, it co-purifies larger amounts of lipoproteins, thus it is maybe more suitable for non-serum/plasma starting material. Asymmetric flow field-flow fractionation (AF4) is ideal to separate EVs and ENPs by their size, showing a great precision, but its set-up is difficult, it is not suitable for big preparations and it requires a pre-isolation step of the sample for the low volume that can be injected.

The density gradient is another frequent method used, allowing a high rate of purity in its preparations of EVs and even more, being able to separate subpopulations of different densities. Depending on where the sample is seeded (bottom or top), they can be classified in bottom-up that separates by floatation and dense particles remain at the bottom, and top down that pellets by density and light particles remain at top. Both can lead to preparations with different contaminants. Bottom-up density gradient showed to be more effective than top-down ones to purify EVs from protein contaminants (Dhondt et al., 2020).

The most specific separation can be achieved by immunoprecipitation (IP) using specific antibodies to a given surface protein of EVs. But it has to be taken in consideration that only one component on EVs used for the IP so a limited proportion of the EVs will be purified.

3. Therapeutic applications of EVs

EVs have been proposed as therapeutic agents because of their particular properties as carriers and involvement in the intercellular communication. They have the potential to be combined with artificial products as liposomes or nanoparticles (György et al., 2015).

They can be used by their intrinsic therapeutic activity, like mesenchymal stem cells (MSCs) EVs for anti-inflammatory or anti-fibrosis effect. They are able to enhance the recovery of various injuries, suppress inflammation induced by hypoxia and hypertension (C. Lee et al., 2012), reduce damage after myocardial ischemia/perfusion (Arslan et al., 2013) and be neuroprotective in brain injuries (Xin et al., 2012).

EVs can be used as vaccines against infectious diseases. One approach consists in pulsing APCs with purified antigens or peptides and recovering their EVs, so that APC derived EVs bear MHC loaded with the introduced peptides. Another approach consists in using a tag that will

direct antigens specifically to EVs produced by another cell type, thus leading to production of EVs carrying the antigenic protein. The use of these EVs often is more effective than the soluble proteins and elicit a Th1 immune response. DCs treated with *Mycobacterium tuberculosis* proteins or diphtheria toxoid are examples of this (Cheng & Schorey, 2013; Colino & Snapper, 2006).

DC-EV were also proved to be efficient for vaccination against cancer in mice models of OVA expressing melanoma (Gehrmann et al., 2013). Similarly, vaccination with vesicles obtained by sonication of melanomas was able to decrease the tumor growth and metastasis (E. Y. Lee et al., 2012).

EVs from cells genetically engineered to carry inflammatory cytokines receptors (TNF- α and IL-6) were able to act as decoys and ameliorate systemic inflammation, neuroinflammation and intestinal inflammation in mouse models (Gupta et al., 2021).

In addition, EVs can be mediators for the delivery of biomolecules as nucleic acids, drugs or other cargos. They have a lipid composition that maintain their content for some time in circulation before being degraded or phagocytosed, and at least some contain inhibitors of the complement (CD55 and CD59) that prevent their lysis (Clayton et al., 2003; H. S. Kim et al., 2012). Moreover, they have the potential of carrying transmembrane protein that can target specific recipient cells.

The potential of EVs as decoys and immune modulators are the main focus of this Thesis and they are further described in the next sections.

Extracellular vesicles as decoys

EVs are secreted by all domains of life Archaea, Bacteria and Eukarya and they are involved in the intercellular communication, not only inside the organism, but also interspecies. That includes the context of pathologic infections, in which, sometimes, both EVs from the pathogen or the host could have evolved to perform adaptative functions for the inductor organism. For example, in the case of mammals as hosts, they can produce EVs that fight the infection, or be manipulated by pathogen to secrete EVs that disseminate or enhance the infection. Conversely, pathogens can produce EVs that deliver toxins to host cells, signaling molecules or DNA between the pathogens, favoring its establishment and dissemination, this in balance with the energetic cost and the spreading of danger signal that it implies (McMillan & Kuehn, 2021; White et al., 2021). The involvement of EVs in the interaction pathogen/host is complex and includes numerous strategies. I will focalize here in the role of EVs as decoys.

We consider for an EVs to be a decoy if it interferes physically in a regular process of communication between cells or between a cell and an external signal. This means that EVs bind either to a cell or to a component of the extracellular environment (soluble molecule, virus, other pathogen) and prevents interaction of this with its target (i.e. the cell or other).

Since EVs have the same membrane orientation as cells, they expose at their surface the extracellular domains of transmembrane proteins that can bind to nearby or long-distance targets. By specifically binding to different proteins and protein-containing structures, EVs can act as a decoy.

It was shown that EVs from metastatic melanomas carried PD-L1 at their surface that suppresses the function of CD8 T cells and promotes tumor growth (G. Chen et al., 2018) and can also act as decoys of anti-PD-1 antibodies (J. Chen et al., 2022).

HER2-overexpressing breast carcinoma cell lines secreted EVs bearing HER2 that bound Trastuzumab, interfering with the therapeutic activity of the humanized antibody (Ciravolo et al., 2012)

Both Gram-negative and Gram-positive bacteria can produce EVs. The Gram-negative have an inner and outer membrane separated by a thin peptidoglycan cell wall and can generate EVs from the latter one named outer membrane vesicles (OMVs). Gram-positive bacteria have only one membrane inside of a thick peptidoglycan cell wall and the production of EVs from these cells was discovered later so to include both we can refer to bacterial EVs (bEVs) (Ñahui Palomino et al., 2021).

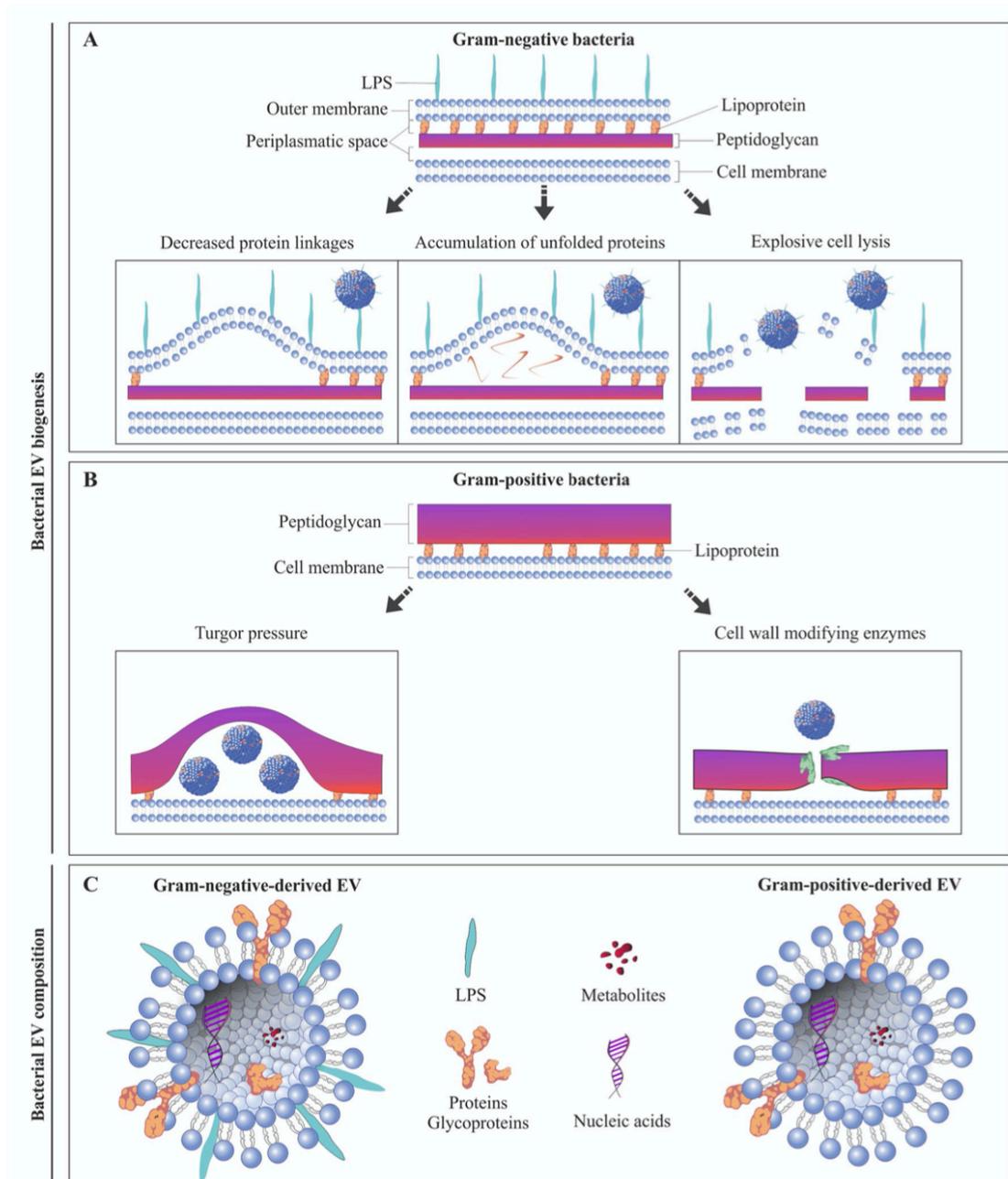


Figure 9: Bacterial EVs.

Source: (Ñahui Palomino et al., 2021).

bEVs can serve as immune decoy. For example, they can bind in the serum C3 of the complement system and inactivate it (Thuan et al., 2007). *Pseudomonas aeruginosa* can incorporate released OMVs that had fished iron through the iron-binding Pseudomonas quinolone signal (PQS) in mediums with iron chelators (Lin et al., 2017). bEVs can also serve as decoys of antibiotics, by capturing them or carrying enzymes that degrade them (Karthikeyan et al., 2020), for example carrying β -lactamase that degrades amoxicillin (Schaar et al., 2014; Stentz et al., 2015).

On the counterpart, the host cells can increase the EV production upon infection by bacteria, viruses, fungi and parasites, by detection of a given component (e.g.: CpG) (Andreoni et al.,

2019; Antwi-Baffour et al., 2020; M. Kim et al., 2012; Mehanny et al., 2020). And these EVs may have, in some cases, beneficial effects in the fight against the pathogen. For example, immune cells can release ADAM10 bearing EVs that protect the host from methicillin-resistant *Staphylococcus aureus* α -toxin that induce plasma membrane disruption (Keller et al., 2020). In viral infection as well, EVs from CD4⁺ T cells inhibit the infection by HIV-1 by binding to the virus via its receptor CD4 exposed on the EV surface, and preventing its binding to CD4 on target cells. The virus evolved to counteract this mechanism, by encoding for Nef, an accessory protein, which reduces the incorporation of CD4 and MHC-I in the EVs, promoting the infection (De Carvalho et al., 2014). The potential of EVs as decoys of viruses suggest the potential use as therapeutic agents. They can be generated either purified from wild type cell lines expressing the appropriate receptor or by introducing it by genetic modification.

Tumor extracellular vesicles as immune modulators and antigen carriers

Intercellular communications are crucial in all tissues, and especially in the tumor microenvironment, where cancer cells must exchange information with the surrounding stromal and immune cells to eventually form a tumor.

In the tumor microenvironment, tumor cells promote changes in surrounding cells of the stroma and the immune system (Gajewski et al., 2013; Whiteside, 2008). In this crosstalk, the EVs and ENPs are also involved (Han et al., 2019), however, the actual functions of EVs/ENPs coming from the tumor cells in relation with the immune system are controversial: there is evidence showing both immune response activation because of tumor antigen transfer or DAMPs delivery, and on the other side, immunosuppressive functions (Greening et al., 2015; Robbins & Morelli, 2014; Théry et al., 2009).

Tumor EVs as activators of the immune system

Immature DCs are professional antigen presenting cells (APC) that surveil the peripheral tissues and have a high uptake capacity. When they capture an extracellular antigen, this can be processed in peptides and the peptides presented by the classical pathway loaded in MHC-II molecules or cross-presented loaded in MHC-I molecules. They can also present directly their own processed peptides from the cytoplasm loaded in MHC-I or cross-present these peptides loaded in MHC-II molecules. After they have captured antigens and sensed the microenvironment, they can eventually mature and migrate to lymph nodes, especially if they

have detected damage, pathogen or inflammatory molecules. There, they can present the antigens they have captured and respectively activate or tolerize T cells, depending if the previous sensed signals were mainly danger/damage or anti-inflammatory, and if the antigen is a foreign molecule or an autoantigen. Conventional T cells that recognize autoantigens are either negatively selected in the thymus or become regulatory T cells. So, in general, if a foreign antigen is presented, conventional T cells will recognize it and start the immune response, while if it is an autoantigen, regulatory T cells will recognize it and inhibit the immune response. But it exists exceptions to the rule and the avidity of the TCR, the abundance of the presented antigens and the costimulatory molecules are some of other important factors that may determine the actual responses of T cells. In some pathological cases as autoimmunity autoantigens can activate conventional T cells and on the contrary, in cancer transformed cell can active the tolerogenic responses of regulatory T cells (Banchereau & Steinman, 1998). Exosomes from B lymphoblastoid cells, an APC, contain MHC-II molecules and can induce by themselves an antigen-specific CD4 T cell response (Raposo et al., 1996). After this discovery the potential role of antigen presenting cells EVs as antitumor therapeutic agent was studied, in particular coming from DCs, the professional APCs that are able to cross-present peptides in MHC-I molecules and elicit an antitumor response (Kowal & Tkach, 2019) (**Figure 10**).

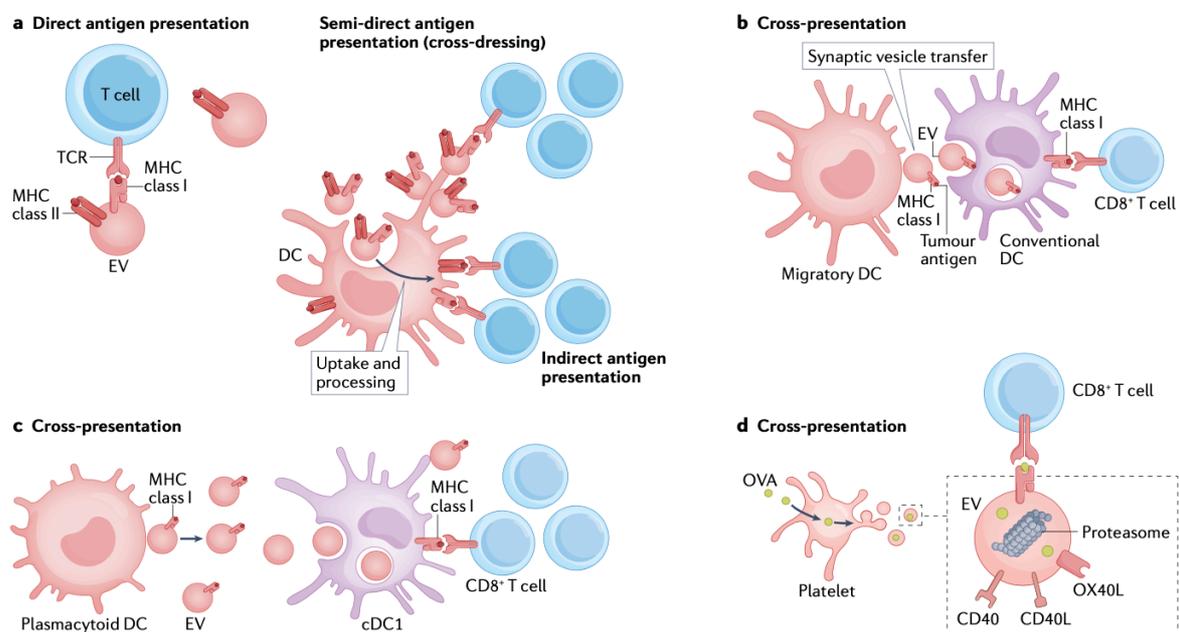


Figure 10: Mechanisms of EVs for antigen presentation.

EVs can present directly (a); do cross-dressing, which means that they attach to the membrane of DCs and can present their MHC-I or MHC-II to T cells and also use co-stimulatory molecules from the DC (a); be endocytosed and their content processed and presented (b and c); or crosspresent by platelets EVs (d). Source: (Buzas, 2022).

EVs coming from peptide-pulsed DCs expose MHC-I, MHC-II and costimulatory molecules and are able to prime specific cytotoxic T cells and eradicate or suppress the growth of tumors in mice models (Zitvogel et al., 1998). However, the direct activation of T cells or presentation of MHC-II⁺ EVs to naïve T cells was shown to be poorly efficient, and it required the presence of DCs and their co-stimulatory molecules (André et al., 2004; Théry et al., 2002; Vincent-Schneider et al., 2002). The potency of DC-derived EVs' effect is also related with the state of the producing DC: EVs from mature DCs are the most powerful, due to the enrichment on MHC-II and ICAM-1 (Segura et al., 2005). The activity of DC-EVs may depend also on the immunogenicity of the peptide: viral peptides loaded into EVs are able to activate human T cells directly (Admyre et al., 2006). Interestingly, by comparing the effects of DCs-derived EVs loaded either directly with OVA₃₂₃₋₃₃₉ peptide on MHC-II, or by indirect loading giving the whole OVA protein to DCs and then purifying their EVs, the group of Susanne Gabrielsson saw that both were able to activate CD4 T cells *in vitro*, however, the OVA peptide direct loading led to more effective EVs in *in vitro* assays, but *in vivo*, only the indirect loaded EVs were able to elicit a specific T cell response that was dependent on B cells, it was shifted to a Th1-type response and secretion of antibodies (Qazi et al., 2009). This is due, probably, to the fact that by giving the complete OVA protein, that was present inside and on the surface of EVs, some native epitopes could be recognized by B cells, and so a coordinated response between B cells, T cells and DCs could arise and synergize the effect. Even more, when evaluating the CD8 T cell response and the tumor growth, the whole OVA indirect loading led to better outcome than the peptide, and the effector response of CD8 T cells was dependent both on CD4 T cells and on B cells (Näslund et al., 2013).

It was shown that tumor-derived EVs can carry antigens naturally present in the producing cells (Wolfers et al., 2001) or artificially introduced by transforming cells to express the OVA antigen on the EVs (Zeelenberg et al., 2008b). These EVs can then transfer the antigen, either native or as peptides presented in MHC-I complexes on the EV membrane, to dendritic cells (DCs), leading to a T cell-mediated antitumor response and tumor rejection. DCs can capture EVs from circulation through MFG-E8, CD11a, CD54, PS and tetraspanins and present processed peptides in the MHC-II (Morelli et al., 2004).

In addition to containing antigens, it has been proposed that EVs possess an adjuvant-intrinsic effect due to the presence of damage-associated molecular patterns (DAMPs), and specially when they are under stress conditions (Abu et al., 2021; Murao et al., 2020). DAMPs are key factors for the immune system to recognize the danger coming from self-threats, and as it was proposed by Matzinger, changing the paradigm in immunology: the danger signals are the ones

that drive the immune system to react and destroy the dangerous agents, regardless if they are self or non-self (Matzinger, 1994). This includes damaged and dying cells. In tumors, an inflammatory and stressed microenvironment, with lack of enough nutrients and oxygen, trigger the secretion of DAMPs and induce the immunogenic cell death (ICD) (Krysko et al., 2012). The ICD, opposite to apoptosis, is immunogenic and is a desirable effect in cancer immunotherapies. Some of its DAMPs include exposed calreticulin, Hsp70, Hsp90, extracellular ATP, HMGB1, type I IFNs and IL1 cytokine family (Ahmed & Tait, 2020). However, the type of inflammation induced is also important, since for some kinds such as chronic inflammation, instead of driving elimination of the cancer cells, it promotes their survival and growth of the tumor (Balkwill & Mantovani, 2001). The presence of several types of DAMPs has been reported in EVs, such as HMGB1, HSP chaperones, S100 proteins and miRNAs (Abu et al., 2021). This would explain, at least partially, why EVs are more efficient than soluble proteins at transferring antigens to APCs and inducing an immune response (Zeelenberg et al., 2008a).

The apoptotic EVs (ApoEVs) were described as carriers of DAMPs, together with signals that favors their clearance by phagocytic cells (Caruso & Poon, 2018) (**Figure 11**). However, several subtypes of EVs also exist in cells undergoing apoptosis, and whether the antigen transfer capacities and the DAMPs are carried by all EV subtypes or ENPs, or only some specific types, in these cells or healthy ones, is unknown.

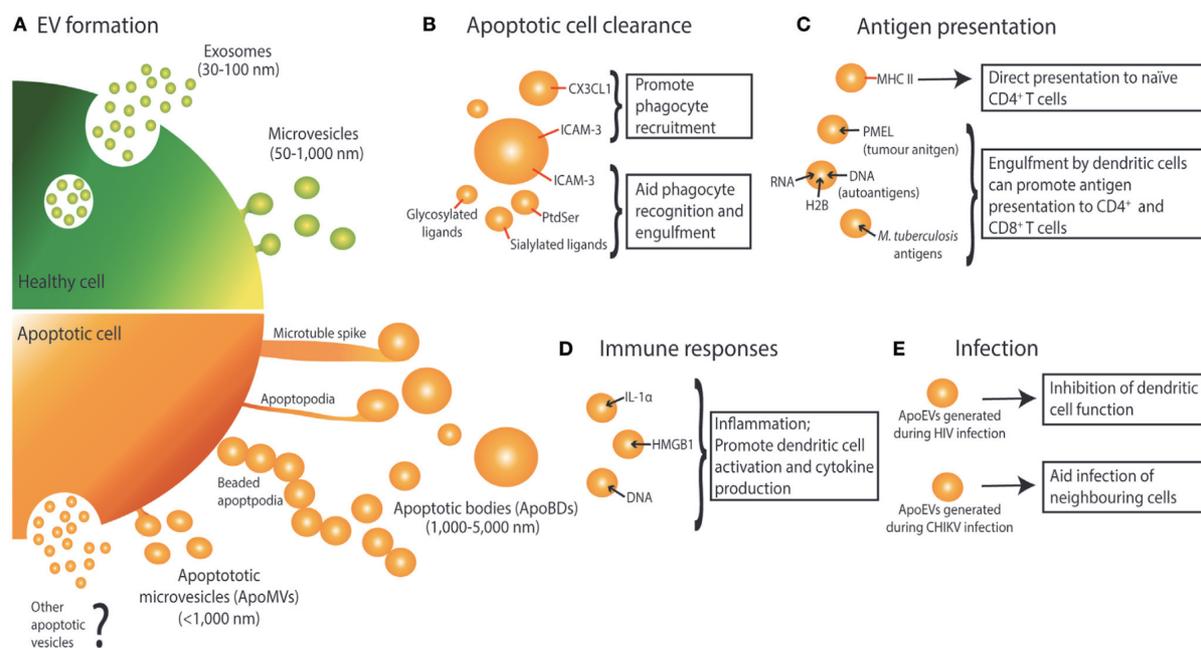


Figure 11: EVs from healthy and apoptotic cells and the immune functions of ApoEVs.

ApoEVs can harbour signals to be eaten by phagocytic cells (B), carry antigens (C), DAMPs (D) or promote infections (E). Source: (Caruso & Poon, 2018)

Another cell type responsible for the antitumor immune response driven by tumor EVs is the NK cells, even if NK cells' activity can also be suppressed by EVs depending on the signals that they may harbor (Soriani et al., 2020). Some of the molecules that activate the antitumor capacities of NK cells are HSPs (Gastpar et al., 2005; Lv et al., 2012; Noman et al., 2016), BAG-6 (Pogge von Strandmann et al., 2007) and IL-15R α (Borrelli et al., 2018).

Tumor EVs as inhibitors of the immune system

The bibliography supporting the idea that tumor EVs are immunosuppressive agents is more abundant, and this can be due to the fact that, as one of the mechanisms for intercellular communication, it is favorable for the tumor cell to co-opt the EVs for the immune evasion. If this happens, it will be positively selected and so observable in several cancer types and individuals and through different mechanisms (Théry et al., 2009).

Some of these discovered mechanisms of EV-mediated immunosuppression are the induction of T cell apoptosis through FASL (Andreola et al., 2002; Huber et al., 2005) or galectin 9 (Klibi et al., 2009). Tumor EVs can also turn the phenotype of T cells to regulatory and impair conventional T cells by depleting IL-2 (Clayton et al., 2008), reduce the cytotoxic capacity of NK cells and CD8 T cells (Clayton et al., 2008; C. Liu et al., 2006), impair the differentiation of myeloid precursors into DCs (Yu et al., 2007) and instead induce the differentiation into myeloid-derived suppressor cells (MDSCs) (Valenti et al., 2006) by the presence of TGF- β and NKG2D ligands. Tumor EVs from metastatic melanomas carry PD-L1 and cause the inactivation of CD8 T cells (Chen et al., 2018).

Also, tumor EVs can modify the immune cells through the microenvironment, that is for example, transforming fibroblast to become cancer-associated fibroblasts that secrete pro-inflammatory cytokines (such as IL-1 α , IL-6, and TNF- α), growth factors (such as G-CSF and GM-CSF) and angiogenic factors (VEGF) and thus favoring tumor growth (Dörsam et al., 2018).

An alternative explanation to the discrepancy that both antitumor and immunosuppressive effects were seen in tumor EVs could be because the different isolation methods and conditions used in each work can lead to a particular mix of heterogenous EVs, ENPs and other contaminants that may have individually diverse and sometimes opposite effects.

HYPOTHESIS AND OBJECTIVES

The work done during my PhD focused on the potential as therapeutic tools of different EVs subtypes, non-EV extracellular particles and proteins in 2 different contexts: as decoys in viral infection; and as immune modulators and antigen carriers in tumor immune responses. As it, I will present the next sections: Hypothesis and objectives, Results and Discussion divided in two parts according to the context:

Part 1: Extracellular vesicles and non-EV extracellular proteins as decoy for viral infection.

Part 2: Subtypes of extracellular vesicles and particles as antigen carriers and antigen presenting cells modulators.

1. Extracellular vesicles and non-EV extracellular proteins as decoy for viral infection

Hypothesis

EVs are lipid bilayer-enclosed structures containing transmembrane proteins with the same orientation as cells, they can expose at their surface the extracellular domains of transmembrane proteins that can bind to nearby or long-distance targets. By specifically binding to different proteins and protein-containing structures, EVs can act as a decoy, i.e. capture these structures and prevent their natural action or function. Our hypothesis was that EVs containing the receptor for the SARS-CoV-2, ACE2, could act as decoy agents and eventually prevent the viral infection into target cells, with better efficacy than soluble ACE2. Moreover, concomitant presence of an active serine-protease TMPRSS2 on the same EVs could further interfere with viral infectivity, by forcing viral fusion on the EV membrane, rather than on target cells.

Objectives

The objectives of the part 1 of my work have been to:

- 1- Obtain EVs bearing or not the human ACE2 receptor and with or without the protease TMPRSS2

- 2- Asses the capacity of ACE2-EV and ACE2-TMPRSS2-EV to inhibit the infection by SARS-CoV-2 virus.

2. Subtypes of extracellular vesicles and particles as antigen carriers and antigen presenting cells modulators.

Hypothesis

The hypothesis was that distinct subtypes of EVs and ENPs act as antigens and DAMP carriers delivering information to DCs in different manners, resulting in different outcomes in terms of adaptive immune responses. These different EV functions may be due to distinct protein compositions endowing them with specific abilities to interact and modify the DCs.

Objectives

The objectives of the part 2 of my work have been to:

- 1- Explore and characterize the heterogeneity of EVs and ENPs secreted by mouse tumor cell lines. Ideally set up a protocol to separate them, that will allow the study of their specific composition and function.
- 2- Uncover the phenotypic changes and immune modulation induced on DCs by the previously described and eventually separated tumor EVs and ENPs.
- 3- Determine the efficiency of the transfer of the cargo by each of these EVs/ENPs subtypes and their antigen presentation and activation capacities of T cells when given to DCs.

RESULTS

1. Extracellular vesicles and non-EV extracellular proteins as decoy for viral infection

Summary

SARS-CoV-2 entry is mediated by binding of the spike protein (S) to the surface receptor ACE2 and subsequent priming by host TMPRSS2 allowing membrane fusion. Here, we produced extracellular vesicles (EVs) exposing ACE2 and demonstrated that ACE2-EVs are efficient decoys for SARS-CoV-2 S protein-containing lentivirus. Reduction of infectivity positively correlated with the level of ACE2, was much more efficient than with soluble ACE2 and further enhanced by the inclusion of TMPRSS2.

Background

SARS-CoV-2 is the infectious causative agent of the COVID-19 pandemic (Zhou et al., 2020). Viral entry into host cells is mediated by the interaction of the spike (S) protein on the surface of SARS-CoV-2 with the surface receptor angiotensin-converting enzyme 2 (ACE2) (Walls et al., 2020). After binding to ACE2, the S protein is cleaved by Transmembrane protease serine 2 (TMPRSS2) and becomes fusogenic thus allowing viral entry (Hoffmann et al., 2020).

Soluble recombinant ACE2 neutralizes SARS-CoV-2 by binding the S protein and has been proven to reduce entry of SARS-CoV-2 into Vero-E6 cells and engineered human organoids (Monteil et al., 2020). ACE2, however, is synthesized as a transmembrane protein, like TMPRSS2. We postulate that ACE2 could be present on the surface of extracellular vesicles (EVs), which could result in better efficacy as a decoy to capture SARS-CoV-2. Furthermore, concomitant presence of an active serine-protease TMPRSS2 on the same EVs could further interfere with viral infectivity, by forcing viral fusion on the EV membrane, rather than on target cells.

Results and discussion

We transduced 293FT cells with lentivirus containing ACE2 alone (293FT- ACE2) or in combination with TMPRSS2 (293FT-ACE2-TMPRSS2). 293FT cells transduced with lentivirus containing empty plasmids were used as a control (293FT-mock).

We isolated EVs and soluble protein fractions by SEC from the 3 293FT cell lines. Overexpression of ACE2 in 293FT cells led to the incorporation of this molecule into EVs with higher levels found on EVs from 293FT-ACE2 than from 293FT-ACE2-TMPRSS2. 293FT-mock and 293FT-ACE2 cells expressed TMPRSS2, but the predicted full-length 54 kDa form was not detected in their EVs (Zmora et al., 2015). Importantly, EVs from 293FT cells overexpressing TMPRSS2 contained higher levels of the full-length TMPRSS2 protein and less of a cleaved form than EVs from 293FT-mock or 293FT-ACE2 cells.

We then analysed the capacity of ACE2- and ACE2-TMPRSS2-containing EVs to reduce the infection of target cells by a lentivirus containing SARS-CoV-2-S protein. We infected target cells with SARS-CoV-2-S-pseudotyped virus in the presence or the absence of EVs isolated from 293FT- mock (mock-EVs) or 293FT-ACE2 (ACE2-EVs) or 293FT-ACE2-TMPRSS2 cells (ACE2-TMPRSS2-EVs). Infection of 293FT-ACE2 and Caco-2 cells (the latter being a colon epithelial cell expressing ACE2, thus target of the SARS-CoV-2 virus) in the presence of ACE2-EVs and ACE2-TMPRSS2-EVs was reduced in a dose-dependent manner while infection remained unaffected by mock-EVs.

When re-plotting the results obtained before as a function of the absolute amount of ACE2 measured for these same samples by ELISA, we observed that EVs from cells overexpressing the full length TMPRSS2 together with ACE2 were more efficient at inhibiting SARS-CoV-2-S-pseudotyped viral infection than those from cells overexpressing ACE2 alone. Moreover, to achieve similar levels of inhibition of lentiviral infection as those observed with ACE2- or ACE2-TMPRSS2-EVs, 500 to 1500 times more of the soluble recombinant human ACE2 had to be used.

Conclusions

Altogether, these findings highlight the increased efficiency of EVs containing full-length ACE2 to inhibit SARS-CoV-2-S-pseudotyped viral entry when compared to the soluble protein alone. The enhanced efficiency of EVs from cells overexpressing TMPRSS2 could be due to the presence of TMPRSS2 together with ACE2, leading to fusion of the virus with the EV thus impairing their capacity to infect cells, and/or to other modifications of the EV composition induced by overexpression of TMPRSS2 in the EV-secreting cells.

Thus, ACE2-EVs represent a potential versatile therapeutic tool to block not only SARS-CoV-2 infection but also other coronavirus infections that use the ACE2 receptor for host cell entry, such as SARS-CoV (W. Li et al., 2003) and NL63 (Hofmann et al., 2005). Further studies to determine the efficacy of the ACE2/TMPRSS2-EVs in experimental models of SARS-CoV-2

virus need to be conducted to validate their therapeutic use for COVID-19, but also the lack of side-effects.

The results summarized here are described in more detail in the following article “Extracellular vesicles containing ACE2 efficiently prevent infection by SARS-CoV-2 Spike protein-containing virus” Article 1, published the 28/12/20 in Journal of Extracellular Vesicles (JEV).

Article 1: Extracellular vesicles containing ACE2 efficiently prevent infection by SARS-CoV-2 Spike protein-containing virus

SHORT COMMUNICATION

Extracellular vesicles containing ACE2 efficiently prevent infection by SARS-CoV-2 Spike protein-containing virus

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Abstract

SARS-CoV-2 entry is mediated by binding of the spike protein (S) to the surface receptor ACE2 and subsequent priming by host TMPRSS2 allowing membrane fusion. Here, we produced extracellular vesicles (EVs) exposing ACE2 and demonstrate that ACE2-EVs are efficient decoys for SARS-CoV-2 S protein-containing lentivirus. Reduction of infectivity positively correlates with the level of ACE2, is much more efficient than with soluble ACE2 and further enhanced by the inclusion of TMPRSS2.

KEYWORDS

ACE2, EV therapy, SARS-CoV-2, TMPRSS2

1 | INTRODUCTION

SARS-CoV-2 is the infectious causative agent of the COVID-19 pandemic (Zhou et al., 2020). Viral entry into host cells is mediated by the interaction of the spike (S) protein on the surface of SARS-CoV-2 with the surface receptor angiotensin-converting enzyme 2 (ACE2) (Walls et al., 2020). After binding to ACE2, the S protein is cleaved by Transmembrane protease serine 2 (TMPRSS2) and becomes fusogenic thus allowing viral entry (Hoffmann et al., 2020).

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ACE2 is expressed at the surface of pneumocytes and intestinal epithelial cells which are potential target cells for infection (Ziegler et al., 2020). A soluble form of the ACE2 ectodomain can be released after cleavage by ADAM10 or ADAM17 in different physiological conditions (Jia et al., 2009). In addition, TMPRSS2 can compete with the metalloproteases for ACE2 cleavage (Heurich et al., 2014). Soluble recombinant ACE2 neutralizes SARS-CoV-2 by binding the S protein and has been proven to reduce entry of SARS-CoV-2 into Vero-E6 cells and engineered human organoids (Monteil et al., 2020). ACE2, however, is synthesized as a transmembrane protein, like TMPRSS2. We postulate that ACE2 could be present on the surface of extracellular vesicles (EVs), which could result in better efficacy as a decoy to capture SARS-CoV-2. Furthermore, concomitant presence of an active serine-protease TMPRSS2 on the same EVs could further interfere with viral infectivity, by forcing viral fusion on the EV membrane, rather than on target cells.

EVs are lipid bilayer-enclosed structures containing transmembrane proteins, membrane-associated proteins, cytosolic proteins and nucleic acids that are released into the environment by different cell types (Mathieu et al., 2019). Since EVs have the same membrane orientation as cells, they expose at their surface the extracellular domains of transmembrane proteins that can bind to nearby or long-distance targets. By specifically binding to different proteins and protein-containing structures, EVs can act as a decoy for virus (De Carvalho et al., 2014) and bacterial toxins (Keller et al., 2020), thus suggesting a potential role as therapeutic agents.

2 | RESULTS

In order to explore the hypothesis that EVs can be used as SARS-CoV-2 decoy agents, we first assessed whether ACE2 can be present in EVs from cell lines derived from tissues expressing ACE2. As cell lines endogenously expressing ACE2, we used the human lung epithelial cell line Calu-3 and the epithelial colorectal cell line Caco-2 which are known targets for SARS-CoV-2 infection (Hoffmann et al., 2020). In a pilot experiment, Calu-3 and Caco-2 were cultured in medium without fetal bovine serum (FBS) for 24 h and EVs were isolated from the cell conditioned medium (CCM) by size-exclusion chromatography (SEC). This technique allows the separation of EVs from soluble proteins (Figure 1a). We analysed side-by-side fractions enriched in EVs (pooled fractions 7–11), in soluble factors (pooled fractions 17–21), and the fractions in-between (pooled fraction 12–16) (Figure 1a). Particle quantification by nanoparticle tracking analysis (NTA) confirmed that the majority of particles released by Calu-3 and Caco-2 cells were isolated in EV-containing fractions (Supplementary Figure A). In this experiment, Caco-2 cells released less EVs but of similar mode size than Calu-3 (Figure 1b). These EVs contained ACE2 protein as well as known EV markers (CD63, CD81 ADAM10) (Figure 1c). In addition, although Caco-2 and Calu-3 expressed TMPRSS2, this protease was not released in EVs (Figure 1c). To obtain EVs with high amounts of ACE2 and TMPRSS2 to be tested as decoy agents, we switched to 293FT cells that could be easily genetically-edited. We transduced 293FT cells with lentivirus containing ACE2 alone (293FT-ACE2) or in combination with TMPRSS2 (293FT-ACE2-TMPRSS2). 293FT cells transduced with lentivirus containing empty plasmids were used as a control (293FT-mock). The three 293FT cell lines were cultured in FBS-containing EV-depleted medium and EVs were isolated from concentrated CCM by SEC. We observed a high count of particles with comparable mode sizes in EV fractions from all 293FT cell lines (Figure 1d, Supplementary Figure A) coincident with the presence of CD63, CD81, ADAM10 and HSP70 EV markers (Figure 1e). Overexpression of ACE2 in 293FT cells led to the incorporation of this molecule into EVs (Figure 1e) with higher levels found on EVs from 293FT-ACE2 than from 293FT-ACE2-TMPRSS2 (Supplementary Figure B). 293FT-mock and 293FT-ACE2 cells expressed TMPRSS2, but the predicted full-length 54 kDa form was not detected in their EVs: a 30 kDa N-terminal fragment devoid of the serine protease domain (Zmora et al., 2015) was mainly detected, together with a low level of the glycosylated full-length 70 kDa form. Importantly, EVs from 293FT cells overexpressing TMPRSS2 contained higher levels of the full-length TMPRSS2 protein and less of a cleaved form than EVs from 293FT-mock or 293FT-ACE2 cells (Figure 1e, Supplementary Figure C) (Afar et al., 2001). Since the ACE2 ectodomain can be released after cleavage (Jia et al., 2009), we evaluated whether cells overexpressing ACE2 release soluble ACE2 in addition to EV-associated ACE2. To do this, we analysed the presence of ACE2, the EV marker CD81 and the non-EVs component AChE (Liao et al., 2019) by WB on EV fractions (1×10^9 particles) and the intermediate and soluble fractions obtained from the same amount of CCM. We detected some particles in intermediate and soluble fractions from the three 293FT cell lines, that probably came from the depleted medium since they did not contain EV markers by WB (Supplementary Figure A, D, E). AChE was enriched in soluble fractions whereas CD81 was mainly found in EV fractions validating our isolation protocol for EVs and soluble components (Figure 1f). Importantly, ACE2 was found as a full-length transmembrane form in the EV fractions, as two shorter cleaved forms in the soluble fractions, and a mixture of all forms in the intermediate fractions (Figure 1f). Intermediate fractions thus represent a mixture of EVs and soluble proteins and for this reason were not further analysed. Low levels of ACE2 were found in the soluble SEC fractions of 293FT-ACE2 cells when compared to the levels present on the EV-containing fraction (Figure 1f, 1g). By contrast, 293FT-ACE2-TMPRSS2 cells released similar amounts of EV-associated as of soluble ACE2 (Figure 1f, 1g), suggesting that the overexpression of the protease cleaves ACE2 as previously described (Heurich et al., 2014), favouring its secretion to the extracellular space as a soluble protein instead of associated to EVs.

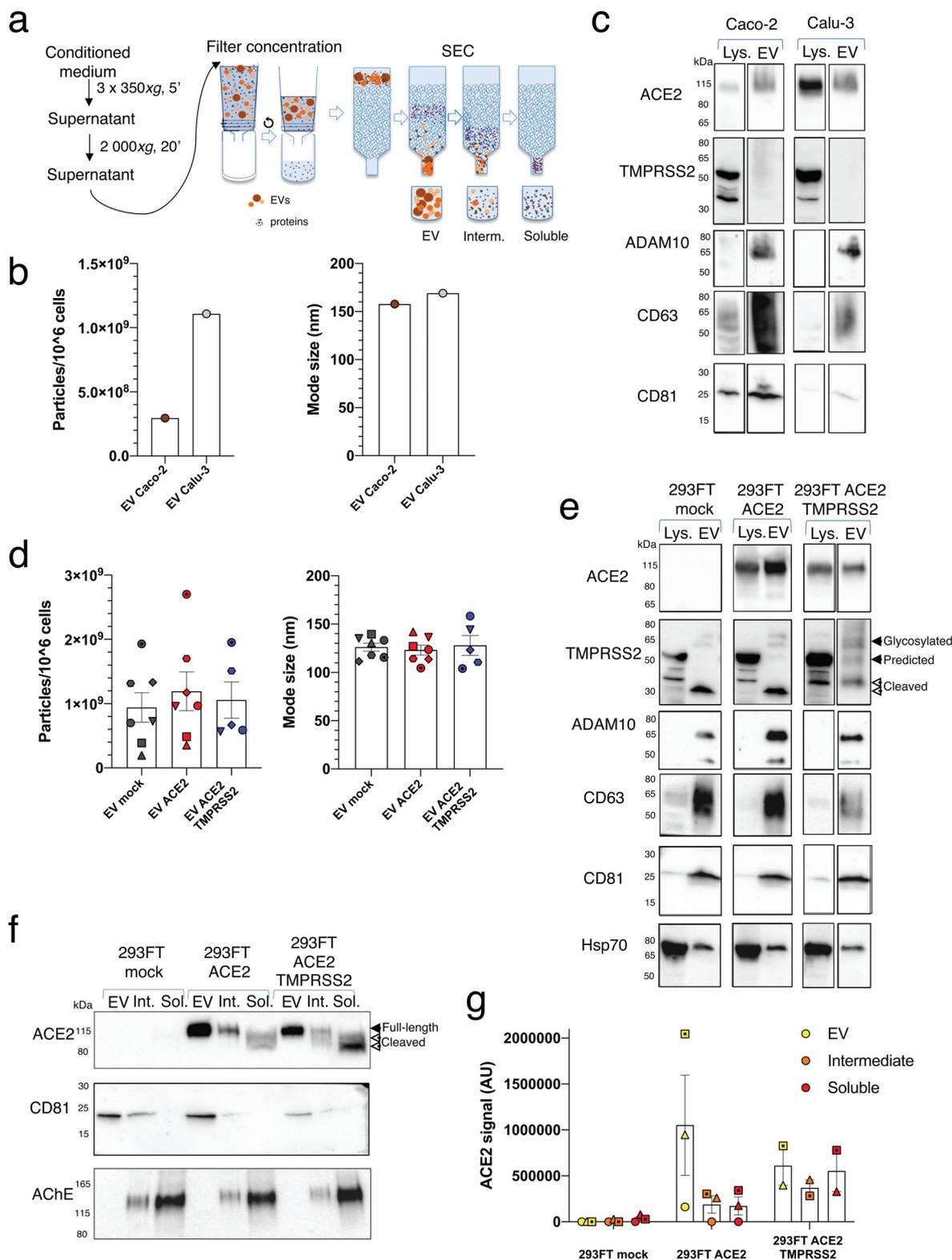


FIGURE 1 Isolation and characterization of EVs containing ACE2 and TMPRSS2. (a) Scheme of EV isolation and separation from soluble components by SEC. (b) NTA quantification and mode size of the particles in EV-containing SEC fractions obtained from Caco-2 and Calu-3 cells. (c) WB analysis of ACE2, TMPRSS2 and different EV markers in lysates from 4×10^5 cells and 1×10^{10} 2020 (Caco-2) or 0.5×10^{10} 2020 (Calu-3) particles obtained from EV SEC fractions. One experiment. (d) NTA quantification and mode size of the particles in EV-containing fractions obtained from 293FT-mock, 293FT-ACE2 and 293FT-ACE2-TMPRSS2 cell lines. Different symbols correspond to independent experiments. Error bars indicate SEM. (e) WB analysis of ACE2, TMPRSS2 and different EV markers in lysates from 4×10^5 cells and 1×10^{10} 2020 particles from EV SEC fractions obtained from the three 293FT cell lines. (f-g) WB analysis of ACE2, CD81 and AChE on EV, intermediate and soluble fractions from the three 293FT cell lines. 1×10^9 particles from the EV fraction or material recovered from the same corresponding volume of CCM, for the intermediate or soluble fractions, were loaded on the gel. (f) Representative WB. (g) Quantification of ACE2 signal (pooled full-length and cleaved forms) in 2–3 independent WB

We then analysed the capacity of ACE2- and ACE2-TMPRSS2-containing EVs to reduce the infection of target cells by a lentivirus containing SARS-CoV-2-S protein. The virus used in this study was produced using an HIV packaging vector pseudotyped with SARS-CoV-2 Spike protein and including a GFP-coding sequence, which is expressed in infected cells. A variant of SARS-CoV-2 Spike protein bearing a region of the tail of the HIV gp41, instead of the Spike tail, previously shown to enhance infection of pseudotyped virus (Moore et al., 2004) was used. First, we determined the infectivity of the target cells Caco-2, Calu-3 and 293FT-ACE2 by SARS-CoV-2-S-pseudotyped lentivirus and observed that each of these cell lines is infected similarly in a concentration-dependent manner (Figure 2a). To assess the ability of ACE2-containing EVs to decrease virus infectivity *in vitro*, we infected target cells with SARS-CoV-2-S-pseudotyped virus in the presence or the absence of EVs isolated from 293FT-mock (mock-EVs) or 293FT-ACE2 (ACE2-EVs) or 293FT-ACE2-TMPRSS2 cells (ACE2-TMPRSS2-EVs) (Figure 2b). Infection of 293FT-ACE2 cells in the presence of ACE2-EVs and ACE2-TMPRSS2-EVs was reduced while infection remained unaffected by mock-EVs (Figure 2c and quantification in 2d). Importantly, this inhibition was dependent on the dose of EVs (Figure 2d). Caco-2 infection was also reduced in the presence of ACE2-EVs and ACE2-TMPRSS2-EVs (Figure 2e). We then quantified by enzyme-linked immunosorbent assay (ELISA) the amount of ACE2 released by these cell lines. As previously documented by western blot (Figure 1f and 1g), we observed that 293FT-ACE2 cells released high levels of ACE2 mainly associated with EVs while 293FT-ACE2-TMPRSS2 cells released lower ACE2 levels that were equally distributed between EV and soluble fractions (Figure 2f). Strikingly, ACE2 in the soluble fractions from these latter cells could not inhibit SARS-CoV-2-S-pseudotyped virus infection as compared to a comparable amount of ACE2 associated with EVs (Figure 2g). When re-plotting the results obtained in Figure 2d as a function of the absolute amount of ACE2 measured for these same samples by ELISA (Figure 2f), we observed that EVs from cells overexpressing the full length TMPRSS2 together with ACE2 were more efficient at inhibiting SARS-CoV-2-S-pseudotyped viral infection than those from cells overexpressing ACE2 alone (Figure 2h). Moreover, to achieve similar levels of inhibition of lentiviral infection as those observed with ACE2- or ACE2-TMPRSS2-EVs, 500 to 1500 times more of the soluble recombinant human ACE2 had to be used (Figure 2h) in accordance with previous publications (Monteil et al., 2020). Altogether, these findings highlight the increased efficiency of EVs containing full-length ACE2 to inhibit SARS-CoV-2-S-pseudotyped viral entry when compared to the soluble protein alone. The enhanced efficiency of EVs from cells overexpressing TMPRSS2 could be due to the presence of TMPRSS2 together with ACE2, leading to fusion of the virus with the EV thus impairing their capacity to infect cells, and/or to other modifications of the EV composition induced by overexpression of TMPRSS2 in the EV-secreting cells.

3 | DISCUSSION

Our data demonstrate that EVs containing ACE2, alone or in combination with TMPRSS2, block SARS-CoV-2 Spike-dependent infection in a much more efficient manner than soluble ACE2. Thus, ACE2-EVs represent a potential versatile therapeutic tool to block not only SARS-CoV-2 infection but also other coronavirus infections that use the ACE2 receptor for host cell entry, such as SARS-CoV (Li et al., 2003) and NL63 (H et al., 2005). Further studies to determine the efficacy of the ACE2/TMPRSS2-EVs in experimental models of SARS-CoV-2 virus need to be conducted to validate their therapeutic use for COVID-19, but also the lack of side-effects. The use of engineered EVs as therapeutic agents has been proposed several years ago and is currently being explored in humans (Wiklander et al., 2019), suggesting that well-designed EV therapeutics against COVID-19 may be feasible to prevent initial infection or further internal dissemination of the virus, and thus reducing the virus burden and disease severity. However, as recently highlighted by the International Societies for EV (ISEV) and for Cellular Therapies (ISCT) (Börger et al., 2020), despite the urgency induced by the current pandemic, EV-based therapeutic developments for COVID-19 will have to meet as strong criteria of manufacturing processes, quality controls and compliance to safety regulation as any other therapies, before they can be implemented in human subjects.

Note: a speculative article discussing the idea that we demonstrate experimentally here was published while we were preparing this article, thus showing concomitant emergence of similar scientific ideas (Inal, 2020).

4 | METHODS

4.1 | Cells

Human Caco-2 (HTB-37) and Calu-3 (HTB-55) were purchased from ATCC and maintained at 37°C in a humidified atmosphere with 5% CO₂. Caco-2 and Calu-3 cells were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (Gibco), 100 U/ml penicillin-streptomycin (Thermo Fisher Scientific) and 1% non-essential amino acids (Thermo Fisher Scientific). For Calu-3 cells the medium was also supplemented with 1 mM sodium pyruvate (Thermo Fisher Scientific) and 10 mM HEPES (Thermo Fisher Scientific). 293FT cells were cultured in DMEM medium (Thermo Fisher Scientific) supplemented with 10% FBS (Eurobio) and 100 U/ml penicillin-streptomycin (Thermo Fisher Scientific). 293FT-mock, 293FT-ACE2 and 293FT-ACE2-TMPRSS2 cells were generated by stable double transduction with pTRIP-SFFV-tagBFP-2A and

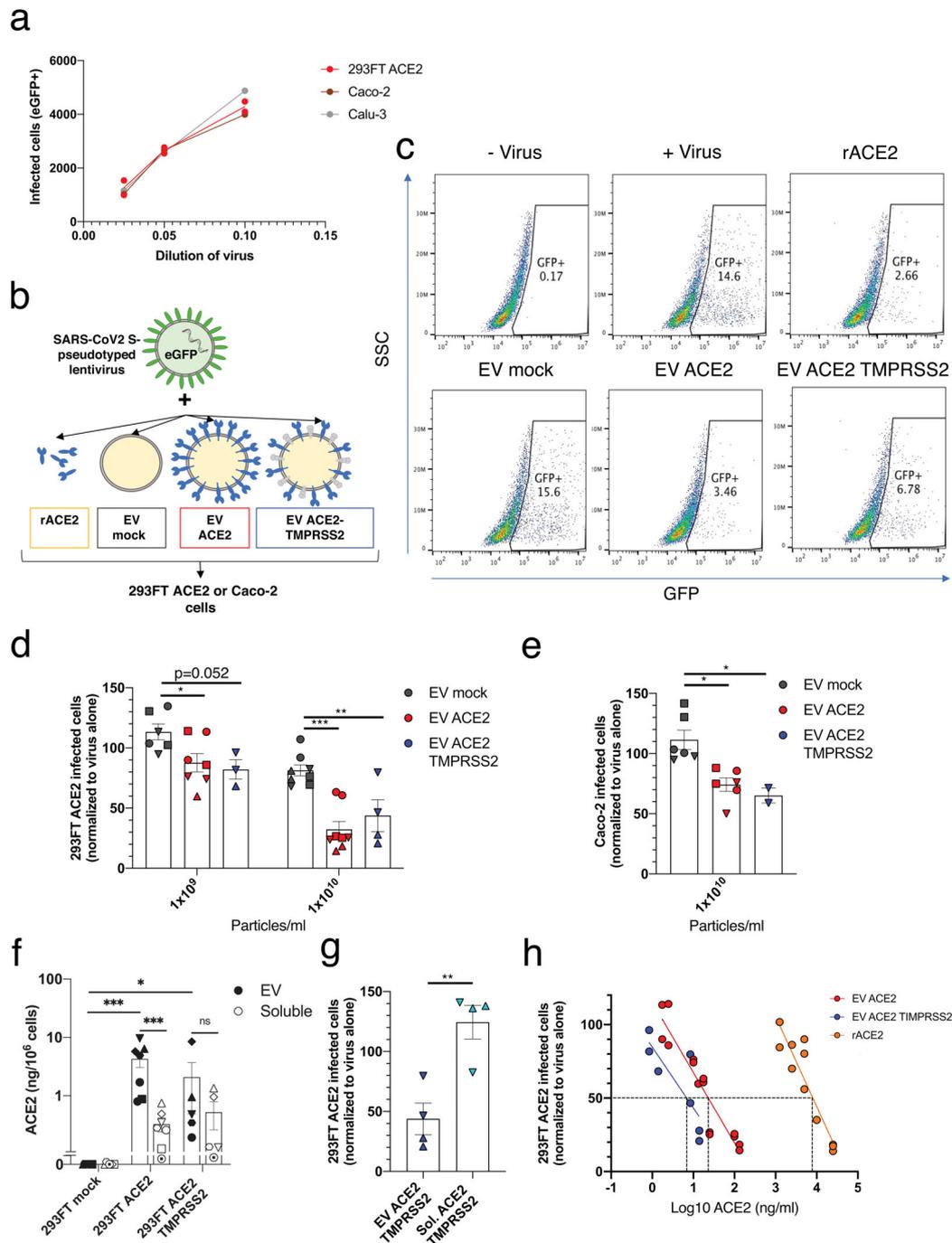


FIGURE 2 Inhibition of SARS-CoV-2-S-pseudotyped virus infection with ACE2 EVs. (a) Infection of 293FT-ACE2, Caco-2 and Calu-3 cells with different dilutions of a SARS-CoV-2-S-pseudotyped lentivirus encoding for eGFP. The number of infected cells was calculated by multiplying the percentage of GFP-positive cells by the initial number of cells. (b) Scheme of the infectivity assay with different treatments. (c) Dot plots showing the percentage of infected (= eGFP+) 293FT-ACE2 cells obtained after incubation with viruses alone (0.05 dilution), in combination with 1×10^{10} 2020 EVs from the different 293FT cell lines or in combination with rACE2 (25 $\mu\text{g/ml}$). This rACE2 level represents 250 and 2960 times more ACE2 than the one contained in ACE2-EVs and ACE2-TMPRSS2-EVs, respectively, measured by ELISA. (d) Quantification of the number of infected 293FT-ACE2 cells in the presence of EVs. The percentage of eGFP+ cells was measured by FACS and normalized to infection with the virus alone (100%). Results from three independent experiments are shown. All replicates from each experiment are included. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Dunnett's test). (e) Caco-2 infection in the presence of ACE2-EVs and ACE2-TMPRSS2-EVs. * $P < 0.05$ (Dunnett's test). (f) ACE2 quantification by ELISA in EV and soluble fractions obtained from the three different 293FT cell lines. Results are expressed as ng per million of secreting cells. * $P < 0.05$; *** $P < 0.001$ (non-parametric ANOVA with Kruskal-Wallis test for comparison among all groups). *** $P < 0.001$ (Mann-Whitney test for comparison among EV vs soluble for each cell line). (g) Comparison of the effect on 293T-ACE2 infection of 1×10^{10} 2020 EVs and soluble fractions from an equivalent volume of CCM of 293FT-ACE2-TMPRSS2 cells. ** $P < 0.01$ (t-test) (h) Percentage of infectivity from Figure 2d related to the amount of EV-associated ACE2, quantified by ELISA in Figure 2f. As a comparison, cell infection rates in the presence of increasing amount of recombinant ACE2 (rACE2) were also determined ($n = 4$). Lines represent results of linear regression analysis. Comparison of slopes and intercepts using Prism indicated that the three regression lines are distinct but parallels. ACE2-TMPRSS2 is different from ACE2 ($P = 0.0017$) and rACE2 is different from ACE2-TMPRSS2 ($P < 0.0001$) and from ACE2 ($P < 0.0001$)

pTRIP-SFFV-TagRFP657-2A, pTRIP-SFFV-tagBFP-2A-hACE2 and pTRIP-SFFV-TagRFP657-2A, or pTRIP-SFFV-tagBFP-2A-hACE2 and pTRIP-SFFV-TagRFP657-2A-TMPRSS2, respectively.

4.2 | Plasmids

The plasmids psPAX2, CMV-VSVG, pTRIP-SFFV-tagBFP-2A (Cerboni et al., 2017) and pTRIP-SFFV-eGFP-NLS (Raab et al., 2016) were previously described. pTRIP-SFFV-TagRFP657-2A was generated by PCR from a synthetic gene coding for TagRFP657. pTRIP-SFFV-tagBFP-2A-hACE2 and pTRIP-SFFV-TagRFP657-2A-TMPRSS2 constructs were obtained by PCR from pLenti6-hACE2-BSD (hACE2 sequence from Addgene #1786 subcloned into pLenti6-BSD) and pCSDest-TMPRSS2 (Addgene #53887) respectively. A codon optimized version of the SARS-CoV-2 S gene (GenBank: QHD43416.1) was transferred into the pHCMV backbone (GenBank: AJ318514), by replacing the VSV-G gene (pHCMV-SARS-CoV-2-Spike) (Grzelak et al., 2020). pHCMV-SARS-CoV-2-S-H2 was obtained by PCR from pHCMV-SARS-CoV-2-Spike in order to include the membrane-proximal region of the cytoplasmic domain of HIV-1 gp160 (NRVRQGYs, amino acid sequence) (Mammano et al., 1995) after residue 1246 of the S protein (Moore et al., 2004).

4.3 | Preparation of EV-depleted medium

EV-depleted medium was obtained by overnight ultracentrifugation of DMEM supplemented with 20% FBS at 100,000 $\times g$ in a Type 45 Ti rotor (Beckman Coulter, K-factor 1042.2). After ultracentrifugation, EV-depleted supernatant was carefully pipetted from the top and leaving 7 ml in the bottom. Supernatant was filtered through a 0.22 μm bottle filter (Millipore) and additional DMEM and antibiotics were added to prepare complete medium (10% EV-depleted FBS medium).

4.4 | EV isolation by size-exclusion chromatography

239FT-mock, 293FT-ACE2 and 293FT-ACE2-TMPRSS2 cells were cultured in FBS EV-depleted medium for 24 h. Caco-2 and Calu-3 cells were cultured in FBS-free DMEM for 24 h. Cell conditioned medium (CCM) was harvested by pelleting cells at 350 $\times g$ for 5 min at 4°C three times. Supernatant was centrifuged at 2,000 $\times g$ for 20 min at 4°C to discard 2K pellet and concentrated on a Millipore Filter (MWCO = 10 kDa, UCF701008) to obtain concentrated CCM. Medium was concentrated to 1 ml from 12 to 41 ml for Caco-2 and Calu-3 and from 75 ml for 293FT cells and overlaid on a 70 nm qEV size-exclusion column (Izon, SPI). 0.5 ml fractions were collected and EVs were recovered in fractions 7 to 11 following manufacturer's instructions. We additionally collected intermediate fractions 12 to 16 and soluble factors in fractions 17 to 21. The three pools of fractions were concentrated using 10 kDa filter (Amicon, UCF801024) to reach a final volume of approximately 100 μl . Samples were stored aliquoted at -80°C.

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV200117) (Van Deun et al., 2017).

4.5 | Nanoparticle tracking analysis

NTA was performed to analyse EV fractions, intermediate fractions and soluble fractions using ZetaView PMX-120 (Particle Metrix) with software version 8.04.02. The instrument was set a sensitivity 77 and shutter of 70. Measurements were done at 11 different positions (three cycles per position) and frame rate of 30 frames per second.

4.6 | Western blotting (WB)

Cell lysate was prepared using lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton, pH 8) supplemented with Protease Inhibitor Cocktail (Sigma) at a concentration of 4×10^6 cells in 100 μl of buffer. After incubation for 20 min on ice, samples were centrifuged at 18,500 $\times g$ for 20 min. The pellet was discarded and the supernatant was kept for further analysis. Cell lysates, EVs and the other SEC fractions were resuspended in Laemmli Sample Buffer (Bio-Rad). Cells lysates corresponding to 4×10^5 cells, the number of particles indicated in figures legends for EV fractions and the intermediate and soluble fractions obtained from the same volume of conditioned medium were loaded on 4%–15% Mini-Protean TGX Stain-Free gels (Bio-Rad), under non-reducing conditions. Transferred membranes (Immuno-Blot PVDF Bio-Rad) were incubated with antibodies and developed using Clarity Western ECL substrate (Bio-Rad) and the ChemiDoc Touch imager (Bio-Rad). Antibodies for WB were anti-human: ACE2 (clone EPR4435 against extracellular domain, Abcam 108252), TMPRSS2 (clone EPR3681 against cytoplasmic domain, Abcam 92323), ADAM10 (clone 163003, R&D Systems MAB1427), CD63 (clone H5C6, BD Bioscience 556019), Syntenin-1 (clone C2C3,

Genetex GTX10847), CD81 (clone 5A6, Santa Cruz sc-23692), HSP70 (clone C92F3A-5, Enzo LifeScience, ADI-SPA-810-D) and AChE (Abcam, ab31276). Secondary antibodies included HRP-conjugated goat anti-rabbit IgG (H+L) (Jackson 111-035-144), goat anti-mouse IgG (H+L) (Jackson 111-035-146) and donkey anti-goat IgG (Jackson, 705-035-147).

4.7 | Viral production

SARS-CoV-2-S-pseudotyped lentiviruses were produced by transient transfection of 293FT cells in 150 cm² flask with 5 µg pHCMV-SARS-CoV-2-S-H2, 13 µg psPAX2 and 20 µg pTRIP-SFFV-eGFP-NLS and 114 µl of TransIT-293 (Mirus Bio). SARS-CoV-2-S-pseudotyped viruses' supernatant was centrifuged at 300xg for 5 min to remove dead cells, filtered with a 0.45 µm filter (Millipore) and loaded on top of a 20% sucrose gradient for concentration. Viral concentration was achieved by ultracentrifugation at 120,000 x g for 1 h 30 min at 4 °C in a SW32i rotor. The pellet containing concentrated SARS-CoV-2 S-pseudotyped virus from three 150 cm² flasks was resuspended in 1 ml EV-depleted DMEM and 100 µl aliquots were stored at -80°C.

4.8 | Infectivity assay

20,000 Caco-2 or Calu-3 cells or 10,000 293FT-ACE2 cells were seeded in a 96 well plate 6 h before infection with SARS-CoV-2 S-pseudotyped virus. Infection was performed in the absence or in the presence of different amount of EVs or human recombinant ACE2 (Abcam, 151852) by spinoculation at 1200 x g for 1 h 30 min at 25°C. 48 h after infection, cells were washed, trypsinized and fixed. Infection was measured by analysing eGFP expression using a CytoFLEX LX cytometer. Data were analysed using FlowJo software.

4.9 | ACE2 enzyme-linked immunosorbent assay

Quantification of the amount of human ACE2 in the different EV preparations and other SEC fractions was done using the human ACE2 ELISA kit (Abcam, ab235649) following manufacturer's instructions.

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AUTHOR CONTRIBUTIONS

Federico Coccozza, Ester Piovesana, Nathalie Névo, Xavier Lahaye performed the experiments. Federico Coccozza, Ester Piovesana, Mercedes Tkach, Lorena Martin-Jaular, Clotilde Théry analysed the data. Lorena Martin-Jaular, Clotilde Théry, Mercedes Tkach designed the experiments. Ester Piovesana, Federico Coccozza, Mercedes Tkach, Clotilde Théry, Lorena Martin-Jaular wrote the paper. Xavier Lahaye, Nicolas Manel, Julian Buchrieser, Olivier Schwartz designed plasmids. Xavier Lahaye, Nicolas Manel generated cells overexpressing ACE2 and TMPRSS2 and developed the infection assay.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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2. Subtypes of extracellular vesicles and particles as antigen carriers and antigen presenting cells modulators.

Summary

Tumor-derived EVs have been proposed to act as a cell-free source of tumor antigens and to carry PAMPs and DAMPs that may induce immune priming of antigen presenting cells, possibly resulting in immune responses against tumors. Conversely, tumor-derived EVs have been also proposed as immunosuppressive agents promoting tumor immune escape. Here, we aimed to exhaustively characterize biochemically and functionally the array of secreted EVs and ENPs of murine tumor cell lines. We compared the protein composition and the functional interaction with target dendritic cells of sEVs, VLPs, ENPs and a mixture of large/small (ls)EVs/VLPs. These subpopulations were differentially uptaken, induced different phenotypic changes, and displayed different efficacies to induce antigen-specific responses by dendritic cells (DCs). Our results call for re-evaluation of previous studies of mouse tumor-derived EVs to decipher the respective proportions and functions of non-viral EVs and virus-like particles and their contribution to anti-tumor immune responses and to tumor progression.

Background

In addition to soluble proteins, cells secrete membrane-enclosed extracellular vesicles (EVs) and non-vesicular nanoparticles (ENPs) that are thought to play a role in intercellular communications. Tumor cells secrete extracellular vesicles (EVs) and nanoparticles (ENPs) that have been associated with a plethora of immune regulatory functions. In particular, EVs have been proposed to act as a cell-free source of tumor antigens and to carry together PAMPs and DAMPs that may induce immune priming of antigen presenting cells (APC) possibly resulting in immune responses against tumors. However, the EV composition and their cargo can be remarkably diverse, and different EVs and ENPs may have varying abilities to interact and modify recipient cells. Therefore, understanding the mechanisms by which the different types of EVs and ENPs modulate the responses of APCs in the context of tumor progression is crucial, and may identify suitable EV-based candidates to use as immunotherapeutic agents against tumors.

Results and discussion

Here we characterize the heterogeneity of the secreted EVs and ENPs of EO771 cells, a murine mammary adenocarcinoma. Unexpectedly, we identified viral-like particles (VLPs) of infectious endogenous murine leukemia virus in preparations of small (s)EVs/ENPs produced by two mammary tumor or dendritic cell lines, but not by fibroblasts or primary cells. We established a robust protocol to separate sEVs from VLPs. We compared the protein composition and the functional interaction with target dendritic cells of sEVs, VLPs, ENPs and a mixture of large/small (ls)EVs/VLPs. These subpopulations were differentially uptaken and induced different phenotypic changes in a dendritic cell line and primary DCs. ENPs were poorly captured and did not affect DCs. sEVs specifically induced DC death. The mixed lsEV/VLP preparation was the most efficient to induce DC maturation and antigen presentation, followed by VLPs.

Conclusions

EVs isolated by regular protocols from mouse tumor cells contain VLPs coming from endogenous MLV with infectious potential. Combination of filter concentration, centrifugation and density gradient allows to separate 10k, sEV, VLP and ENP. These subtypes of particles contribute differently to modification of target immune myeloid cells *in vitro*.

Our results call for re-evaluation of previous studies of mouse tumor-derived EVs to decipher the respective proportions and functions of non-viral EVs and virus-like particles and their contribution to anti-tumor immune responses and to tumor progression.

The results summarized here are described in more detail in the following manuscript “Subtypes of tumor-derived extracellular vesicles, co-isolated enveloped viruses and non-vesicular particles differently modulate and transfer antigen to dendritic cells”, Article 2, in preparation.

Article 2: Subtypes of tumor-derived extracellular vesicles, co-isolated enveloped viruses and non-vesicular particles differently modulate and transfer antigen to dendritic cells

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DISCUSSION

1. Extracellular vesicles and non-EV extracellular proteins as decoy for viral infection

EVs as decoy for SARS-CoV-2

In the context of the COVID-19 pandemic, we demonstrated that ACE2-carrying EVs and not EVs without it were able to inhibit pseudotyped SARS-CoV-2 virus infectivity on target cells *in vitro* (Section: **Article 1**, (Cocozza et al., 2020)). A few months later, thus practically concomitantly, the group of Robert Coffey published a work where they probed the binding of the spike protein of SARS-CoV-2 to EVs and exomeres bearing ACE2 (Q. Zhang, Jeppesen, Higginbotham, Franklin, et al., 2021). And after this, several groups published similar results showing the infection inhibition with different cell models *in vitro* (El-Shennawy et al., 2022; H. K. Kim et al., 2022; Wu et al., 2022; Xie et al., 2021), thus validating our observations. Moreover, some artificial nanoparticles sharing properties with the EVs were also capable to neutralize the SARS-CoV-2 virus (Z. Li et al., 2021; C. Wang et al., 2021; H. Zhang et al., 2021). Also nanoparticles enveloped with membrane of ACE2-expressing cells have been used to neutralize SARS-CoV-2 or pseudotyped SARS-CoV-2 virus (M. Chen et al., 2021; Z. Wang et al., 2022).

Although these last approaches allow obtaining a higher yield of vesicles containing ACE2, the membrane orientation of these artificial vesicles is not controlled, and thus can be the opposite of the desired (i.e. outside exposure of the ACE2). Different strategies are needed to ensure the right-side membrane orientation, and for clinical applications, the proportion of wrong-side vesicles should be estimated for each batch. On the other hand, natural EVs are always of the right orientation.

In a few studies, EVs containing ACE2 have been tested for their capacity to inhibit SARS-CoV-2 infection *in vivo* in mouse models expressing human ACE2, leading to promising results. However, these studies required high amounts and/or multiple doses of EVs to neutralize the infection (El-Shennawy et al., 2022; H. K. Kim et al., 2022; Xie et al., 2021) and were often reporting single experiments with a limited number of mice (H. K. Kim et al., 2022; Wu et al., 2022; Xie et al., 2021; J. Zhang et al., 2021). In addition, our study includes TMPRSS2 that has not been proposed by others. We think that the presence of TMPRSS2

together with the presence of a high amount of ACE2 on EVs is essential to ensure an efficient inactivation of the virus in particular by promoting its fusion to the decoy EVs.

To understand the molecular mechanisms of this activity of EVs bearing ACE2, the group of Richard Wong performed a real time visualization by high-speed atomic force microscopy (HS-AFM) of the interaction between the S protein and the hACE2 in the membrane of sEVs (Lim et al., 2021). They showed that a single ACE2-containing EV could interact with several S proteins in a reversible way, but when it interacted with the S2 subunit alone, the part of the protein that is exposed after cleavage by surface serine proteases (TMPRSS2) or endosomal cysteine proteases (CatB/L) of the host, the S2 subunit fused with the membrane of the EV and induced its shrinking. This observation supports the hypothesis that the SARS-CoV-2 could interact with EVs containing the receptor ACE2 by binding to it and then, after cleavage by a protease such as TMPRSS2, of the S protein and exposure of the S2 subunit, induce the entrance of the virus into the EV.

On the contrary, other groups have shown an enhancement on the infectivity of SARS-CoV-2 virus, through the action of extracellular ACE2. In particular, a study screening for cellular factors involved in the SARS-CoV-2 infection found as important factors the receptors involved in the renin-angiotensin system signaling (AT1 and AVPR1B) (Yeung et al., 2021). They further characterized a mechanism by which the S protein of the virus can bind the soluble form of ACE2 (sACE2) alone or in complex with vasopressin and be endocytosed by AT1 or AVPR1B receptors respectively. However, this concerns the sACE2, and not necessarily the transmembrane form included on EVs, the capacity of the entire ACE2 to be recognized by AT1 or AVPR1B remains to be evaluated. These results may explain why we do not obtain any inhibitory effect, and even a tendency to enhance it, elicited by the soluble fraction of the ACE2-TMPRSS2 transfected 293FT cells. One study did find an infection enhancing effect of ACE2-EVs, using a 293T (similar to our 293FT) transfected with mock or ACE2, to produce the EVs (Tey et al., 2022). Moreover, they propose that this is mediated by the endocytosis of the EVs-virus complexes suggesting the previous described renin-angiotensin receptors. However, some conclusions are not strongly supported and others are not demonstrated. First, the infection increasing effect is shown comparing mock-EVs and ACE2-EVs with no control of the infection without EVs. Second, they showed the inhibition of the uptake of EVs using a macropinocytosis inhibitor (EIPA), lipid raft-mediated endocytosis (filipin), impairers of the endosomal acidification (BafA1) and endosomal-lysosomal system (cytochalasin D), but when they added the virus they showed the inhibition of the infection only with the last 2 drugs, that have been previously demonstrated to diminish the infectivity of the virus by themselves

(Yeung et al., 2021), so the conclusion that the inhibition of uptake of EVs drives the decrease of the infectivity cannot be done. Finally, the proposal of the mechanism involving the AT1 and AVPR1B was not tested. Anyway, the effect of increasing of the infectivity by ACE2-EVs compared with mock-EVs in their conditions, using Vero E6 cells is clear. This could be explained because these EVs contain more TMPRSS2 compared with ours and the protease could cleave and prime the S protein to become fusogenic as it was demonstrated to be performed by human mucosal sEVs bearing TMPRSS2 and ACE2, favoring the infectivity of the SARS-CoV-2 (Berry et al., 2022). Another explanation could be due to the fact that they add EVs in 2 steps, first together with the virus and once again alone, like this, cells might capture and incorporate the ACE2 from the EVs to their membranes as it was shown to be possible (J. Wang et al., 2020) and increase their susceptibility to be infected.

The importance of the abundance of the TMPRSS2 in the EVs and in the target cell was suggested by the fact that, in the work from F. Berry *et al.*, they obtained enhancement of the infection using human nasal epithelial cells (HNECs) as targets; and reduction using A549 and Vero-E6 cells, which express low levels of TMPRSS2 and might rather use the late endosomal path of SARS-CoV-2 entry (Berry et al., 2022).

So, we agree that, besides more evidence support the fact that the ACE2-EVs inhibit the infection, in certain contexts this could not be the case. In order to further explore a possible therapeutic use of the ACE2-EV, deeper understanding of the role of these EVs on the SARS-CoV-2 infection is needed. Together with the validation in physiological relevant models, remarkably *in vivo*.

Another strategy to neutralize the recognition of the S protein of the SARS-CoV-2 virus to its receptor and block the entrance are antibodies, either naturally formed by the host or developed in laboratories. Since the beginning of the COVID-19 pandemic it was understood that patients generated protective antibodies specific for the receptor-binding domain (RBD) of the S protein (Ju et al., 2020). And the plasma from convalescent patients was proposed as a therapy (Bloch et al., 2020; L. Chen et al., 2020). However, in clinical trials this strategy showed no overall clinical benefit, probably because the vast majority of the patients already had neutralizing antibodies against SARS-CoV-2 (Gharbharan et al., 2021). The developed antibodies have shown different degrees of success in preclinical and clinical trials and 2 neutralizing antibody cocktails have been approved by the FDA (D. Li et al., 2022). However, they have the disadvantage of the possibility of escape through mutations in the RBD by the virus (Weisblum et al., 2020), and the potential of enhancement of infection by the Fc dependent endocytosis or inflammation. By contrast, ACE2-EVs lack of these antibody related properties and offer a

different strategy, that would lead to a different outcome in the pathophysiologic development of the SARS-CoV-2 infection in patients, that might result in better clinical benefits and worth to be explored.

Conclusions and perspectives

Our data demonstrates that EVs containing ACE2, alone or in combination with TMPRSS2, reduce SARS-CoV-2 Spike-dependent infection in a much more efficient manner than soluble ACE2. Thus, ACE2-EVs represent a potential versatile therapeutic tool to block not only SARS-CoV-2 infection but also other coronavirus infections that use the ACE2 receptor for host cell entry, such as SARS-CoV (W. Li et al., 2003) and NL63 (Hofmann et al., 2005). Further studies to determine the efficacy of the ACE2/TMPRSS2-EVs in experimental models of SARS-CoV-2 virus need to be conducted to validate their therapeutic use for COVID-19, but also the lack of side-effects. The use of engineered EVs as therapeutic agents has been proposed several years ago and is currently being explored in humans (Wiklander et al., 2019), suggesting that well-designed EV therapeutics against COVID-19 may be feasible to prevent initial infection or further internal dissemination of the virus, and thus reducing the virus burden and disease severity. However, as recently highlighted by the International Societies for EV (ISEV) and for Cellular Therapies (ISCT) (Börger et al., 2020), despite the urgency induced by the current pandemic, EV-based therapeutic developments for COVID-19 will have to meet as strong criteria of manufacturing processes, quality controls and compliance to safety regulation as any other therapies, before they can be implemented in human subjects.

One possible risk of failure if used as therapy in human result from the fact that 293FT cells were transfected with a lentivirus to produce the ACE2-EVs and ACE2-TMPRSS2-EVs. This could induce an immune response when given *in vivo*, possibly with antibodies which may shield or remove EVs quickly after injection if repeated injections are used, or if the patient has been treated with other lentiviral based treatments previously. However, the lentiviruses used were replication defective, decreasing almost completely the possibilities of finding lentiviral derived viral components in the EVs isolated from these cells. Even though, for the use of these EVs in human, it would be recommended to use stable cell lines and check the absence of traces of lentiviruses.

Moreover, the possible therapeutic use of the ACE2-EVs does not present any presumable contraindication in treating patients who were vaccinated against SARS-CoV-2, since they would recognize also the S protein as the antibodies generated by the vaccine. Being more

likely a complementation for severe cases, or for variants eventually not recognized by the antibodies, that still use the ACE2 as a receptor for the infection.

In the context of HIV, bioengineered nanoparticles covered with plasma membrane of CD4⁺ T cells are able to neutralize the HIV viral infection *in vitro* (Wei et al., 2018), showing that the concept of EVs as particle decoys for virus can be extended to multiple types of viruses.

For our specific approach of ACE2- and TMPRSS2-containing EVs to be used in the future as therapeutic or preventive treatment for SARS-CoV-2 infection, we will need 1) to demonstrate their efficacy *in vivo*, and 2) to further increase the decoy efficacy of the EVs, by increasing the amount of available ACE2 and protease. For these purposes, we envisage two simultaneous strategies for development of this project.

1. We will need to test efficacy of our first generation ACE2-EVs in preventing infection by the real virus SARS-CoV-2 *in vitro* (since we have only so far used a Spike-pseudotyped lentivirus for the *in vitro* infectivity assays), and in a mouse model *in vivo*: we have access to transgenic mice expressing the human ACE2 in epithelial cells (K18-hACE2 transgenic mice) and a BSL3 animal facility, through collaborators in Marseille, France. These mice are efficiently infected by intranasal instillation of the virus, but recover if a low level of virus is used. The presence of the virus can also be exhaustively analysed post-mortem by immunohistochemistry or sensitive PCR-based assays, and the effect on immune cells infiltrating lungs can also be measured by flow cytometry. This model is thus perfect to understand the cellular mechanisms that ACE2-EVs can promote *in vivo*.

2. We will have to use molecular biology approaches to obtain an improved variant (second generation) of ACE2*-EVs with higher *in vitro* efficacy. For this, we will want to increase the amount of ACE2 on EVs together with the protease that cleaves the Spike protein, first by mutating ACE2 to make it resistant to cleavage by TMPRSS2 and by other proteases also potentially involved in its cleavage, such as matrix metalloproteases (ADAM10, which is an abundant component of small EVs, and ADAM17). We could mutate the cleavage sites of the ACE2 protein used to engineer the 293FT producing cells, or eliminate ADAM10/17 in the secreting cells, or use small molecule inhibitors of their activity. Alternatively, we could test other proteases that TMPRSS2 to be combined with ACE2 on EVs, such as TMPRSS4, and determine whether it cleaves less ACE2 than TMPRSS2. This will, also, eventually diminish the amounts of sACE2 generated, that was proven to increase the infectivity of the SARS-CoV-2, preventing a possible side effect *in vivo* (Yeung et al., 2021). Finally, the versatile decoy EV approach would be easily implemented to engineer ACE2 to increase its affinity for the S protein, or the S variants observed in the new variants of SARS-CoV-2 viruses.

The ultimate goal of such project would therefore to find an engineered ACE2*-EV able to prevent infection by different SARS-CoV-2 variants *in vivo* reducing SARS-CoV-2 viral titers and improving the outcome of the infection. This could then be proposed as a new therapy, easily delivered, for instance by intranasal nebulization, as preventive strategy before likely exposure to SARS-CoV-2, or in already infected patients to reduce internal spreading of the virus.

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ANNEX

Résumé substantiel en français

Les travaux réalisés au cours de mon doctorat ont porté sur le potentiel thérapeutique de différents sous-types de VE, de particules extracellulaires non VE et de protéines dans deux contextes différents: comme leurres dans les infections virales et comme modulateurs immunes et porteurs d'antigènes dans les réponses immunitaires aux tumeurs. Pour cela, je présente mon travail divisé en deux parties selon le contexte :

Partie 1 : Vésicules extracellulaires et protéines extracellulaires non-EV comme leurre pour l'infection virale.

Partie 2 : Sous-types de vésicules et de particules extracellulaires comme transporteurs d'antigènes et modulateurs des cellules présentatrices d'antigènes.

Vésicules extracellulaires et protéines extracellulaires non-EV comme leurre pour l'infection virale

Résumé

L'entrée du SRAS-CoV-2 est médiée par la liaison de la protéine de pointe (S) au récepteur de surface ACE2 et l'amorçage ultérieur par le TMPRSS2 de l'hôte permettant la fusion membranaire. Nous avons produit des vésicules extracellulaires (VE) exposant l'ACE2 et démontré que les VE-ACE2 sont des leurres efficaces pour les lentivirus contenant la protéine S du SARS-CoV-2. La réduction de l'infectivité est positivement corrélée au niveau d'ACE2, est beaucoup plus efficace qu'avec l'ACE2 soluble et est encore améliorée par l'inclusion de TMPRSS2.

Contexte

Le SARS-CoV-2 est l'agent infectieux responsable de la pandémie de COVID-19 (Zhou et al., 2020). L'entrée virale dans les cellules hôtes est médiée par l'interaction de la protéine spike (S) à la surface du SARS-CoV-2 avec le récepteur de surface de l'enzyme de conversion de l'angiotensine 2 (ACE2) (Walls et al., 2020). Après s'être liée à l'ACE2, la protéine S est clivée

par la protéase transmembranaire sérine 2 (TMPRSS2) et devient fusogène permettant ainsi l'entrée du virus (Hoffmann et al., 2020).

L'ACE2 recombinante soluble neutralise le SARS-CoV-2 en se liant à la protéine S et il a été prouvé qu'elle réduit l'entrée du SARS-CoV-2 dans les cellules Vero-E6 et les organoïdes humains artificiels (Monteil et al., 2020). Cependant, l'ACE2 est synthétisée sous la forme d'une protéine transmembranaire, comme la TMPRSS2. Nous postulons que l'ACE2 pourrait être présente à la surface des vésicules extracellulaires (VEs), ce qui pourrait se traduire par une meilleure efficacité en tant que leurre pour capturer le SARS-CoV-2. De plus, la présence concomitante d'une sérine-protéase active, la TMPRSS2, sur les mêmes EVs pourrait interférer davantage avec l'infectivité virale, en forçant la fusion virale sur la membrane de l'EV, plutôt que sur les cellules cibles.

Résultats et discussion

Nous avons transduit des cellules 293FT avec un lentivirus contenant ACE2 seul (293FT-ACE2) ou en combinaison avec TMPRSS2 (293FT-ACE2-TMPRSS2). Les cellules 293FT transduites avec un lentivirus contenant des plasmides vides ont été utilisées comme contrôle (293FT-mock).

Nous avons isolé les VE et les fractions protéiques solubles par SEC à partir des 3 lignées cellulaires 293FT. La surexpression de l'ACE2 dans les cellules 293FT a conduit à l'incorporation de cette molécule dans les VEs avec des niveaux plus élevés trouvés sur les VEs de 293FT-ACE2 que sur ceux de 293FT-ACE2-TMPRSS2. Les cellules 293FT-mock et 293FT-ACE2 ont exprimé TMPRSS2, mais la forme prédite de 54 kDa n'a pas été détectée dans leurs VE (Zmora et al., 2015). Il est important de noter que les VE des cellules 293FT surexprimant TMPRSS2 contenaient des niveaux plus élevés de la protéine TMPRSS2 pleine longueur et moins de forme clivée que les VE des cellules 293FT-mock ou 293FT-ACE2.

Nous avons ensuite analysé la capacité des VEs contenant ACE2- et ACE2-TMPRSS2 à réduire l'infection des cellules cibles par un lentivirus contenant la protéine SARS-CoV-2-S. Nous avons infecté les cellules cibles avec le virus SARS-CoV-2-S-pseudotype en présence ou en l'absence d'VEs isolées de cellules 293FT- mock (mock-VEs) ou 293FT-ACE2 (ACE2-VEs) ou 293FT-ACE2-TMPRSS2 (ACE2-TMPRSS2-VEs). L'infection des cellules 293FT-ACE2 et Caco-2 (cette dernière étant une cellule épithéliale du côlon exprimant l'ACE2, donc la cible du virus SARS-CoV-2) en présence des ACE2-VEs et des ACE2-TMPRSS2-VEs a été réduite de manière dose-dépendante alors que l'infection n'a pas été affectée par les mock-VEs.

En reportant les résultats obtenus précédemment en fonction de la quantité absolue d'ACE2 mesurée pour ces mêmes échantillons par ELISA, nous avons observé que les VE provenant de cellules surexprimant la longueur complète de TMPRSS2 en même temps que l'ACE2 étaient plus efficaces pour inhiber l'infection virale SARS-CoV-2-S-pseudotypée que celles provenant de cellules surexprimant l'ACE2 seul. De plus, pour obtenir des niveaux d'inhibition de l'infection lentivirale similaires à ceux observés avec les VE d'ACE2 ou d'ACE2-TMPRSS2, il fallait utiliser 500 à 1500 fois plus d'ACE2 humaine recombinante soluble.

Conclusions

Dans l'ensemble, ces résultats soulignent l'efficacité accrue des VE contenant de l'ACE2 pleine longueur pour inhiber l'entrée du virus pseudotypé SRAS-CoV-2-S par rapport à la protéine soluble seule. L'efficacité accrue des VE provenant de cellules surexprimant TMPRSS2 pourrait être due à la présence de TMPRSS2 en même temps que l'ACE2, ce qui entraîne la fusion du virus avec les VE et réduit leur capacité à infecter les cellules, et/ou à d'autres modifications de la composition des VE induites par la surexpression de TMPRSS2 dans les cellules sécrétant les VE.

Ainsi, les VE ACE2 représentent un outil thérapeutique polyvalent potentiel pour bloquer non seulement l'infection par le SARS-CoV-2 mais aussi d'autres infections par des coronavirus qui utilisent le récepteur ACE2 pour entrer dans la cellule hôte, comme le SARS-CoV (W. Li et al., 2003) et le NL63 (Hofmann et al., 2005). D'autres études visant à déterminer l'efficacité des ACE2/TMPRSS2-VE dans des modèles expérimentaux du virus SARS-CoV-2 doivent être menées pour valider leur utilisation thérapeutique pour le COVID-19, mais aussi l'absence d'effets secondaires.

Les résultats résumés ici sont décrits plus en détail dans l'article suivant "Extracellular vesicles containing ACE2 efficiently prevent infection by SARS-CoV-2 Spike protein-containing virus", publié le 28/12/20 dans Journal of Extracellular Vesicles (JEV).

Sous-types de vésicules et de particules extracellulaires comme transporteurs d'antigènes et modulateurs des cellules présentatrices d'antigènes.

Résumé

Il a été proposé que les VE dérivés des tumeurs agissent comme une source acellulaire d'antigènes tumoraux et transportent des PAMPs et des DAMPs qui peuvent induire un

amorçage immunitaire des cellules présentatrices d'antigènes, ce qui peut entraîner des réponses immunitaires contre les tumeurs. Inversement, les VE dérivées des tumeurs ont également été proposées comme agents immunosupresseurs favorisant l'échappe immunitaire des tumeurs. Ici, nous avons caractérisé de manière exhaustive, biochimiquement et fonctionnellement, l'ensemble des VE et des ENP sécrétés par les lignées cellulaires tumorales murines. Nous avons comparé la composition protéique et l'interaction fonctionnelle avec les cellules dendritiques cibles des VEs, des VLPs, des ENPs et d'un mélange de grands/petits (gp)VEs/VLPs. Ces sous-populations ont été absorbées de manière différentielle, ont induit des changements phénotypiques différents et ont montré des efficacités différentes pour induire des réponses spécifiques aux antigènes par les cellules dendritiques (DCs). Nos résultats appellent à une réévaluation des études précédentes sur les VE dérivées de tumeurs de souris afin de déchiffrer les proportions et les fonctions respectives des VE non virales et des particules de type viral et leur contribution aux réponses immunitaires anti-tumorales et à la progression tumorale.

Contexte

En plus des protéines solubles, les cellules sécrètent des vésicules extracellulaires (VE) et des nanoparticules non-vésiculaires (ENP) qui jouent un rôle dans les communications intercellulaires. Les cellules tumorales sécrètent des vésicules extracellulaires (VE) et des nanoparticules (ENP) qui ont été associées à une pléthore de fonctions de régulation immunitaire. En particulier, il a été proposé que les VE agissent comme une source acellulaire d'antigènes tumoraux et qu'elles transportent ensemble des PAMPs et des DAMPs qui peuvent induire un amorçage immunitaire des cellules présentatrices d'antigènes (CPA), entraînant éventuellement des réponses immunitaires contre les tumeurs. Cependant, la composition des VE et leur cargaison peuvent être remarquablement diverses, et différents VE et PNE peuvent avoir des capacités variables d'interaction et de modification des cellules réceptrices. Par conséquent, il est crucial de comprendre les mécanismes par lesquels les différents types de VE et de ENP modulent les réponses des CPA dans le contexte de la progression tumorale, ce qui pourrait permettre d'identifier des candidats appropriés à base de VE à utiliser comme agents immunothérapeutiques contre les tumeurs.

Résultats et discussion

Nous caractérisons ici l'hétérogénéité des VEs et des ENPs sécrétés par les cellules E0771, un adénocarcinome mammaire murin. De manière inattendue, nous avons identifié des particules

de type viral (VLP) du virus infectieux de la leucémie murine (MLV) endogène dans des préparations de petits (p)VEs/ENPs produits par deux lignées de cellules tumorales ou dendritiques mammaires, mais pas par des fibroblastes ou des cellules primaires. Nous avons établi un protocole robuste pour séparer les pVEs des VLPs. Nous avons comparé la composition protéique et l'interaction fonctionnelle avec les cellules dendritiques cibles des pVEs, VLPs, ENPs et d'un mélange de grands/petits (gp)EVs/VLPs. Ces sous-populations ont été capturées de manière différentielle et ont induit différents changements phénotypiques dans une lignée de cellules dendritiques et dans des cellules DC primaires. Les ENP ont été faiblement capturés et n'ont pas affecté les DCs. Les pVEs ont spécifiquement induit la mort des DCs. La préparation mixte gpEV/VLP a été la plus efficace pour induire la maturation des DC et la présentation des antigènes, suivie par les VLP.

Conclusions

Les VEs isolés par des protocoles réguliers à partir de cellules tumorales de souris contiennent des VLPs provenant de MLV endogènes avec un potentiel infectieux. La combinaison de la concentration du filtre, de la centrifugation et du gradient de densité permet de séparer 10k, pVE, VLP et ENP. Ces sous-types de particules contribuent différemment à la modification des cellules myéloïdes immunitaires cibles *in vitro*.

Nos résultats appellent à une réévaluation des études précédentes sur les VE dérivées de tumeurs de souris afin de déchiffrer les proportions et fonctions respectives des VE non virales et des particules de type viral et leur contribution aux réponses immunitaires anti-tumorales et à la progression tumorale.

Notre travail a des implications dans l'application à l'immunothérapie du cancer, pour le raffinement de l'utilisation des VE tumorales comme source d'antigènes. Nos résultats illustrent l'importance d'identifier le(s) sous-type(s) de particule(s) portant la fonction d'intérêt, avec le but d'optimiser de futures utilisations de VEs à visée thérapeutique. Finalement, ces résultats pourraient conduire à l'utilisation future des sous-types VEs pour l'immunothérapie contre les tumeurs. Nous proposons que le 10k soit le candidat le plus approprié. Néanmoins, cela devrait être testé pour les différentes sources de cellules productrices, et la présence de retroparticules virales infectieux systématiquement testée, comme un facteur pertinent potentiel. Alternativement, les résultats de notre travail ouvrent la voie à une stratégie alternative, en bloquant l'activité immunosuppressive des VEs tumoraux cytotoxiques.

Les résultats résumés ici sont décrits plus en détail dans le manuscrit suivant "Subtypes of tumor-derived extracellular vesicles, co-isolated enveloped viruses and non-vesicular particles differently modulate and transfer antigen to dendritic cells", en préparation.

Article 3: SnapShot: Extracellular Vesicles

SnapShot: Extracellular Vesicles

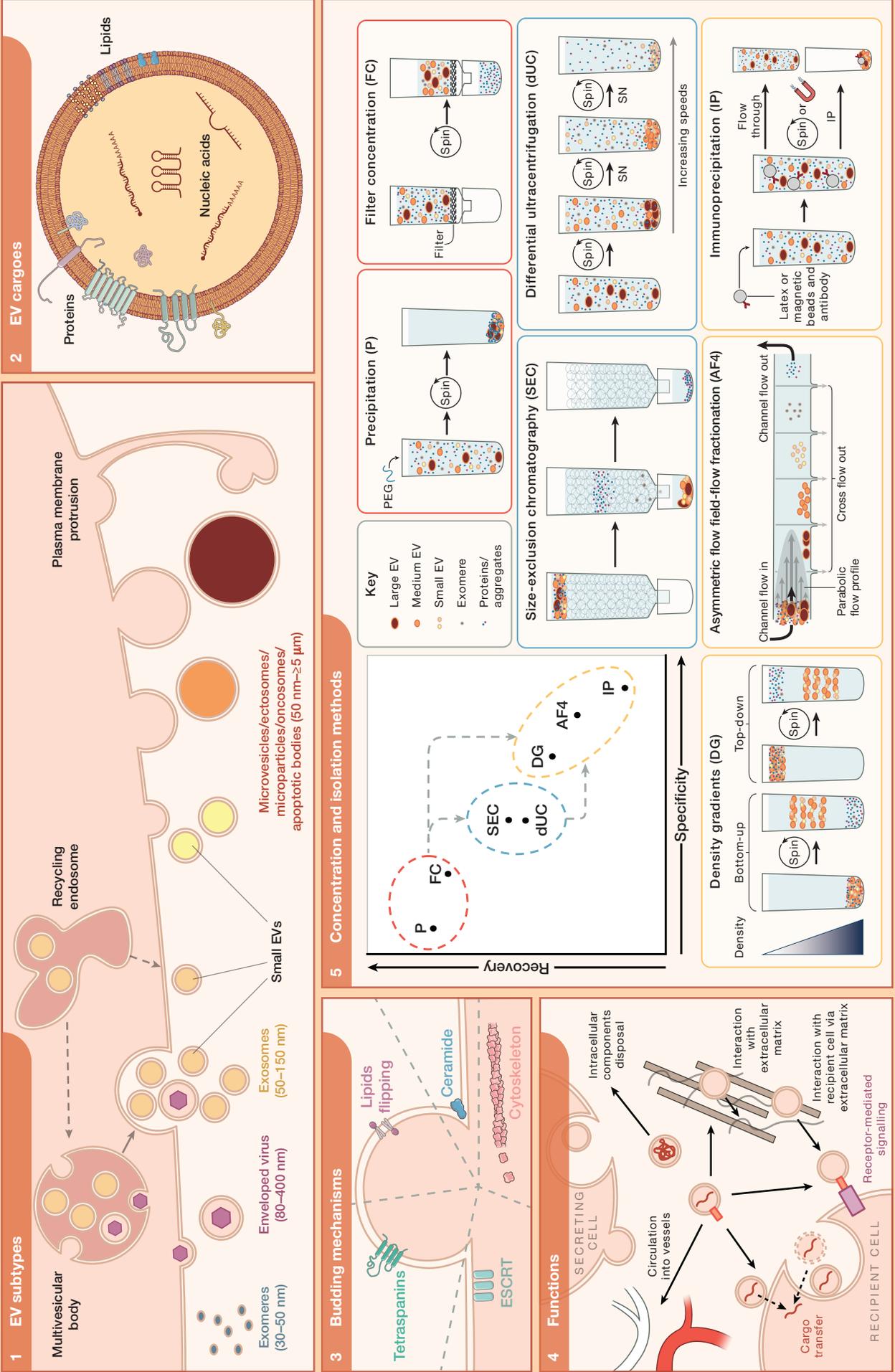
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(1 and 2) What Are Extracellular Vesicles?

Extracellular vesicles (EVs) are structures released into their environment by all cells. They are delimited by a lipid-bilayer and contain components from the cells that release them. EVs can form first as intraluminal vesicles (ILVs) in multivesicular compartments (generally of endocytic nature) and be secreted upon fusion of these compartments with the plasma membrane (exosomes). Other EVs can be released directly from the plasma membrane (ectosomes, microvesicles, microparticles, large oncosomes, and apoptotic bodies). Because prokaryotic cells do not have internal compartments, their EVs can only originate from their limiting membrane. Enveloped viruses that hijack membranes from the infected cell for release can also be considered as a type of EV. EVs have the same membrane orientation as the cells: they expose at their surface lipids and the extracellular domains of transmembrane proteins and enclose mainly cytoplasmic components, including proteins and nucleic acids. Since they enclose components from the originating cells, EVs are also explored as sources of circulating biomarkers in biological fluids. Several comparative proteomic studies have recently provided lists of proteins that could be specific for determined EV subtypes. However, because of the difficulty in finding consistent markers across studies, specific EV subtype marker proteins are not yet clearly identified.

(3) What Do We Know about the Mechanisms of Biogenesis of EVs?

Several mechanisms of EVs budding can take place, involving either one or a combination of the following machineries: ESCRT complex, tetraspanins, sphingomyelinases generating cone-shaped lipids (ceramide), relocation of phospholipids toward the leaflet of the membrane where budding takes place, and depolymerization of the actin cytoskeleton. Some of these mechanisms have been described specifically in exosome or ectosome formation but their potential involvement in the budding of both cannot be excluded. Therefore, specific biogenesis mechanisms are not yet clearly known.

(4) What Do We Know about the Function of EVs?

EVs have been identified as a means, for the secreting cell, of disposing harmful or useless intracellular components but also as important mediators of communication with other cells. EVs carry various cargoes that can deliver signals to induce physiological changes in recipient cells. For example, EVs expose ligands that can bind to cognate receptors on the target cells mediating signaling cascades. Internalized EVs can be degraded, becoming a source of nutrients for recipient cells. EVs' content can also be transferred into and present activity in the cytoplasm of target cell. Transfer mechanisms for non-viral EVs are yet unclear. EVs can mediate the interaction of secreting cells with the surrounding extracellular matrix (ECM). Moreover, EVs deposited in ECM can also serve as indirect communication to nearby cells. EVs also function in long-distance communication, as they can be released into blood or lymphatic vessels and deliver their content to distant target cells.

(5) How Can We Separate Different Types of EVs and Co-isolated Components?

EVs are isolated from conditioned medium of cultured cells, or from biological fluids, after elimination of cells by low-speed centrifugation. The different types of EVs display overlapping biophysical properties. The diameter of ILVs of endocytic compartments is around 100 nm: exosomes are therefore in the diameter range of 50–150 nm. EVs formed at the plasma membrane can be of this size range or larger (up to 5 μ m). Other extracellular nanoparticles (ENPs) with unknown site of origin and mode of formation, for instance, "exomeres," can be found together with EVs. Exomeres contain proteins, nucleic acids, and lipids and are in the lowest range of EV sizes (around or below 50 nm). Various non-vesicular lipoproteins released by some cell types and in biological fluids also overlap with EVs in size and density. Because of these overlapping properties, most EV isolation methods co-isolate to various degrees some of the other extracellular structures or do not separate the different EV subtypes. Here, we present some of the most commonly used techniques, ranking them in a recovery versus specificity grid. Specificity can be considered either for EVs (as opposed to other ENPs) or for a specific EV subtype.

Global concentration methods by filtration (FC) or polymer-based precipitation (P) (e.g., with PEG, *polyethylene glycol*) can recover EVs, ENPs, and a majority of other secreted products. EVs can be separated from soluble components and some lipoproteins by size-exclusion chromatography (SEC, soluble components and the smallest lipoproteins remain longer in the column bed). In these settings, EV subtypes display slightly different but overlapping properties, and therefore separation of EV subpopulations is inefficient. Separation of EV subtypes can be performed based on size and/or weight using differential centrifugation (dUC) with increasing g force/time. This approach enriches for (but does not purify) EVs of a selected range of sizes, and ENPs as well as soluble components are non-specifically co-isolated, especially with the smallest EVs. Further separation of EV subtypes and ENPs can be achieved by bottom-up flotation into a density gradient (DG): non-lipidic structures do not float upward. Alternatively, they can be separated by top-down DG or density cushions: soluble components do not enter the gradient. Separation of EV subtypes and ENPs can also be achieved based on size via asymmetric flow field-flow fractionation (AF4). Finally, the most specific separation can be achieved by immunoprecipitation (IP) using antibodies specific to a given surface protein of EVs. In general, a combination of techniques is necessary to first concentrate EVs and then achieve better specificity of isolation.

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