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THERAPEUTIC POTENTIAL OF EXTRACELLULAR VESICLES

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To my family and friends

“Oscar, you choose. You don’t have to do the extra home work, but if you do and study hard, then you can always choose what you want to do.”

Tommy Wiklander, 1994

ABSTRACT

Extracellular vesicles (EVs) are nanometer-sized, lipid membrane enclosed, vesicles that are secreted by most, if not all, cells and contain macromolecular material of the source cell including lipids, proteins and various nucleic acid species. Over the last two decades, EVs have been recognized as important mediators of cell-to-cell communication that influence both physiological and pathological conditions. Owing to their ability to transfer bioactive components and surpass biological barriers, EVs are increasingly explored as therapeutics, both as natural delivery vectors and in its own right, as improved cell based therapies.

In paper I, the great potential of EVs as therapeutic entities is explored by equipping EVs with the brain targeting rabies viral glycoprotein peptide and load them with siRNA against alpha-synuclein (α-Syn). The findings demonstrate that EVs efficiently deliver the siRNA to the target with subsequent reduction of α-Syn pathology in vitro as well as in the brains of α-Syn overexpressing transgenic mice. Thus, this indicates that targeted EVs can be employed as efficient vectors for siRNA therapy against Parkinson's disease and other α-Syn related pathological conditions.

In pursuance of using EVs for therapeutic purposes, the fate of injected EVs must be understood. Consequently, the aim of paper II was to elucidate the biodistribution of injected EVs and to investigate factors that may influence the tissue distribution of exogenous EVs. The use of the fluorescent lipophilic dye DiR was thoroughly assessed and found to be a suitable labelling method for biodistribution studies that allowed for in vivo EV tracing with high sensitivity. EVs displayed a general distribution pattern with high accumulation in liver, lung and spleen, which is in line with previous findings of mononuclear phagocyte system (MPS)-associated EV uptake. In addition, the biodistribution profile of EVs was, to a varying degree, influenced by the administration route, cell source, dosing and targeting. These variables may thus be adopted for future EV-based therapies to reflect the preferred biodistribution and/or pharmacokinetic profile for a given therapeutic approach.

Furthermore, EVs have been found to convey the beneficial immunomodulatory effects of mesenchymal stromal cell (MSC)-based cell therapy. Based on these findings and studies demonstrating that EVs can be engineered to display surface moieties, the objective of paper III was to produce MSC-derived EVs that express therapeutic proteins. A chimeric construct, with an EV sorting domain fused to a non-signalling cytokine receptor, was introduced to the parental cell to produce EVs that can sequester cytokines, termed decoy EVs. By targeting the central inflammatory pathways of TNFα and IL-6 trans-signalling, these decoy EVs significantly ameliorate systemic inflammation and neuroinflammation in vivo. This novel concept thus combines the beneficial effects of stem cell therapy, EVs as delivery agents and cytokine targeted biologics.

Taken together, the findings in this thesis suggest that EVs have the potential to be utilized as a future platform of highly potent multifaceted biopharmaceuticals.

LIST OF SCIENTIFIC PAPERS

I. Cooper JM, **Wiklander OPB**, Nordin JZ, Al-Shawi R, Wood MJA, Vithlani M, Schapira AH, Simons JP, EL Andaloussi S, Alvarez-Erviti L. **Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice.** *Movement Disorders*. 2014 Oct 1;29(12):1476-85.

II. **Wiklander OPB**, Nordin JZ, O'Loughlin A, Gustafsson Y, Corso G, Mäger I, Vader P, Lee Y, Sork H, Seow Y, Heldring N, Alvarez-Erviti L, Smith CIE, Le Blanc K, Macchiarini P, Jungebluth P, Wood MJA, EL Andaloussi S. **Extra-cellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting.** *Journal of extracellular vesicles*. 2015 Apr 20;4:26316.

III. **Wiklander OPB**, Nordin JZ, Gupta D, Seow Y, Balusu S, Liang X, Corso G, Feldin U, Conceicao M, Vandenbroucke R, Wood MJA, Görgens A, EL Andaloussi S. **Engineered Extracellular vesicles as Therapeutic Decoys.** *Unpublished Manuscript*.

LIST OF ADDITIONAL PUBLICATIONS

IV. Stenler S, **Wiklander OPB**, Badal-Tejedor M, Turunen J, Nordin JZ, Hallengard D, Wahren B, EL Andaloussi S, Rutland MW, Smith CIE, Lundin KE, Blomberg P. **Micro-minicircle Gene Therapy: Implications of Size on Fermentation, Complexation, Shearing Resistance, and Expression**. *Molecular therapy - nucleic acids* 2014 3: e140.

V. Aswad H, Forterre A, **Wiklander OPB**, Vial G, Danty-Berger E, Jalabert A, Lamaziere A, Meugnier E, Pesenti S, Ott C, Chikh K, EL Andaloussi S, Vidal H, Lefai E, Rieusset J, Rome S. **Exosomes participate in the alteration of muscle homeostasis during lipid-induced insulin resistance in mice.** *Diabetologica* 2014 57:10 2155-64.

VI. Nordin JZ, Lee Y, Vader P, Mäger I, Johansson HJ, Heusermann W, **Wiklander OPB**, Hällbrink M, Seow Y, Bultema JJ, Gilthorpe J, Davies T, Fairchild PJ, Gabrielsson S, Meisner-Kober NC, Lehtiö J, Smith CIE, Wood MJ, EL Andaloussi S. **Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties**. *Nanomedicine: nanotechnology, biology, and medicine*. 2015 11:4 879-83.

VII. Li J, Lee Y, Johansson HJ, Mäger I, Vader P, Nordin JZ, **Wiklander OPB**, Lehtiö J, Wood MJ, EL Andaloussi S. **Serum-free culture alters the quantity and protein composition of neuroblastoma-derived extracellular vesicles**. *Journal of extracellular vesicles* 2015 4: 26883.

VIII. Rocha CS, **Wiklander OPB**, Larsson L, Moreno PMD, Parini P, Lundin KE, Smith CIE. **RNA therapeutics inactivate PCSK9 by inducing a unique intracellular retention form.** *Journal of molecular and cellular cardiology* 2015 82: 186-93.

IX. Simonson OE, Mougiakakos D, Heldring N, Bassi G, Johansson HJ, Dalén M, Jitschin R, Rodin S, Corbascio M, EL Andaloussi S, **Wiklander OPB**, Nordin JZ, Skog J, Romain C, Koestler T, Hellgren-Johansson L, Schiller P, Joachimsson PO, Hägglund H, Mattsson M, Lehtiö J, Faridani OR, Sandberg R, Korsgren O, Krampera M, Weiss DJ, Grinnemo KH, Le Blanc K. **In Vivo Effects of Mesenchymal Stromal Cells in Two Patients With Severe Acute Respiratory Distress Syndrome**. *Stem cells translational medicine* 2015 4:10 1199-213.

X. Jungebluth P, Holzgraefe B, Lim ML, Duru AD, Lundin V, Heldring N, **Wiklander OPB**, Nordin JZ, Chrobok M, Roderburg C, Sjöqvist S, Anderstam B, Beltrán Rodríguez A, Haag JC, Gustafsson Y, Roddewig KG, Jones P, Wood MJ, Luedde T, Teixeira AI, Hermanson O, Winqvist O, Kalzén H, EL Andaloussi S, Alici E, Macchiarini P. **Autologous Peripheral Blood Mononuclear Cells as Treatment in Refractory Acute Respiratory Distress Syndrome Respiration.** *Respiration* 2015 90:6 481-92.

XI. Sork H, Nordin JZ, Turunen JJ, **Wiklander OPB**, Bestas B, Zaghloul EM, Margus H, Padari K, Duru AD, Corso G, Bost J, Vader P, Pooga M, Smith CIE, Wood MJ, Schiffelers RM, Hällbrink M, EL Andaloussi S. **Lipid-based Transfection Reagents Exhibit Cryo-induced Increase in Transfection Efficiency.** *Molecular therapy Nucleic acids* 2016 5: e290.

XII. Jalabert A, Vial G, Guay C, **Wiklander OPB**, Nordin JZ, Aswad H, Forterre A, Meugnier E, Pesenti S, Regazzi R, DantyBerger E, Ducreux S, Vidal H, EL Andaloussi S, Rieusset J, Rome S. **Exosome-like vesicles released from lipid-induced insulinresistant muscles modulate gene expression and proliferation of beta recipient cells in mice**. *Diabetologia* 2016 59:5 1049-58.

CONTENTS

LIST OF ABBREVIATIONS

α-Syn	Alpha-synuclein
AD	Alzheimer's disease
Aex	Ascites fluid-derived exosomes
AFM	Atomic force microscopy
AGO2	Argonaute 2
ALIX	ALG-2-interacting protein X
APC	Antigen presenting cell
ARF6	ADP-ribosylation factor 6
B16-BL6	Mouse melanoma cell line
B16-F10	Mouse melanoma cell line
BBB	Blood brain-barrier
BM	Bone marrow
C2C12	Mouse myoblast cell line
CEA	Carcinoembryonic antigen
CHMP4	Charged multivesicular body protein 4
CNS	Central nervous system
CSF	Cerebrospinal fluid
CDE	Caveolin-dependent endocytosis
CIE	Clathrin-independent endocytosis
CM	Conditioned cell culture medium
CME	Clathrin-mediated endocytosis
CT	Clinical trial
DC	Dendritic cell
DDS	Drug delivery systems
Dex	DC-derived exosomes
DiR	Fluorescent dye, 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanineiodide
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalitis

EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
FasL	Fas ligand
FCS	Fluorescence correlation spectroscopy
GFP	Green fluorescent protein
gLuc	Gaussia luciferase
g/ml	Gram per milliliter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
HEK293	Human embryonic kidney cell line
Hsp	Heat shock protein
IL	Interleukin
IL6R	Interleukin-6 receptor
IL6ST	Interleukin-6 signal transducer
ILV	Intraluminal vesicle
imDC	Immature dendritic cell
i.p.	Intraperitoneal
ISEV	International Society of Extracellular Vesicles
i.v.	Intravenous
IVIS	In Vivo Imaging System
Luc	Luciferase
mDC	Mature dendritic cell
MHC	Major histocompatibility complex
miRNA	MicroRNA
MLCK	Myosin light-chain kinase
MP	Macropinocytosis
MPS	Mononuclear phagocyte system
mRNA	Messenger RNA
MSC	Mesenchymal stromal cell

MV	Microvesicle
MVB	Multivesicular body
ncRNA	Non-coding RNA
NF-κB	Nuclear factor kappa B
NIH	National Institute of Health (of the United States)
nm	Nanometer
nSMase2	Neutral sphingomyelinase 2
nt	Nucleotides
NTA	Nanoparticle tracking analysis
OLN-93	Rat oligodendrocyte cell line
OMVs	Bacterial outer membrane vesicles
PC3	Human prostatic carcinoma cell line
PD	Parkinson's disease
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol 3-phosphate
PLD	Phospholipase D
PS	Phosphatidylserine
qPCR	Quantitative polymerase chain reaction
RILP	Rab-interacting lysosomal protein
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPS	Resistive pulse sensing
RVG	Rabies viral glycoprotein
s.c.	Subcutaneous
SEC	Size exclusion chromatography
SH-SY5Y	Mouse neuroblastoma cell line
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SR-A	Scavenger receptor class A

STAT3	Signal transducer and activator of transcription 3
TEM	Transmission electron microscopy
tEV	Tumor-derived EV
TFF	Tangential flow filtration
Tg	Transgenic
TGFβ	Transforming growth factor‑β
Tim4	T cell immunoglobulin and mucin domain protein 4
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TRAIL	TNF-related apoptosis-inducing ligand
TSG101	Tumor susceptibility gene 101
UC	Ultracentrifugation
VAMP7	Vesicle-associated membrane protein 7
WB	Western blot

1 INTRODUCTION

1.1 HISTORY OF EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) hold great potential to be the next medical breakthrough as an emerging platform of highly potent and multifaceted biopharmaceuticals. The EV research field has grown almost exponentially in terms of the number of published scientific articles over the last decades, which has led to an increased understanding of EVs' biogenesis, content and biological function. Figure 1 shows the number of published articles over time and highlights the breakthrough findings, publications and events of the field.

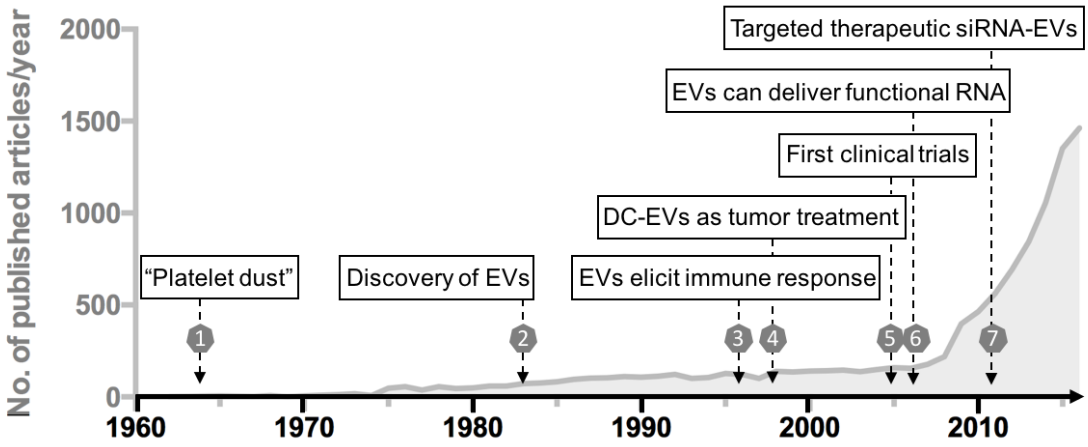

Figure 1 – Background graph showing number of published articles per year on PubMed with the search term "extra cellular vesicles". Important breakthrough articles are indicated by numbers (1-7) over time [1-9].

Extracellular vesicles include plasma membrane shed vesicles, such as microvesicles and apoptotic vesicles, as well as exosomes, which are derived from the endosomal pathway (see classification below) [10]. The field of EV research springs from the findings on coagulation from the 1940-50s, where it was discovered that even platelet-free plasma possesses a small coagulation component that can be sedimented. In an article published in 1964, Peter Wolf coined the term "platelet dust" to describe this small plasma component originating from platelets and pelleted down by high-speed centrifugation, which could be identified by electron microscopy and was later defined as EVs [1]. Furthermore, studies and findings of cellular compartments including the endosome and lysosome, which were granted the 1974 Nobel prize in Medicine to Albert Claude, Christian de Duve, and George E. Palade, serve as the fundamental basis for understanding the biogenesis of exosomes. In 1983 two groups described formation and secretion of exosomes while investigating the transferrin recycling cycle [2, 3]. Those groups showed that labelled transferrin was internalized, redistributed via endosomes to a multivesicular compartment and later externalized via vesicles, thus partially describing what was later identified as EVs.

The initial hypothesis was that EVs carried cellular waste and served as a disposal bin to maintain cellular homeostasis. However, about two decades ago, Raposo et. al. presented the first evidence that EVs have other important biological functions. The groundbreaking article "B Lymphocytes Secrete Antigen-presenting Vesicles", published in 1996, showed that EVs derived from B lymphocytes can induce an immune response [4]. In 1998, Zitvogel et. al. published the first therapeutic approach using EVs in Nature Medicine with the title "Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes" [5]. Following these findings, two clinical trials using autologous dendritic cell-derived EVs pulsed with tumor antigens were conducted in 2005, for the treatment of metastatic melanoma and non-small cell lung cancer, respectively [6, 7]. A decade ago, in 2007, the importance of EVs was further appreciated through a study by Valadi et. al. published in Nature Cell Biology, which was the next groundbreaking study showing that EVs take part in cell-to-cell communication and that EVs can deliver functional mRNA and miRNA to recipient cells [8]. Another pioneering development was published in 2011 by Alvarez-Erviti et al, showing that engineered EVs can be targeted to the brain and used for delivery of functional siRNA [9]. This important article highlights the great potential of EVs as natural delivery vectors for therapeutics.

1.2 CLASSIFICATION

The term extracellular vesicles is a hypernym covering different classes of vesicles derived from eukaryotic and prokaryotic cells. EVs are enclosed by a lipid bilayer, with a size ranging from 30-2,000 nm in diameter and contain proteins, lipids and nucleic acids originating from the source cell [10]. Still being in its youth, the EV field has had somewhat inconsistent nomenclature where the terms microparticles, microvesicles, exosomes, and EVs have been used interchangeably to describe vesicles derived from cells. Others have used terms based on the origin of the vesicles, such as platelet dust [1] (platelet-derived vesicles), prostasomes [11] (derived from prostate epithelium) and dexosome [7] (dendritic cell released vesicles), etc. The most common definition of the different classes of EVs is based on biogenesis, density, size, and/or differential centrifugation properties, i.e. the required gravitational force for pelleting [12]. Here, I am following the suggestions by Gould and Raposo, whom recommend the term EVs to cover the different forms of cell-derived vesicles and to define the denominations used to describe different vesicles [12]. Furthermore, I am employing the definitions of the classes of EVs based on their biogenesis as described by EL Andaloussi et. al. [10], which states that there are three main classes: exosomes, microvesicles and apoptotic bodies. The most studied type of EVs is exosomes, which are formed through the endosomal system, with a size of about 40-120 nm in diameter. Microvesicles are more heterogeneous with sizes ranging between 50-1000 nm and derive from the direct outward budding of the plasma membrane. Similar to microvesicles, apoptotic bodies are shed directly from the cell membrane and are larger in size, measuring 500-2000 nm in diameter, but are formed by blebbing of apoptotic cells and may contain diverse parts of the dying cell source. As mentioned by EL Andaloussi et. al [10] and emphasized by van der Pol et. al. [13] as well as Witwer et. al. [14], there is an overlap between the different EV classes in terms of size,

density and protein content. This heterogeneity has further been strengthened by findings of exosome subpopulations, indicating that some exosomes may contain only certain exosome characteristics but lack others [15]. Despite the muddled nomenclature, basic requirements have been established within the EV-community [16]. In this work, the focus lays on the EV types commonly classified as exosomes and smaller microvesicles, but as discussed above the distinction between the classes are still unclear and hence the term EV will be used subsequently.

1.3 BIOGENESIS OF EXTRACELLULAR VESICLES

EVs are formed either as exosomes from the endocytic pathway where invagination of the endosomal membrane forms multivesicular bodies (MVBs) that can fuse with the plasma membrane to release exosomes into the extracellular milieu. MVs, on the other hand, arise from the outward budding and fission of the plasma membrane [10, 17, 18]. Apoptotic bodies, which will not be further emphasized in this work, are formed by blebbing of apoptotic cells [10]. An overview of the biogenesis is illustrated in Figure 2.

1.3.1 MVB Formation

The endosomal system comprises early to late endosomes, MVBs and recycling endosomes and act as a sorting network that direct various intraluminal vesicles to appropriate destination, including lysosomal degradation, cellular recycling or exocytosis [19-21]. After deposition of content destined for recycling into recycling endosomes, the early endosomes transform into late endosomes. During this maturation, inward budding of the endosomal membrane occurs, giving rise to intraluminal vesicles (ILVs) and subsequent formation of the MVB [20, 22, 23]. The main process governing the creation of ILVs and the maturation of the late endosome into MVB is through the endosomal sorting complex required for transport (ESCRT), consisting of four protein complexes known as ESCRT-0, -I, -II, -III, which are recruited to the site of ILV formation [24-26]. Ubiquitinated proteins on the cytosolic side of the endosome, presence of phosphatidylinositol 3-phosphate (PIP3), which is abundant on early endosomes, and membrane curvature have all been shown to play a role in the recruitment of ESCRT-0, -I and –II [27]. These ESCRTs are believed to initiate the intraluminal membrane budding by binding and sequestering ubiquitinated proteins, whereas ESCRT-III completes this process through membrane fission and abscission of ILVs. ESCRT-III becomes associated via ALG-2-interacting protein X (ALIX) that simultaneously binds tumor susceptibility gene 101 (TSG101), which is part of the ESCRT-1 complex, and charged multivesicular body protein 4 (CHMP4), which is included in ESCRT-III [28, 29].

Furthermore, different ESCRT-independent pathways have been identified and cells with inactivated ESCRTs can still form MVBs [30-33]. Other pathways that act in parallel to, or cooperate with, the ESCRT system include ceramide-dependent ILV formation and enrichment of membrane proteins, known as tetraspanins. The sphingolipid ceramide, which is present in exosomes, has in some settings, been shown to facilitate the invagination of

ILVs, possibly by its cone-shaped configuration [34]. Trajkovic et. al. showed that inhibition of neutral sphingomyelinase, an enzyme necessary for ceramide production, decreases the yield of exosomes. In addition, clustered enrichment on the endosome membrane of tetraspanins, such as CD63 and CD9, which are commonly found in exosomes, is believed to initiate the formation of ILVs via specific protein-protein interactions [20].

1.3.2 Formation of Exosomes

The formation of MVBs with the invagination of ILVs constitutes the start of exosome biogenesis. [21]. MVBs and their content are either directed to the lysosome for degradation or toward fusion with the plasma membrane and subsequent release of the ILVs that now become exosomes as they enter into the extracellular milieu [20]. The process that dictates the fate of MVB to either fuse with the lysosome or the plasma membrane is still being dissected [35]. It has been proposed that endosomes are being directed to the different fates by a series of different Rab GTPases. The members of the Rab GTPase family display distinct intracellular membrane localization patterns and regulate membrane traffic between organelles, including vesicle movement along actin and tubulin networks, and are also associated with the formation of vesicles and membrane fusion [35-39]. For instance, late endosomes have distinct RAB7 and RAB9 membrane domains that guide them towards lysosmal degradation via the RAB7 effector Rab-interacting lysosomal protein (RILP) or towards plasma membrane fusion via RAB9 and TIP47 association [40-43]. In addition, differences in cholesterol levels of MVBs have been shown to govern the direction towards plasma membrane or lysosome fusion, where cholesterol enriched MVBs have been shown to be destined for membrane fusion and vice versa [44]. The Rab GTPases have further been indicated to play an important role in the MVB to plasma membrane-fusion and release of exosomes. Suppression of RAB11, RAB27a, RAB27b and RAB35 or their effector proteins have all been demonstrated to negatively affect exosome release [21, 45-47]. It has furthermore been suggested that there is an association of Rab GTPases and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) complexes [48-51], which are known to be involved in membrane fusion events [52, 53]. More specifically, a SNARE protein known as vesicle-associated membrane protein 7 (VAMP7), has been demonstrated to take part in the release of exosomes [54]. In conclusion, several modes of action have been proposed for exosome biogenesis and release and it is likely that several mechanisms operate in parallel, which makes the study of these cellular events rather intricate.

1.3.3 Biogenesis of Microvesicles

Microvesicles (MVs) arise from a completely different pathway which – as compared to exosome biogenesis – is even less well characterized. MV formation comes about from outward budding and fission of the plasma membrane, which is believed to be accomplished by a combination of mechanisms including phospholipid rearrangement and activation of cytoskeletal proteins. Phospholipids and proteins are non-uniformly distributed within the plasma membrane. The heterogeneous distribution and formation of clusters is regulated by

scramblases, flippases and floppases, which are aminophospholipid translocases that transfer phospholipids between the outer and the inner leaflet and vice versa [55-57]. The initiating step of the MV biogenesis has been suggested to emanate from surface exposure of phosphatidylserine (PS) by translocation to the outer leaflet [20, 58-60]. Others have shown that increased calcium levels trigger redistribution of phospholipids, which results in MV release [61, 62].

Following phospholipid redistribution, the budding process is completed by cytoskeletal protein contraction, through actin–myosin interactions. The cytoskeletal contraction has been demonstrated to be initiated by a signaling cascade starting with ADP-ribosylation factor 6 (ARF6) that activates phospholipase D (PLD), which leads to activation of myosin light chain via recruitment of extracellular signal-regulated kinase (ERK) that phosphorylates myosin light-chain kinase (MLCK) [21, 63].

The involvement of TSG101, the ESCRT-1 component, which has been associated with exosomal biogenesis, as described above, has also been connected to MV biogenesis [64, 65]. Here, however, TSG101 is believed to interact with a tetrapeptide protein within the Arrestin 1 domain–containing protein 1 as part of MV formation. Furthermore, in addition to calcium, external factors, such as hypoxia, have been shown to induce MV release via another pathway associated to the expression of the small GTPase RAB22A that co-localizes with materializing MVs at the plasma membrane [66].

In summary, the distinct difference between exosome biogenesis, via the endocytic pathway, and MV formation, through membrane budding and fission, is well established. However, the detailed processes of vesicle biogenesis are still not fully understood and the studies are, to some extent, hampered by downstream processes and analytics, including the first step that involves isolating and purifying the formed EVs from the extracellular milieu in an accurate manner.

1.4 ISOLATION OF EXTRACELLULAR VESICLES

EVs have been successfully isolated from conditioned cell culture media [4, 67, 68] and various body fluids, including blood serum [69, 70] and plasma [71, 72], urine [73, 74], semen [75, 76], breast milk [77], cerebrospinal fluid [78, 79], amniotic fluid [80], ascites fluid [81, 82], bile [83] and saliva [84]. Isolating EVs is technically challenging due to their small size, heterogeneity, physiochemical properties and often complex surroundings. There are a number of different considerations that needs to be taken into account when assessing the isolation procedure and the purified EV sample. The optimal isolation technique should give; 1) high recovery of EVs that are 2) pure, i.e. not contaminated by non-vesicular components and 3) have intact integrity and biochemical properties.

1.4.1 Technical Considerations of Different Isolation Methods

The currently considered gold standard isolation technique in the field, differential centrifugation with ultracentrifugation (UC) [4, 85] is limited by low EV recovery, risk of co-sedimentation of non-vesicular macromolecule contaminants, and EV integrity disruption. Furthermore, UC is laborious and time-consuming with limited scalability. It is also associated with EV aggregation due to high gravitational forces [86-89]. Various alternative isolation techniques have consequently been explored to improve the isolation process. These isolation methods can be grouped into categories that include UC, density gradient separation [90, 91], filtration with or without size exclusion based techniques [87, 92, 93], precipitation [94, 95], affinity binding techniques [96, 97] and microfluidics [98, 99].

1.4.1.1 Differential centrifugation with ultracentrifugation (UC)

The classical differential centrifugation process involves a series of increasing centrifugation steps that start with a 300-500 x *g* spin followed by a 2,000 x *g* spin to remove floating cells and cell debris. The supernatant is then spun at 10,000 x g to pellet larger EVs. This fraction is sometimes referred to as the MV pellet or simply the 10,000 x g EV pellet. This is often followed, or replaced, by a sterile 0.2 μm filtration to enrich for smaller EVs, followed by an UC step of about 100,000 x *g* to pellet the small EVs. The UC step is usually repeated after re-suspending the pellet, to increase the purity [85]. Numerous different protocols using different speeds, centrifugation times, and rotors have been employed for the differential centrifugation process. The inconsistency of EV isolation within the field consequently hampers comparability of the findings between different publications with varying centrifugation based-isolation protocols. In fact, comparison of different factors of this process, including EV media viscosity, UC speed, rotor type and angle, etc., has been investigated in order to reach a consensus of a defined isolation protocol using differential centrifugation [85, 100, 101]. In order to overcome the contamination of non-vesicular components in the isolated EV pellet of the differential centrifugation process, an additional step using density gradient to separate the EVs based on their buoyancy, can be employed. Density gradient separation effectively reduces non-EV-associated protein contamination [85, 102]. However, if the EV medium is more complex than cell culture conditioned media, other contaminants with similar density, such as lipoprotein particles in blood plasma, will remain [103].

1.4.1.2 Filtration and/or size exclusion based techniques

In contrast to density-based isolation techniques, size-based isolation techniques are being increasingly employed for EV isolation. Ultrafiltration devices [104], as well as tangential flow filtration (TFF) systems [105], have been used for isolating and concentrating the EV fraction of cell culture conditioned media based on EV size. In order to purify the EVs from co-isolated contaminants a subsequent step is typically added using size exclusion chromatograph (SEC), which separates smaller molecules, by transiently trapping them in pores of a matrix, from larger molecules [72, 87]. Of note, the addition of SEC has been

shown to be associated with improved EV integrity and protein purity as compared to UC-based isolation [87]. Small commercial SEC columns specialized for EV isolation are now available and are used in numerous labs. These are suitable for isolations from relatively small volumes of EV containing media, such as blood plasma, but are not very scalable per se. Further developments of SEC include combining size exclusion with bind-elute chromatography, which, when combined with a filtration step, has been shown suitable for scalable EV isolation [106].

1.4.1.3 Precipitation methods

Polymer-based precipitation methods include commercial isolation kits, such as ExoQuick™ and Total Exosome Isolation™ as well as Polyethylene glycol (PEG)-precipitation that has been adopted for EV isolation and also applied in clinical settings [95]. Precipitation methods have been widely used and demonstrate high recovery of EVs, however the purity is often reported to be rather low with co-precipitation of non-vesicular-associated protein and nucleic acid contaminants that may render invalid conclusions of EV content and function [107].

1.4.1.4 Affinity binding techniques

Other techniques, such as affinity-based capture, utilize known EV composition properties. For instance, immuno-affinity capture by anti-EpCAM and anti-CD63 antibodies, have been used for small EV isolation with high purity [65, 108]. This isolation method will naturally favor EVs with high expression of these EV antigens, resulting in partial EV isolation, which may or may not be advantageous depending on the research approach. In addition, the capturing beads or antibodies may interfere with down-stream analysis. To overcome this, another affinity based approach targets phosphatidylserine, which is exposed on the EV surface, using a calcium dependent binding to a transmembrane protein (T cell immunoglobulin and mucin domain protein 4, Tim4) decorated on magnetic beads. By adding calcium chelating buffer the captured EVs are released from the beads [109].

1.4.1.5 Microfluidics

Microfluidic methods are another attractive group of EV isolation approaches applicable for small scale isolation and high throughput screening of e.g. body fluid samples for diagnostics. Several different microfluidic techniques, sometimes referred to as lab-on-chip devices, including dielectrophoresis, immune-affinity, hydrodynamic based methods and magnetic-based techniques have been used for EV isolation [86, 110].

The large variety of emerging EV isolation techniques with different pros and cons, and the lack of in the field as to which method to use, may result in an increased risk of incomparability. In addition, co-isolation of contaminants including proteins and nucleic acids may result in invalid conclusions of EV content and function. However, the variety of techniques also provides researchers the ability to cherry-pick the isolation method most suitable for their application and down-stream analysis.

1.4.2 Clinical Grade Production of EVs

A large share of EV research is devoted to exploring EVs as therapeutic moieties in a number of applications, as discussed below. For therapeutic purposes, EVs are usually isolated from cell culture conditioned media of a producer cell source with less complexity as compared to body fluids. There are however other challenges to consider. Transitioning EV isolation from in vitro settings and small preclinical studies to clinical settings does not only require a great deal of scalability, high purity, retained integrity and functionality, but also clearly defined components, standard operation procedures for reproducibility, and sterility. A recent position article suggests that the following must be considered for clinical grade production of EVs [89]:

- Isolation techniques and standardization
- Purity and impurities
- Scalability of technology
- Adequate quality of reagents and materials
- In-process controls

The isolation techniques used for EV application in clinical settings until now are ultracentrifugation into a sucrose cushion with a preceding concentration step of the conditioned media using ultrafiltration [6, 7, 111, 112] or purification using PEG-based precipitation [95]. Following the advances made in EV isolation techniques, future clinical trials will most probably require a greater scalability of isolation and an increased level of purity. It appears likely that a combinational approach, utilizing the advantage of different isolation techniques, would be preferable. Currently, filtration based isolation techniques, e.g. TFF, in combination with SEC seem to be very promising for clinical application, owing to high scalability, reproducibility as well as the possibility to be kept in a closed system.

1.5 CHARACTERIZATION OF EXTRACELLULAR VESICLES

The combination of EVs' invisibility (by naked eye and light microscopy) and undetectability by human senses, with their seemingly remarkable functions and complexity has intrigued a whole field of researchers. Naturally, the technical challenges associated with their nanometer size range, heterogeneity and often complex environment, are equally well impacting the characterization of the isolated EVs. Owing to this, as well as the limitations of isolating perfectly pure EV samples and limitations of the analyses, there is no exclusive detection technique available and the characterization of EVs requires a combinational approach [16].

1.5.1 Size Characterization

Transmission electron microscopy (TEM) was the first technique used to detect EVs and is still often included for EV characterization and can be made increasingly EV-specific via immunolabelling of vesicular proteins (immuno-EM) [113]. Atomic force microscopy (AFM) is an alternative microscopy method suitable to detect EVs [114]. Size distribution

measurements, including nanoparticle tracking analysis (NTA), dynamic light scattering (DLS) and/or resistive pulse sensing (RPS), are commonly used to assess particle size and concentration of a sample. All of the methods have individual shortcomings [115], but they also share limitations in specificity as EV sized non-vesicular particles, such as lipid particles and protein complexes, may falsely be detected as EVs. Furthermore, as with microscopy techniques, these techniques include subjective settings of e.g. light intensity, sample view and pre- and post-acquisition detection thresholds, which have an impact on the result and may affect the reliability and reproducibility of the measurement. With an awareness of the technical limitations and when used in a combinational characterization approach, these methods do however contribute with valid and highly important information in EV research.

1.5.2 Density Characterization

In parallel to size and content, EVs can also be characterized by density. Exosomes have been reported to have a slightly higher buoyant floating density (1.13-1.19 g/ml) [116] compared to MVs (1.03-1.08 g/ml) [117] and their density can be assessed using density gradient separation based on e.g. layers of different sucrose concentrations [85]. However, risk of hyperosmotic pressure from the sucrose affecting the EVs' properties; high variability of measurements depending on sample loading techniques; differences associated with centrifugation duration and speed; inconsistencies in protocols used in the field; and inability to distinguish from lipoprotein particles and viral particles, have all been reported as issues related to sucrose density gradient separation for EVs [15, 85, 118, 119]. An alternative density gradient, based on different concentrations of the isosmotic iodixanol (Optiprep^{TM}) has been reported to overcome some of these shortcomings [91].

1.5.3 Content Characterization

In addition to the morphological features, EVs are also characterized based on their content. Similar to the characterization of size, quantity and density, non-EV-associated contaminants may give rise to false positive readings with the risk of artefacts being reported as EV attributes. This further emphasizes the need for appropriate controls, utilizing adequate isolation techniques and an awareness of the limitations in EV purification and characterization.

1.5.3.1 Protein characterization

Protein content assays are commonly utilized to probe for the presence of known EV-associated proteins, including tetraspanins such as CD63, CD9 and CD81 as well as biogenesis-associated components, e.g. TSG101 and ALIX (see below for protein content of EVs). Likewise, detection of non-EV-associated proteins, such as endoplasmic reticulum (ER)-associated calnexin, to indicate their absence, is normally assessed. In addition to basic molecular biology techniques, such as western blot (WB), which has relatively poor detection limit and requires relatively high protein levels, high-throughput mass spectrometry-based proteomics for in-depth proteomic analysis of EVs have demonstrated to be highly sensitive and able to detect thousands of proteins in an EV sample [87]. For detection of EV surface

membrane proteins, antibody coated beads for flow cytometry is increasingly being exploited as a versatile and rapid tool for the EV analysis, but does not allow for absolute quantification [120, 121].

1.5.3.2 Nucleic acid characterization

The presence of nucleic acids is frequently analyzed in EVs, owing to the early findings of the ability of EVs to deliver functional RNA [8]. The main focus is on various RNA species, which have repeatedly been found in EVs, whereas for instance the presence of DNA is still debatable [122]. RNA detection is carried out using molecular biology techniques, such as quantitative polymerase chain reaction (qPCR), northern blotting, microarrays and next generation sequencing. Again, contaminating non-EV-associated RNA, such as extracellular RNA protein complexes or lipoprotein complexes, may interfere with the characterization [123, 124]. Different attempts to address the risk of contaminants have been made, including proteinase and RNase treatments to disrupt ribonucleoproteins outside of EVs. However, possible "truly" EV surface associated RNA will then also be disrupted. Furthermore, the levels of e.g. miRNA have been reported to be as low as less than one molecule of a given miRNA per EV in average and these minute quantities indicate the challenge and need for optimized isolation and characterization [125]. The concerns regarding EV-associated RNA characterization is being appreciated within the EV field and was in fact the focus of a recent position paper from the International Society of Extracellular Vesicles (ISEV) [122].

1.5.3.3 Lipid characterization

A growing part of the EV field is also focusing on the lipid content of EVs, which has been somewhat overlooked as compared to the efforts to dissect the protein and nucleic acid content. In parallel to the other characterization methods, lipidomics faces the same challenges of possible artifacts from non-vesicular impurities. Methods such as high-throughput mass-spectrometry are used to decipher the lipid repertoire of EVs, which will expand our understanding of EVs and most possibly impact on how we exploit EVs as therapeutics [126].

1.5.3.4 Characterization of functionality

In addition to the characterization of EV morphology and components, integrity and functionality assessments are highly important for understanding the impact of isolation and storage methods as well as to explore EV biology and applying EVs as therapeutics. The stability of EVs is often indicated as an advantage for exploiting EVs as drug-modalities. The stability is commonly assessed based on changes over time in regard to size and composition, including proteins and RNA, as well as membrane permeability studies measuring the presence of the EV components in the sample supernatant. Cellular uptake of the isolated EVs, e.g. with a fluorescent label for traceability, is frequently used as a functional readout [127-130]. There is however a risk in this system of merely following the free label and the functionality is limited to uptake and does not convey insight into the effects of EVs in the target cell. A method that recently has been applied for EVs that overcomes some of these

limitations is the Cre/LoxP system, where functional delivery of Cre recombinase mRNA or protein via EVs can be assessed by Cre-reporter cells, which generate a fluorescent protein upon recombination of flanking LoxP sites, both in vitro and in vivo [131, 132]. In addition, numerous other functionality readouts based on the studied EV component-specific actions, including miRNA, mRNA and protein activity, have been described. Furthermore, functional assays based on EV type-specific actions, such as immune stimulation by antigen presenting cell (APC)-derived EVs by T-cell activation assays [5, 133] and the immune modulating functions of mesenchymal stromal cells (MSC)-derived EVs [134], are used to provide evidence for the biological function of EVs.

1.5.4 Future Characterization Considerations

The methods currently used for characterization of EVs are mostly based on a bulk EV sample and often require relatively high numbers of EVs for analysis. Size distribution measurement with NTA or protein detection with WB, for instance, requires about $1x10^8$ – $1x10^{10}$ EVs [115]. As aforementioned, EVs are believed to be relatively heterogeneous with different classes and subpopulations. Hence, the individual vesicles in a bulk EV sample, purified with current isolation techniques, will differ in both morphology and content. In addition, artefacts from contaminants due to insufficient isolation and purification methods, as well as buffer-associated artefacts, e.g. EV-sized calcium phosphate aggregates in PBS [135], which can interfere with size distribution measurements and quantifications, indicate the need for standardized characterization methods.

Moreover, in order to further understand EV biology as well as for applying EVs as therapeutics, there is a need to further dissect the EV bulk sample and move towards single vesicle analysis. In addition to immuno-EM, fluorescence correlation spectroscopy (FCS) has been used for single vesicle analysis [136]. The main drawback of this is the need of a fluorescent tag that is usually introduced by a dye or genetically engineered EV membrane proteins with a fluorescent moiety, which limits the detection to EVs with that particular moiety. Another method that has gained increased focus is flow cytometry for single EV characterization. Regular flow cytometers are not able to detect individual vesicles <300 nm [115], however by optimizing acquisition and analysis parameters of configurable flow cytometers, single particle analysis of EVs has been reported [137, 138]. It is expected that flow cytometry-based systems will offer more robust and enhanced multiparameter analysis at a single vesicle level in the near future [89, 139].

1.6 COMPOSITION OF EXTRACELLULAR VESICLES

Despite the limitations of current isolation and characterization methods, the composition and content of EVs are extensively being unraveled with in-depth nucleic acid characterization, preoteomics and lipidomics studies being undertaken. Databases, such as Exocarta, Vesiclepedia, and EVpedia, have been generated to compile these comprehensive datasets for systematic analysis [140-142]. All subtypes of EVs share a general composition of an outer

lipid bilayer and various proteins, lipids and nucleic acids carried by the vesicles, as illustrated in Figure 2. The specific content of EVs is however largely dependent on biogenesis, cell source and culturing conditions.

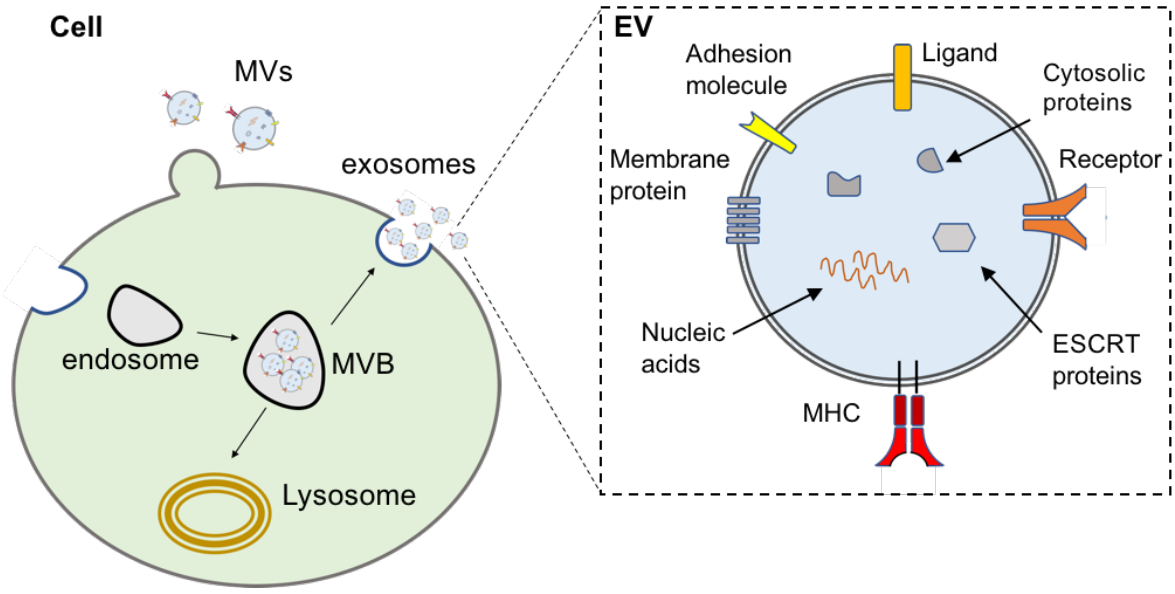

Figure 2 – Illustration of EV (MVs and exosomes) biogenesis and general EV composition.

1.6.1 Protein Content

The protein content of EVs is usually utilized as characterization markers of EVs and their subtypes. Many of the commonly found proteins are involved in the biogenesis and formation of EVs. Exosomes, which are derived from the endocytic pathway, have been shown to generally contain proteins associated with their endosomal origin including major histocompatibility complex class II (MHC II) and tetraspanins. In addition, ESCRT machinery components, such as Alix and TSG101 and chaperone heat shock proteins, such as Hsp70 and Hsp90, as well as RAB27A, RAB11B, associated with exosomes biogenesis as described above, are all commonly found in exosomes, but may also be present in MVs [143]. MVs on the other hand, have been reported to be enriched in glycoprotein Ib, integrins and P-selectin [113]. Many of the EV-mediated effects, attributed to various EV-enriched proteins, are often parental cell-specific. Examples of this include EV-mediated disposal of transferrin receptor via EVs during erythrocyte maturation [2]; MHC II display on the surface of EVs derived from APCs, which can elicit immune responses [4]; immune suppression mediated by placenta-derived EVs via surface expression of Fas ligand (FasL) and TRAIL, which maintain immune-privileged sites [144]; and tumor-associated fibroblasts that shuttle the metalloproteinase ADAM10 via EVs, which promotes the motility of breast cancer cells [145]. In addition to the common and cell type-specific EV proteins, EVs tend to be devoid of proteins associated with non-endosomal intracellular compartments, including mitochondria,

Golgi apparatus and ER [146, 147]. Absence of these non-EV-related proteins can thus serve as confirmation of EV purity in the EV isolation process [148].

1.6.2 Lipid Content

The lipid composition of EVs is essential for the membrane stability, structural rigidity and resistance to physiochemical changes [149, 150]. In addition, there is evidence of lipid-dependent functions in EVs with transfer of bioactive lipids via EVs and the abovementioned role of lipids in the biogenesis of EVs [151]. EVs differ in their lipid composition compared to their parental cells with an EV-specific enrichment of sphingomyelin, cholesterol, PS and glycosphingolipids and a decreased level of phosphatidylcholine and diacyl-glycerol [151], which clearly indicates a sorting mechanism. A number of lipids has been found to be involved in the formation of EVs. In addition to the demonstrated role of ceramide and cholesterol in EV biogenesis as described above, the polyglycerophospholipid BMP, for instance, has been linked to the formation of ILVs by binding of both Alix, an important protein of the ESCRT machinery, and the chaperon protein Hsp70 [152, 153]. Furthermore, some of the EV-mediated cellular responses have been shown to be lipid-dependent. Vesicular displayed prostaglandins can activate intracellular signaling pathways of a target cell [154]; other bioactive eicosanoids, including certain leukotrienes, which are associated with inflammatory asthma, have been shown to be enriched and functional in EVs [155]; EV-mediated sphingomyelin has been shown to play a key role in angiogenesis mediated by tumor-derived EVs [156], to mention just a few examples of EV lipid-dependent cell-to-cell signaling.

1.6.3 Nucleic Acid Content and Loading

The presence of nucleic acids in EVs and the effects mediated by nucleic acids, shuttled via EVs, constitute a great part of the EV field's interest, owing to potential new insight into EV biology and the idea of utilizing EVs as potential novel therapeutic agents. The presence of DNA in EVs has been described by a few groups [157-160]. Whether DNA is truly EV bound and not an isolation artefact is however still controversial within the EV research field and needs further investigation [122]. The main focus has so far been on different RNA-species found in EVs (EV-RNA). The predominant forms found in EVs are small RNA, below 200 nucleotides (nt) in length, although longer, up to 4.5 kb, have been detected [161-164]. Various RNA species have been detected in EVs, including mRNAs, miRNAs and other long and short non-coding RNA (ncRNA). With the majority of the reads being relatively short most of the mRNA and long ncRNA is believed to be fragmented, although some appear to be intact.

The EV-RNA content has been reported to be EV subtype- and cell source-specific [158, 165, 166] and seems, to a certain degree, to reflect the parental cell source with many common transcripts. There is however a disproportional distribution of RNA species in EVs compared to the parental cell and it is evident that specific RNA enrichment in EVs occurs [8, 163, 166, 167]. EVs have for instance been shown to be enriched in retrotransposon sequences,

mRNAs encoding transcription factors and proteins involved in alternative splicing, as compared to the EV source cell. In contrast, other mRNAs, encoding e.g. mitochondrial or cytoskeletal proteins, seem to be relatively low in EVs [158, 163]. Hence, suggesting an EV-RNA sorting mechanism. In fact, certain specific short mRNA motifs have been suggested to be important for mRNA sorting into EVs [168, 169].

A few different miRNA sorting mechanisms have also been proposed. EV enriched miRNA have been demonstrated to often be 3′ uridylated in contrast to 3′ adenylated miRNAs generally found in cells [170]. This observation indicates that 3′ end post-transcriptional modifications are involved in the sorting of miRNA into EVs. miRNA sorting has also been suggested to be associated with a short sorting motif recognized by the RNA binding ribonucleoprotein hnRNPA2B1 [171]. Two other hnRNPs were also shown to bind EV-miRNA, without any identified motifs though, which still however strengthens the concept of RNP-mediated loading [171, 172]. Another RNA binding protein, argonaute 2 (AGO2), associated with the RISC complex involved in RNA silencing, has been linked to miRNA EV sorting. AGO2 sorting has been demonstrated to be dependent on its activation state, as phosphorylated AGO2 seemed to inhibit the miRNA-EV sorting, which further indicates a controlled sorting mechanism [173-175]. In addition, AGO2 knockout resulted in decreased levels of specific EV-miRNA [173]. The presence of AGO2 in EVs is however controversial and the AGO2-miRNA complex has been claimed to rather be a co-isolated artefact [123]. A fourth suggested miRNA sorting mechanism is the neutral sphingomyelinase 2 (nSMase2)-dependent pathway. nSMase2 has been linked to EV biogenesis and overexpression of nSMase2 has been shown to increase miRNA loading, whereas knock-down of nSMase2 decreased miRNA EV content [176].

1.6.4 Cell Source and Cellular State Dependent Differences of EVs

EVs, and exosomes in particular, have been shown to share general characteristics, including certain proteins, such as tetraspanins, the ESCRT machinery-associated proteins Alix and TSG101, and certain heat shock proteins, for instance [143], as well as a general lipid composition that is distinct from the cell source. There is however clear evidence of distinct cell source-dependent differences between EVs in terms of content and function as exemplified above and systematically organized in various EV databases [140-142]. In addition, the state of the parental cell also seems to impact the EVs composition and function. Cellular maturation state, for instance of dendritic cells (DCs), have shown to have an impact on EV composition and function as immature and mature DCs rendered EVs with different features [177, 178]. Cells exposed to stress-induced conditions, such as thermal and oxidative stress [179-182], acidic conditions [183], serum starvation [184], hypoxia [66, 185], UV-light [180], or cell stimulating substances [186-188], generate EVs with a different composition and function as compared to EVs isolated from cells under normal conditions. It is however questionable how representative or physiologically relevant the common cell flask culturing conditions are. In fact, three-dimensional cell culturing in bioreactors or on spheres, which reflects the physiological cell conditions better than two-dimensional cultures, seems to give

rise to EVs with altered properties compared to corresponding EVs derived from flat plastic dishes [189, 190].

Intense research into protein, lipid and nucleic acid content of EVs is ongoing. With continuous developments in isolation methods and characterization techniques and an increased awareness of their respective limitations as well as the impact of the cellular state and microenvironment, the EV compositions are being unraveled, which will provide further insight into the complex functions of EVs.

1.7 INTERCELLULAR COMMUNICATION OF EXTRACELLULAR VESICLES

The immense interest in the field of EVs during the last decade springs from the recognition of EVs as important mediators of cell-to-cell communication. Via transfer of bioactive components, EVs take part in physiological conditions and maintenance of homeostasis, but also influences various pathological conditions.

1.7.1 Cellular Uptake of Extracellular Vesicles

A few different mechanisms have been proposed for how EVs convey their messages and in particular how EVs are taken up by the recipient cell. Numerous publications have shown uptake of EVs and the evidence of cellular uptake is now indisputable. Experimental designs with a variety of different membrane fluorescent dyes, such as PKH26 [191, 192], PKH67 [129, 130], DiI [193, 194], and DiD [194, 195], as well as fluorescent protein fused to EV-proteins, such as TSG101-GFP [196] and mCherry-CD63 [136], have shown cellular uptake of EVs when observed via e.g. fluorescent microscopy or flow cytometry. The great number of observations as well as controlled experiments suggest that there is specific uptake of EVs rather than artefacts by free dye or free fluorescent protein. Additional information of EV uptake is based on the functional delivery of luminal EV cargo, such as RNA, which must be exposed to the recipient cell's machinery, suggesting a plasma- or intracellular membrane fusion, or intracellular disruption of the confining membrane of EVs.

1.7.2 Membrane Fusion

Rationally, one of the initial hypotheses was thus that EVs would fuse with the recipient cell's plasma membrane [183, 197]. The merge of EV membrane and plasma membrane has been observed via fluorescent lipid dequenching of EVs derived from melanoma as well as dendritic cells. The fusion events were increased during acidic conditions, which may indicate that this would mostly occur in an acidic tumorigenic microenvironment or intracellularly in endosomal departments that are known to have acidic pH conditions [183]. Interestingly, a recent publication demonstrated that EVs seem to be rapidly internalized to endosomal compartment as single vesicles via cellular filopodia and further shuttled to rough ER for cargo display followed by fusion with lysosomal compartments [136].

1.7.3 Endocytosis

The majority of studies do however support an endocytic mechanism as the primary route of EV uptake and whether entry via fusion is a rare alternative route or even at all taking place is still under debate in the field. Multiple studies have shown that EV uptake is reduced at 4°C, indicating that internalization of EVs is an energy-dependent process as opposed to passive membrane fusion [130, 146, 194, 198, 199]. Of note, inhibition of the endocytic pathway, by depolymerization of actin filament network via Cytochalasin D, has been shown to reduce EV internalization, further strengthening the concept of endocytosis-mediated uptake [127, 129, 130, 197, 200]**.** Endocytosis includes a variety of different internalization processes and EV uptake has been proposed to be mediated by phagocytosis [197, 200], macropinocytosis (MP) [129, 199], clathrin-mediated endocytosis (CME) [198, 201] and/or clathrin-independent endocytosis (CIE) [199, 202].

1.7.4 Phagocytosis

Phagocytosis is a receptor-mediated cellular engulfment of particles. The evidence supporting phagocytosis-mediated EV uptake includes observations of inhibited EV internalization of macrophages following inhibition of phagocytosis-associated phosphoinositide 3-kinase (PI3K), an enzyme that is essential in the formation of phagosomes [200]. In addition, EVs labelled with a phagosome tracer, pHrodo that becomes fluorescent at phagosome pH, was active in recipient dendritic cells, thus indicating phagocytosis-mediated EV internalization [197]. The technical limitations associated with these findings, including specificity of the PI3K inhibitors and PI3K, which is also involved in MP, as well as the tracer pHrodo's ability to distinguish phagocytosis compared to other low pH associated endosomal pathways, emphasize the need for further validation of the phagocytosis-mediated EV uptake theory.

1.7.5 Clathrin Dependent and Independent Endocytosis

In contrast to phagocytosis that is associated with specialized phagocytes, CME, CIE and MP are endocytosis mechanisms occurring in all cell types. Inhibition of essential components of the CME process including inhibition of dynamin2, a GTPase required for CME or treatment with chlorpromazine, which prevents clathrin-coated pit formation at the plasma membrane, have been shown to decrease EV uptake, thus indicating a role of CME in EV internalization [128, 198]. In addition, clathrin-independent endocytosis, such as caveolin-dependent endocytosis (CDE), has been described for EV uptake. Of note, dynamin2 is required for both CME and CDE. Thus, the findings of decreased EV uptake following dynamin2-inhibition cannot be applied to distinguish between these endocytic pathways. In fact, a recent publication found that CIE, but not CME, is important for EV internalization [199]. By utilizing chemical inhibitors of CIE and siRNA knockdown of caveolin-1, flotillin-1, and RhoA, all representing different CIE subclasses, significant reduction of EV uptake was observed, whereas knockdown of clathrin heavy chain, representing CME, as well as CME

dependent chemical inhibitor, did not affect the EV internalization [199]. The authors further demonstrated MP-dependent EV uptake using the same approach.

1.7.6 Macropinocytosis

MP resembles phagocytosis, but without the need of direct contact with the internalized material, and involves the inward folding of the cell membrane that is then pinched off into the intracellular compartment. In addition to the abovementioned study indicating MP-dependent uptake, alkalization of the receiving cell microenvironment abrogated EV uptake, which supports an MP-associated pathway since MP requires acidification of vacuoles [129]. Furthermore, the MP pathway might be cell type-specific, since, when using the same specific MP-inhibitor, a decreased EV internalization has been observed in HeLa cells and microglia, but not in in macrophages [129, 199, 200].

1.7.7 EV Content and Recipient Cell Dependent Uptake

EV uptake has been demonstrated to vary depending on recipient cell type. Possible mechanisms include cell type-dependent uptake mechanisms, e.g. phagocytes may utilize phagocytosis whereas other cells may use MP or CIE as exemplified above. Cell-specific attributes important for EV internalization have also been described. For instance, expression levels of heparin sulphate proteoglycans, lectin receptors, lipids and protein-protein interactions have been shown to govern cell-specific EV uptake [146, 198, 203-205]. Differences in uptake have also been reported depending on EV cell source, indicating a sensory mechanism in the recipient cell to certain properties displayed by EVs that become internalized. A variety of different EV-displayed molecules including tetraspanins, integrins and immunoglobulins as well as lipid rafts, sphingolipids, PS and saccharides, have been demonstrated to take part in the uptake process [205-209].

To summarize, most evidence point toward an endocytosis-mediated cellular internalization of EVs, including phagocytosis, macropinocytosis**,** clathrin-mediated and clathrin-independent endocytosis, where the specific pathways in play appear to be dependent on the receiving cell type. Furthermore, there seems to be an EV cell source-receiving cell type relationship dependent uptake mechanism presumably associated with the displayed lipid and protein content of the EVs and receiving cells.

1.8 BIODISTRIBUTION OF EXTRACELLULAR VESICLES

The many findings of the cellular uptake mechanisms have increased our understanding of EVs fascinating capacity to modulate recipient cells. The majority of these important studies are however based on in vitro findings. To further understand EV trafficking and to evaluate EVs as potential therapeutic agents, investigating the fate of EVs in vivo is of uttermost importance.

1.8.1 General Tissue Distribution of EVs

Relatively few studies have been specifically conducted to evaluate the biodistribution of EVs. These studies do, however, generally indicate a distribution pattern associated with the mononuclear phagocyte system (MPS) with accumulation in, among other tissues, liver, spleen, and lungs of EVs derived from lymphoma cells [210], melanoma cells [211], breast cancer cell lines [212] and prostatic cancer cells (PC3) [212] as well as HEK293T cells [213, 214]. The MPS is part of the innate immune system with different subsets of APCs patrolling the circulation for detection of foreign substances. These findings are coherent with studies of other nanoparticles, such as liposomes, which share some EV characteristics, such as similarities in size and confinement of a lipid bilayer [215-217]**.** The impact of MPS on EV biodistribution was in fact observed in a recent publication indicating the role of scavenger receptor (SR-A)-mediated EV uptake in macrophages. Inhibition of SR-A by dextran sulphate dramatically reduced accumulation of EVs in liver [189]. Furthermore, red blood cell-derived EVs have been demonstrated to be removed from the circulation by SR-A of MPS-associated Kupffer cells [218]. When EVs were injected in a mouse strain with impaired innate immune system, a slower uptake in liver and lung was observed compared to mice with impaired adaptive immune system and normal mice, further strengthening the role of MPS in EV biodistribution [212]. Studies on small nanoparticles (less than 100 nm in diameter) have however found less pronounced MPS uptake due to decreased opsonisation by pattern recognition receptors of phagocytes, because of the small size and curvature of such nanoparticles. Small particles have furthermore been demonstrated to penetrate the fenestrations in hepatic and splenic sinusoidal endothelium with subsequent uptake by parenchymal cells [219]. Thus, this indicates hepatocyte and splenocyte uptake rather than MPS-associated cell uptake. Of note, EVs injected in macrophage depleted mice were found to still mainly accumulate in liver and spleen, suggesting non-MPS-associated uptake. The clearance of EVs from the circulation was however dramatically decreased in the macrophage depleted mice, supporting the importance of macrophages for the uptake of EVs. EVs have previously been shown to be cleared from the circulation quite rapidly. Studies with EVs derived from melanoma cells [211], splenocytes [186], and erythrocytes [218] reported a more than 90% clearance within 30 minutes. However, thrombocyte-derived EVs remained in the circulation with a half-life of 5.5 hours [220], indicating differences in clearance between different types of EVs. Attempts to increase EV circulation, such as PEGylation, which has been demonstrated to increase circulation time of injected EVs, indicate the importance of the biophysical properties of EVs in relation to distribution and clearance. Furthermore, the isolation of EVs may impact the biodistribution. UC-based isolation, as aforementioned, carries a risk of causing EV aggregation and has been shown to result in a higher accumulation in pulmonary tissue, most probably due to the lung capillaries being the first capillary bed being subject to EV exposure following intravenous injection, compared to EVs isolated with ultrafiltration and SEC [87]. A subsequent publication comparing different isolation techniques' impact on clearance did, however, not see any isolation- dependent differences [221]. In line with the analytics methods discussed above, isolation technique

may, however, have an impact on EV tissue distribution and is a factor that must be considered when assessing the uptake of EVs in vivo.

In summary, it is likely that there are both MPS-associated and non-MPS-associated mechanisms governing EV uptake and biodistribution, which might additionally be EV-type dependent.

1.8.2 EV Type-Specific Biodistribution

The observed impact of EV cell source-dependent properties and the receiving cell type differences on EV uptake has led to the subsequent hypothesis that different EVs would have a preferential tissue homing capacity, possibly governed by EV type-specific surface properties. Surface expression of CD55 and CD59 on EVs has been demonstrated to protect from lysis and allow for longer survival in the circulation, offering the ability of EVs to distribute in tissues [222]. Clearance of EVs has also been demonstrated to be associated with display of PS on EVs [223]. Display of the CD169 ligand (α2,3-linked sialic acid) on certain B-cell-derived EVs, have been shown to direct them towards spleen and lymph node accumulation by internalization of CD169 positive macrophages residing in these sites. The finding that CD169 controls the access of EVs to lymphoid organs was further strengthened by the finding that the distribution was altered in the absence of this route in $CD169^{-/-}$ mice [224]. Others have demonstrated that EV-displayed saccharides and C-type lectin interaction mediate EV uptake in DCs [205]. The homing potential of EVs linked to the display of certain integrins was nicely demonstrated by Hoshino, et al. Tumor-derived EVs from different malignant cell lines, with known metastatic tissue pattern, were shown to home to specific tissues and prepare the pre-metastatic niche. The importance of EV homing was further demonstrated by the ability to alter the metastatic tissue pattern of a bone-tropic tumor cell towards lung-tropism by injecting EVs derived from a lung-tropic cell line. Proteomic analysis, and subsequent knockdown, demonstrated the importance of certain EV-expressed integrins in mediating the specific tissue homing properties of these tumor EVs. The distribution differences of EVs depending on cell source, as well as dose and injection route was further studied in this thesis and is discussed in more detail below in chapter 4.3. In a comparison of EVs derived from five different mouse cell lines (melanoma, myoblast, fibroblast, endothelial and macrophage-like cells) there were however no significant differences in serum clearance, with all EVs having a serum half-life of about four minutes.[225] The EVs were engineered to express a fusion protein, Gaussia luciferase (gLuc) fused with lactadherin, and the luminescent reporter moiety was also used for biodistribution evaluation. Live imaging was conducted 5 minutes after intravenous injection and EVs from all cell lines displayed accumulation mainly in liver. The authors concluded that the physicochemical and pharmacokinetic properties of EVs from these different cell sources are comparable [225]. The sensitivity of assessing the biodistribution based on a two-dimensional image of the whole mouse at a single time point is however questionable and this approach may not enable detection of small, but possibly significant, cell type-dependent differences in EV biodistribution. The many findings on EV type-specific surface properties

with observed corresponding differences in tissue uptake in vivo, clearly suggests EV-type dependent biodistribution. The degree of impact of these alterations in relation to the general distribution pattern may however be subtle, but yet important.

1.8.3 Technical Considerations of EV Biodistribution Studies

Different techniques of labelling EVs for in vivo tracing have been utilized. Bioluminescent fusion proteins have been employed with gLuc that has been shown to have a 1000-fold higher intensity compared to the commonly used firefly and renilla luciferases [226]. The high sensitivity makes it attractive for tracing small particles in vivo where the signal from a relatively small number of luminescent molecules needs to be strong enough to penetrate tissues for detection. gLuc has been used in a handful of EV biodistribution publications [211, 212, 227, 228]. gLuc is normally a secreted protein, but for EV tracing it has been expressed as a fusion protein with lactadherin [211, 227] or the transmembrane domain of platelet derived growth factor (PDGF)-receptor [228], for EV incorporation. While the use of an EV sorting moiety has advantages with high specificity, i.e. the luminescent protein should only be present in EVs with the respective EV sorting domain and thus minimizing the risk of signal from non-EV elements, as with lipophilic dyes, this specificity may also be disadvantageous, since it will only reflect the EV population carrying the respective EV sorting domain. In fact, different biodistribution patterns were reported using different sorting constructs. However, the different publications were utilizing EVs from different cell sources from different species (HEK293T [228] and B16-BL6 [211]) and at different doses, which is possibly the main reasons for the observed differences. The gLuc-lactadherin fusion protein was further developed by introducing a biotin acceptor domain that could be utilized for conjugation with labelled streptavidin, e.g. with a fluorophore, to offer dual imaging possibilities with both bioluminescence and fluorescence of the same EVs [228]. Although an elegant approach, it suffers from the same limitations as using gLuc fusions only.

Other studies have utilized fluorescent lipophilic dyes, such as red and green PKH dyes (PKH26 [191, 192] and PKH67 [129, 130], respectively), which have previously been used in cell labeling and tracing studies, to label and trace EVs. The most commonly used fluorescent lipophilic dye is however, the near-infrared DiR [87, 212, 213, 229], which is highly fluorescent when incorporated into membranes and offers high optical tissue penetrance owing to near-infrared fluorescence spectrum [230]. Unlike fluorescent or luminescent chimeric proteins fused with an EV sorting protein, the lipophilic dyes will most likely distribute equally among the EV populations. There are however, other potential limitations of using dyes including the risk of having excess or unbound dye as well as reported risks of these dyes to contaminate microenvironments, i.e. being transferred to a neighboring membrane, which in turn may give rise to artefacts that need to be controlled for [231]. In addition, there is a potential risk that the introduced fluorescent/luminescent protein or dye will influence the natural biodistribution of EVs, since other displayed properties have been shown to alter cellular uptake and tissue distribution.

Another approach to trace the EV biodistribution overcomes this latter issue by following RNA species believed to be residing in the lumen of EVs. In one study, B-cell-derived EVs were loaded with miR-155 mimic by electroporation and injected in miR-155 knock-out mice [232]. Perfused and subsequently homogenized tissues were analyzed by real-time PCR, displaying a distribution profile with the highest levels of miR-155 in liver, adipose tissue and lungs (spleen was not analyzed). In addition, plasma clearance of the EV-associated miR-155 was in line with previous reports, with a reported 90% clearance within 30 minutes. Prior to injections, the EVs were RNase treated in order to reduce the possibility of miR-155 being on the outside of EVs and the re-isolated EVs, following RNase treatment, did show a decreased amount of miR-155. The authors concluded that free floating miR-155 mimics thus were degraded. Although the risk of analyzing free miRNA was addressed, possible limitations include incomplete degradation of all the free miRNA, redistributed miRNA during re-isolation and risk of continuous diffusion out of the EVs of the electroporated miR-155 mimic. A few publications have utilized the cre/loxP system, described above, to explore functional delivery of Cre-mRNA to reporter tissues, including tumors [233] and brain [234], with seemingly successful recombination and generation of detectable fluorescence, in vivo. These studies indicate the potential of this highly elegant system, which however needs further developments if it is to be used for complete biodistribution studies, owing to it being quite inefficient even under in vitro settings and not being able to distinguish between different cargos, e.g. Cre-mRNA and –protein, to mention a few of the limitations.[233]

In conclusion, EV biodistribution studies have shown a general distribution pattern associated to the MPS with accumulation of EVs often being reported to be highest in liver, spleen and lung tissue, although non-MPS dependent tissue distribution and uptake have been demonstrated in parallel. Cell source differences and EV type-specific properties seem to impact the biodistribution, at least to a certain degree. In addition, as generally in the EV research field, there may be an influence on the tissue distribution associated with isolation and manipulation of the EVs.

1.9 EVs IN PATHOLOGICAL AND PHYSIOLOGICAL SETTINGS

When initially discovered, EVs were postulated to take part in sustaining homeostasis and function as a mean for cellular disposal of excess proteins, lipids and nucleic acids. The last two decades intense research within the EV field, has however revealed that EVs play an important role in various other physiological as well as pathological processes.

1.9.1 EVs and the Immune System

EVs were first shown to be involved in immune regulation in the breakthrough publication from Raposo et. al., where the authors demonstrated that B-lymphocyte-derived EVs expressed antigen-presenting MHC II molecules and that these EVs could induce a T-cell response [4]. Following this, a number of studies have shown that different APC-derived EVs (APC-EVs) carries peptide–MHC I or –MHC II complexes that can directly stimulate CD8+

and CD4+ T cells, respectively [235-237]. However, the potency of APC-EVs to induce a cellular response is relatively poor compared to APCs and relatively high concentrations of these APC-EVs are required to generate a T-cell response [238, 239]. Hence, indicating that this may not be a main pathway of T-cell activation, at least not for the activation of naïve T-cells, which have been demonstrated to be poorly stimulated by free APC-EVs in vitro [239, 240]. Nonetheless, EVs have also been shown to generate T-cell response (including naïve T-cell activation) via APCs, by APC uptake and transfer of EV-associated antigen peptides to the MHC molecules of the receiving APCs or by being retained on the APCs' surface for EV presentation of peptide–MHC complexes directly to T cells [240]. For instance, it was demonstrated that mycobacterium-infected macrophages generate EVs with mycobacterial antigens can promote T-lymphocyte immunity in mice, via DCs [241], thus indicating a role of EVs in the communication between infected cells and the immune system.

Numerous other EV-associated immune regulations have been proposed. EVs derive from infected cells can mediate toll-like receptor-dependent inflammatory response [242]; mast cell-derived EVs can promote DC maturation [243]; mast cell-dependent B- and T-cell activation is, to some extent, mediated by EVs [244]; and MSC [93, 245] and immature DC-derived EVs [246, 247] have immunosuppressive properties, to mention a few examples of findings that illustrate the numerous roles EVs play in the immune system. EVs have furthermore been demonstrated to have the ability to convey various cytokines, such as interleukin‑1β (IL-1β) [248], IL-6 [249], CXCL8 [250], tumor necrosis factor (TNF) [251], and TNF superfamily members including FasL [252], TRAIL [252] and CD40 ligand [244, 253], that induce a functional immune response, or immune suppression depending on the cytokine. It has been suggested that cytokines, when associated to EVs, are more resistant to proteases and have an increased biological activity [254]. EVs have been implicated as pro-inflammatory mediators in a number of pathological conditions, including rheumatoid arthritis [255, 256], atherosclerosis [257], sepsis [258], type 2 diabetes [259, 260] and pre-eclampsia [261]. In addition, EVs have been shown to play a key role in the important suppression of the maternal immune system during pregnancy. Placenta-derived EVs have been demonstrated to maintain the immune-privileged site by expression of the pro-apoptotic FasL [144], TRAIL [144], and PD-L1 [262], which induce T-lymphocyte killing and subsequent T-cell anergy [263]. NK-dependent cytotoxicity has additionally been shown to be downregulated by placental-derived EVs, which has been suggested to be mediated by the inhibition of NKG2D receptor, by EV presentation of NKG2D ligand [264].

1.9.2 EVs and Malignancies

A great number of publications have shown antitumor immunity in response to tumor-derived EVs (tEVs) through different mechanisms including natural killer cell and cytotoxic T-lymphocyte (CTL) response, which can be mediated by T- and B-cell activation by EVs [5, 81, 265, 266]. For instance, tEVs, carrying tumor antigens, can be efficiently taken up by DCs for antigen processing and presentation to tumor-specific CTLs. NK activation of Hsp70-containing tEVs is another example of EV-mediated antitumor immunity [266]. In

addition, lymph node macrophages have been shown to internalize tEVs and physically prevent tEV dissemination [267]. In contrast, malignant tumors also utilize EVs to foster tumor-promoting immunity, which resembles the mechanism of placental-derived EVs to maintain immune-privilege. Tumor-derived EVs have similarly been demonstrated to promote T-lymphocyte apoptosis by expression of FasL [268] and TRAIL [269] and suppress NK and CD8+ cytotoxicity [269, 270]. In addition, tEVs presentation of transforming growth factor‑β (TGFβ) has been demonstrated to inhibit the maturation of DCs and macrophages in vivo and promote regulatory T-cells, either directly or via recruitment of myeloid-derived suppressor cells, which inhibit antitumor responses [271, 272]. tEVs have furthermore been demonstrated to home to subsequent metastatic sites and induce micro environmental changes that prepare the pre-metastatic niche in a number of different malignancies [208, 273-275].

1.9.3 EVs and the Central Nervous System

Following studies demonstrating the presence of EVs in cerebrospinal fluid (CSF) [78, 79], a number of studies have examined the role of EVs in the central nervous system (CNS). It has been demonstrated that EVs participate in interneuronal communication [276, 277] and in the cross-talk between neurons and glial cells [278], suggesting that EVs participate in maintenance of homeostasis and immune regulation of the CNS. Of note, EVs have been shown to mediate important processes related to neuroinflammation. Brain-derived EVs have been shown to have an altered miRNA profile following traumatic brain injury with increased levels of miR-21, which has been suggested to be neuroprotective [279]. EVs have in addition been implicated in other neuroinflammatory diseases, such as multiple sclerosis [280] and ALS [281], but most evidence of the role of EVs in CNS has been accumulated in relation to neurodegenerative diseases. For instance, α-synuclein, which has been implicated in Parkinson's disease (PD) and other disorders with Lewy body pathology, has been demonstrated to be shuttled by EVs in vitro and in preclinical models, as well as in PD-patients, where increased levels of α-synuclein in plasma-derived EVs have been detected [282]. In addition, several studies have been conducted to investigate the role of EVs in Alzheimer’s disease (AD), demonstrating EV-mediated sequestration and spread of the pathogenic beta amyloid (Aβ) [283, 284] and phosphorylated Tau [285]. In contrast, EVs have also been shown to take part in protective mechanisms related to AD. Neuron-derived EVs have been demonstrated to promote clearance of Aβ by microglia and reduce Aβ burden in vitro and in vivo [191, 286]. Moreover, MSC-derived EVs have been shown to decrease both intracellular and extracellular Aβ levels by presenting enzymatically active neprilysin [287]. Thus, EVs seem to play a dual role in AD, with both protective and detrimental actions, which plausibly relates to EV type and cell source, and a shift of the balance between these two modes of action seems to be associated with disease progression. These findings further imply that EVs may represent a potential therapeutic approach for the treatment of neuroinflammatory diseases, which will be discussed in more detail in chapter 1.10 and 4.3.

Furthermore, several CNS-related infections have been demonstrated to be associated with EVs. Prion proteins can be found in EVs and these EVs are infectious [288], which indicates the role of EVs in prion diseases. Viral infections that affect CNS, including HIV, HTLV and Epstein-Barr virus have been shown to exploit EVs to increase their virulence [289]. HIV spread for instance, is aided by EV-mediated transfer of CCR5-receptor to non-immune cells, to facilitate the entrance to these cells [290].

In addition, EVs have been observed to have the capacity to travel from the blood circulation to the CNS, indicating their ability to cross the blood brain-barrier (BBB) [9, 167], which implies a possible communication route between CNS and peripheral tissues. Of note, inflammatory conditions, which are associated with an increased leakiness of BBB, seem to facilitate the entry of EVs into CNS [234]. The route of EV entry to the CNS is however still unclear and, in addition to BBB-transfer, EVs have been suggested to enter the brain at the choroid plexus [291].

Hence, EVs appear to act as an important messenger and regulator of both physiological and pathological processes within CNS as well as to function as a link between CNS and the periphery.

1.9.4 Extracellular Vesicles as Biomarkers

As discussed above, EVs reflect the cellular state in the body, are involved in pathological and physiological processes, and are present in body fluids, such as blood and urine [10]. The function of EVs to deliver biochemical information can thus be utilized as a biomedical tool to gain information, i.e. as biomarkers. The possibility of taking liquid biopsies and analyze EV properties that can be related to pathological conditions has gained a vast interest. There are currently several clinical trials investigating the use of EVs as biomarkers, with indications including neurological disorders, different types of cancers, liver, lung and kidney diseases and diabetes (http://clincaltrials.gov), to mention a few examples.

In conclusion, EVs have been associated with numerous mechanisms in health and disease, which is only partially covered by the discussion above. In fact, there is a vast number of publications describing the impact of EVs on many other physiological processes in the human body, from embryonic development and tissue repair to vascular biology and liver homeostasis, as well as numerous diseases, such as various infections and metabolic disorders. The many processes demonstrated to be influenced by EVs, likely with varying levels of impact, reflect the importance of EV research and furthermore illustrate the great potential of utilizing EVs as biomarkers and the many opportunities to exploit EVs and EV-mechanisms for therapeutic purposes.

1.10 EXTRACELLULAR VESICLES AS THERAPEUTICS

The multiple observations of the impact of EVs on various processes in the body and their ability to transfer bioactive components over biological barriers, as a mean of intercellular communication, suggest that EVs could be harnessed for use as therapeutic agents. Potential therapeutic approaches include utilizing EVs as drug delivery vectors, immune-modulatory and regenerative therapies, and anti-tumor and pathogen vaccines. In fact, the therapeutic potential of EVs are now being explored in several clinical trials (CTs), see Table 1 for completed and current CTs investigating EV-based therapeutics.

Table 1 - NIH registered CTs of EV-based therapeutics (http://clinicaltrials.gov).

Indication	Phase, patients	EV source	EV manipulation	Results (if any)
Melanoma [6]	Phase I, n=15	imDC, autologous	Pulsed with peptides	Safe, well tolerated. 2 stable disease, 1 minor response (res), 1 partial res, 1 mixed res.
Non-small cell lung cancer [7]	Phase I, n=4	imDC, autologous	Pulsed with peptides	Safe, well tolerated. 4 stable disease (where 2 had initial progression).
Non-small cell lung cancer [112]	Phase II, n=22	mDC, autologous	Pulsed with peptides	32% with stable disease, primary endpoint (>50%) not reached.
Colon cancer [111]	Phase I, n=40	Ascites, autologous	+/- GM-CSF induced CEA	Safe, well tolerated. 1 stable disease, 1 minor res (both in CEA group).
Colon cancer [NCT01294072]	Phase I, n=35	Plant-derived	Loaded with curcumin	-
Radiation and chemotherapy induced oral mucositis [NCT01668849]	Phase I, n=60	Grape-derived		-
Type-1 diabetes [NCT02138331]	Phase I, n=20	MSCs, allogenic	unmodified	-
Malignant pleural effusion [NCT01854866]	II, n=30	Tumor-derived	Loaded with chemotherapeutic drugs	-
Ulcers (wound healing) [NCT02565264]	I, n=5	Plasma, autologous	unmodified	-

NIH, National Institute of Health; CTs, clinical trials; imDC, immature dendritic cells; mDC, mature DCs; GM-CSF, granulocyte-macrophage colony-stimulating factor; CEA, carcinoembryonic antigen. Adopted from [89].

1.10.1 Innate therapeutic potential of EVs

EV composition and content differs depending on type and state of the cell source, as discussed above. The ability of EVs to activate or suppress the immune system, depending on the type of EV, can thus be exploited as e.g. vaccines or immune modifiers. Most studies that aim to elucidate the therapeutic potential of EVs have utilized EVs originating from MSCs

and DCs. The rationale for using MSC-derived EVs is based on the knowledge acquired from the field of MSC-based cell therapy with numerous preclinical studies indicating regenerative and immune modulating properties. Following these findings, MSCs have been used in clinical trials for a wide range of indications, including stroke, myocardial diseases, chronic obstructive pulmonary disease, liver failure and inflammatory bowel disease, to mention a few [134, 292]. The initial hypothesis that MSCs would differentiate to and replace damaged tissue was partially abandoned following observations that very few, if any, cells do engraft for longer periods in the host [293, 294]. The disease-modulating activity was instead suggested to be associated with the secretome of MSCs, which was strengthened by observations that MSC conditioned media could convey the beneficial effects of MSCs [295, 296]. Subsequently, various publications are now demonstrating that MSC-derived EVs convey the regenerative and immunomodulatory effects of MSCs in a great number of animal models of various indications, such as stroke [297], kidney failure [245, 298], acute lung injury [299], myocardial infarction [93, 300, 301], sepsis [302] and liver disease [303, 304]. MSC-EVs have furthermore been given to a patient suffering from steroid-resistant GvHD under passionate care [95], with observed improvements. The therapeutic effect of MSC-EVs has been associated with transfer of different bioactive molecules, such as miR-223 for cardioprotection [302], miR-133b for neuroprotection [305], keratinocyte growth factor for alveolar protection during lung injury [299], neprilysin for Aβ degradation [287], and anti-inflammatory TGFβ and human leukocyte antigen-G [95]. A proteomic study of MSC-EVs indicates that the therapeutic effect is mediated by a combination of surface receptors, signaling molecules, cell adhesion proteins and MSC-associated antigens [306]. Immunosuppression properties of umbilical cord-derived MSC-EVs have additionally been attributed to their ability to inhibit the migration of inflammatory cells [307]. In contrast, pancreas-derived MSC-EVs of diabetic mice have shown immunostimulatory properties, which was suggested to be caused by transfer of autoantigens [308, 309].

Similarly, immature DC-derived EVs have been observed to be immunosuppressive, whereas mature DCs EVs are utilized for their immunostimulatory properties [310, 311]. These opposing features have been contributed to different expression levels of MHC I and II, and co-stimulatory molecules such as CD80, CD86, FasL and PD-L1/2 [312, 313]. These observations further indicate the influence of cell source and cellular state on EV-mediated effects and the importance of choosing appropriate cell source and culturing conditions when applying EVs for therapeutic purposes.

1.10.2 Extracellular Vesicles as Vaccines

The initial approach of EV-based therapies utilized the immunostimulatory properties of EVs to generate an anti-tumor affect. Following successful preclinical results, two phase I clinical trials utilizing autologous DC-derived EVs (Dex) pulsed with tumor antigenic peptides for treatment of melanoma and non-small cell lung cancer, respectively, were conducted in 2005 [6, 7] (Table 1). Both demonstrated feasibility and safety of the EV administration that was given weekly over four weeks. The beneficial effects of the therapy were however minor or

non-existing. The later demonstrations of either tolerogenic or immunostimulatory effects of DC-derived EVs depending on DC maturation, as discussed above, led to a subsequent phase II study in France, targeting non-small cell lung cancer [112]. In this CT, the addition of IFN-γ treatment to the DCs to induce DC maturation and increase immune stimulation was used. The anticipated T-cell activation response, observed in preclinical studies, was not seen in the patients. However, an increased NK cell activity was observed in some patients. A Chinese phase I study, conducted 2008, utilized an alternative antitumor immunotherapy approach by isolating EVs from the patients' ascites fluid (Aex) [111]. Patient suffering from colorectal cancer received Aex, with or without adjuvant treatment of granulocyte–macrophage colony-stimulating factor (GM-CSF), which previously had been found to induce an increased antitumor immunity. The treatment seemed safe and was well tolerated, treatment effect was however only observed in 2 patients. These clinical trials, as well as numerous preclinical studies, indicate that immunostimulatory EV-therapy is a feasible anti-cancer approach and that autologous EVs are safe and well-tolerated.

In addition, EV-based vaccines against pathogens, using pathogen antigen-pulsed or EVs derived from infected cells as well as pathogen-derived EVs, have shown promising results [242, 314-317]. Similar to eukaryotic cells, parasites, helminths, fungi and bacteria release EVs [89]. For instance, bacterial outer membrane vesicles (OMVs) are secreted into the extracellular environment and are, similar to eukaryote-derived EVs, enclosed with a lipid bilayer and carry bioactive proteins, lipids, nucleic acids and virulence factors. OMVs are being assessed as vaccines in clinical trials and are believed to offer an advantage over conventional vaccines and be efficient against infectious diseases such as tuberculosis and enteric diseases, which currently lack efficient treatments [318].

Compared to other biological therapeutics, such as cell therapies, EVs cannot divide and multiply, suggesting that EVs are safer from a tumorigenic and infectious perspective. However, there is a risk of co-isolating pathogens, such as viruses that have similar biophysical properties. In addition, EV-mediated transfer of oncogenic molecules to normal cells has been demonstrated, when derived from tumor cells [319].

In summary, preclinical and clinical observations indicate that EV-based vaccines, as anti-tumor or anti-pathogen treatment, are feasible, well tolerated and render a desired immunostimulatory therapeutic effect. However, the risk of contaminating pathogens and EV-mediated immune tolerance and potential tumor promoting actions need to be considered and controlled for in future clinical trials.

1.10.3 Extracellular Vesicles as Delivery Agents

Owing to their ability to transfer molecular information between cells and tissues, EVs are being explored as natural delivery vectors for different cargos, such as small molecules without suitable pharmaceutical properties and RNA-species, which often have been shown

to have potent action once in contact with the target, but suffer from issues such as low cellular uptake, suboptimal pharmacokinetics, off-target toxicity or stability issues.

Applying EVs as drug delivery systems (DDS) have for instance been explored for a variety of different small molecules, including curcumin, doxorubicin and paclitaxel [320]. Preclinical animal studies indicate enhanced potency of the small molecule treatment with improved pharmacokinetic profiles including improved brain delivery and tumor penetrance, as well as efficient cargo delivery and retention in tumor cells, compared to other vehicles, such as liposomes and polymer-based synthetic nanoparticles [320]. Following these findings, clinical trials with curcumin or chemotherapeutic drug-loaded EVs are being conducted, Table 1.

It is important to note that loading of cargo into EVs often require manipulation of the EVs or the parental cells. The techniques of loading cargo into EVs can be divided into two different approaches; exogenous loading, i.e. loading of isolated EVs and endogenous loading, i.e. loading during EV biogenesis, see Figure 3.

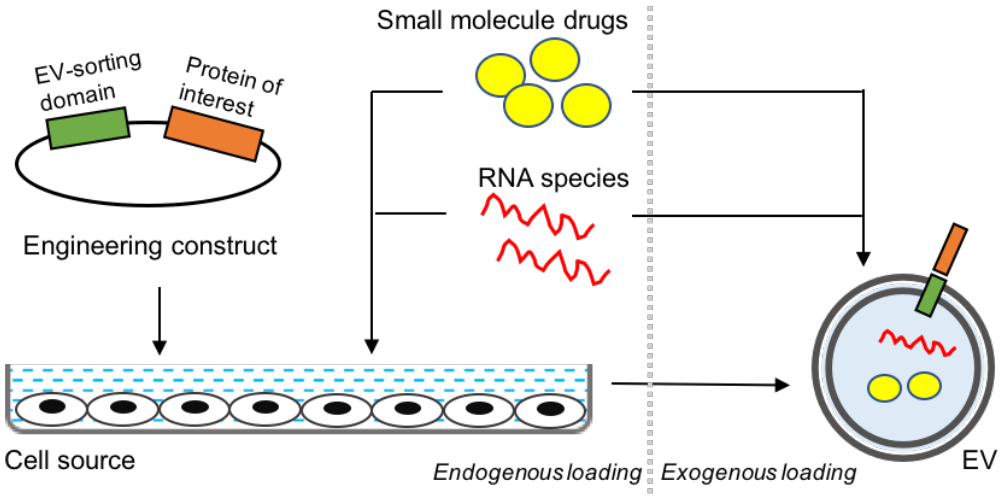

Figure 3 – Illustration of EV loading strategies.

1.10.3.1 Exogenous EV loading

Various techniques have been explored to load isolated EVs with a therapeutic cargo. Incubation of EVs with the anti-inflammatory agent curcumin improved the bioavailability and anti-inflammatory effect in a mouse model of inflammation [210]. Similarly, incubating EVs and the immunosuppressive miR-150 were shown to generate a miRNA-EV association that was functionally active [321]. An interesting improvement in incubation-mediated loading was explored in a recent publication using hydrophobically modified siRNA for huntingtin mRNA silencing, with demonstrated efficient effect in vitro and in vivo [322]. Another approach for EV loading utilizes electroporation to generate transient membrane pores to facilitate entrance of RNA species [9] or small molecules [323]. Permeabilization,

for loading of cargo into EVs, such as therapeutic proteins, has also been demonstrated by saponin, freeze–thaw cycles, sonication, and extrusion [324]. In addition, commercial cationic liposomes have been utilized for EV transfection. However, this was found to be inapplicable due to the inability to separate EVs and micelles and electroporation was suggested to be a superior technique [325]. The different exogenous loading techniques have pros and cons, and whether the cargo is loaded into or onto, only associated to EVs, or just co-isolated, is often debatable. Furthermore, the loading efficiency seems to be quite varying. For instance, electroporation has been suggested to generate as high as 90% loading in some publications [325], whereas others have reported very poor loading efficiency [326], which has been explained by the formation of siRNA aggregates during electroporation that can be misinterpreted as siRNA-loaded EVs. Nevertheless, numerous publications have demonstrated successful cargo loading by electroporation and these differences may be due to different protocol conditions.

1.10.3.2 Endogenous EV loading

In contrast to exogenous loading, endogenous loading implies that cargo is introduced into the producer cell to exploit the cellular machinery for cargo sorting into EVs. Similar techniques as utilized for direct EV loading, including incubation [327] and transfection [213, 328, 329] have been used to load small RNA and small molecules endogenously into EVs via loading into producer cells. The regulated sorting of RNA into EVs as discussed above, will most likely result in varying EV loading efficiency depending on the RNA species. Furthermore, there is a risk that the parental cell will be affected by the RNA intended for EV sorting, which may subsequently lead to unwanted alterations of the produced EVs.

1.10.4 Bioengineered Extracellular Vesicles

In addition to loading EVs with a therapeutic cargo, EVs can be further engineered by manipulating the parental cell to produce EVs with a desired trait. The pioneering publication by Alvarez-Erviti et. al. utilized EVs for brain targeted delivery of siRNA [9]. To enhance the targeting properties of the EVs, a peptide obtained from the rabies viral glycoprotein (RVG) was introduced as a targeting peptide on the EV surface by transfecting the parental cells with a plasmid encoding Lamp2b, an EV membrane protein, fused to RVG. The parental cell was thus engineered to produce EVs with the desired protein, which was sorted onto EVs endogenously by the fusion to an EV sorting domain. A subsequent publication demonstrated increased tumor targeting and antitumor effects by engineered EVs loaded with doxorubicin [323]. The EV source cell was engineered to express Lamp2b fused to αv integrin-specific iRGD peptide, which previously had been demonstrated to have efficient tumor targeting properties [330]. Another study utilized the transmembrane domain of PDGF-receptor fusion to a ligand of epidermal growth factor receptor (EGFR) for the production of engineered EVs that displayed increased efficiency of antitumor miRNA delivery to breast cancer cells [213]. Similarly, EV display of anti-EGFR nanobodies fused with glycosylphosphatidylinositol (GPI)-anchor peptides, for sorting to GPI-rich lipid rafts in EV membranes, was demonstrated to generate nanobodies on EVs with increased binding to EGFR-positive tumor

cells [331]. Similar engineering approaches have furthermore been utilized to display reporter moieties, such as gLuc, on EVs, as aforementioned. Moreover, Sterzenbach et. al. recently showed that a protein of interest could be sorted into EVs endogenously by exploiting the evolutionarily conserved late-domain (L-domain) pathway [332]. The authors tagged Cre recombinase with a WW tag (WW-Cre) that was recognized by the L-domain containing protein Ndfip1, which led to sorting into EVs. Functional delivery of WW-Cre by EVs was demonstrated by the ability of inducing recombination in floxed reporter cells in vitro and in vivo. In addition, engineered hybrid EVs are emerging as an alternative strategy for improved delivery. Adeno-associated virus was incorporated into HEK293T-derived EVs to generate "vexosomes", which were demonstrated to improve transduction efficiency and exhibit lower immunogenicity as compared to the free viral vector [333]. Similarly, EVs have been fused with synthetic liposomes with promising results [334]. A novel strategy of hybrid EVs was recently presented by Votteler et. al., where they introduce the concept of enveloped protein nanocages (EPNs) [335]. By a variety of synthetic proteins, EPNs, similar to EVs, utilize membrane binding, self-assembly, and ESCRT machinery proteins for the biogenesis. The EPNs were able to efficiently delivered their content into the cytoplasm of target cells.

In conclusion, EVs are emerging as highly potent therapeutic entities with innate properties that can be harnessed as cell free cell-based therapies for immunomodulation. These properties can furthermore be combined with loading of bioactive drugs for dual therapeutic actions and exploitation of EVs delivery capacities as a natural vector and DDS. In addition, bioengineering of EVs offers yet another layer to equip EVs with desired properties, such as targeting moieties.

2 AIMS

Building on the knowledge acquired in the field, which covers much details of intercellular communication and cargo delivery capacities of EVs, the overall objective of this thesis is to investigate the therapeutic potential of EVs. In order to further elucidate this, it was important to develop an increased understanding of the biodistribution and factors impacting tissue accumulation of EVs. In addition, the ability of EVs to deliver functional nucleic acids and active proteins in pursuance of improved treatments in disease settings needed further investigation. The specific foci of the overall objective and this thesis are stated as the aims of the respective paper:

2.1 PAPER I

- To evaluate the potential of engineered EVs to improve brain targeting by displaying the rabies viral glycoprotein (RVG) peptide on EVs (RVG-EVs).
- Investigate RVG-EVs potential to convey siRNA against alpha-synuclein (α-Syn).
- Examine siRNA loaded RVG-EVs potential, in a Parkinson's transgenic disease model, to reduce α-Syn and its aggregates in the brains of mice.

2.2 PAPER II

- To find and evaluate a suitable method for biodistribution studies.
- Investigate possible homing capacities and variations in tissue distribution of EVs from different cell sources.
- Explore the influence of a targeting moiety, administration routes and possible dose-dependent variations on the biodistribution of EVs.

2.3 PAPER III

- To generate and optimize EVs that are engineered to display receptors to sequester cytokines and decoy their immunostimulating actions.
- To evaluate the potential of these decoy EVs to act as anti-inflammatory agents in vitro and in vivo in mouse models of systemic inflammation and neuroinflammation.

3 METHODOLOGICAL CONSIDERATIONS

Thorough descriptions of the methods employed in this thesis are presented in the respective paper. The presentation below is thus intended as a brief outline and discussion of the most important methods.

3.1 CELL SOURCES

In paper I, the EVs were isolated from murine DCs that were obtained from bone marrow (BM) of the same mouse strain (C57BL/6J) as the mice used in the subsequent in vivo experiments, to reduce the potential risk of an immune rejection response. The cells were transfected with RVG-Lamp2b or mock plasmid using TransIT LT1 transfection reagent. 24 hours prior to EV harvest, the medium was changed to EV-depleted culture medium. To investigate the potential influence of cell source on EV biodistribution, several different cell types were used in paper II. In addition to the DCs described above, EVs were obtained from human BM-derived MSCs that had been cultured in serum-free media 48 hours prior to EV harvest. The same culturing conditions, 48 hours before EV harvest, was also applied on the four different cell lines included in the comparison: Human embryonic kidney (HEK293T) cells, rat oligodendrocytes (OLN-93), mouse myoblast (C2C12), and mouse melanoma cells (B16-F10). The none-mouse derived cells were included to evaluate possible species-dependent influence on EV biodistribution in mice. Building on the findings from paper I and II, as well as reports from others [134, 336, 337], HEK293T (for in vitro evaluation) and immortalized BM-derived MSCs (for in vitro and in vivo evaluation) were utilized in paper III.

3.2 EV ISOLATION

The isolation procedure was optimized between the studies. The initial steps were however similar with harvest of the cell culture conditioned media (CM) before low speed serial centrifugation at 300-500 x *g* followed by a 2,000 x *g* spin to remove floating cells and cell debris. The supernatant was then filtered through a 0.2 μm filter to enrich for smaller EVs. In paper I and II, the EVs were then pelleted down by an UC step of about 100,000 x *g* followed by a wash spin at 100,000 x *g* after re-suspending the pellet, to increase the purity of the EVs. The final pellet was then re-suspended to desired volume. Based on reports by our group and others [87-89, 106], of the risk of UC-associated contaminations, EV aggregations and negative impact on EVs integrity associated with UC, an optimized isolation procedure was employed in paper III [106]. The 0.2 μm filtered CM was then run through a hollow fiber filter using a TFF system to enrich for and concentrate the EVs. The pre-concentrated CM was subsequently loaded onto bind-elute and size exclusion chromatography columns (CaptoCore 700) to reduce non-EV-associated proteins. The EV sample was subsequently concentrated using a 10 kDa molecular weight cut-off filter to desired volume.

3.3 NANOPARTICLE TRACKING ANALYSIS

As part of characterizing the isolated EVs, NTA was utilized in all papers to measure the size and to quantify the EVs in the samples. NTA is based on the movement of nanometer-sized particles in a solution, known as Brownian motion. The Strokes-Einstein equation is employed to calculate the size of the particles. NanoSight (NS500 nanoparticle analyzer) was employed for NTA in all papers. The instrument is equipped with a laser that that gives rise to light scattering as the beam passes through the sample and hits the particles. The light scatter is visualized and recorded via a CCD camera. In addition, the utilized NS500 is equipped with a 488nm laser and a 500nm long pass filter, which can be turned on for fluorescent readings or off for light scatter measurements. An NTA software is then used to pinpoint the particles and calculate their concentration and size. The instrument can be used for particles ranging from 30-1,000 nm in diameter [338, 339]. For all recordings, samples were diluted in PBS to achieve a particle count of between 2 x 10^8 and 2 x 10^9 per ml, for accurate detection of the software. The camera focus was adjusted to make the particles appear as sharp dots. The script control function of the software was used to run the sample and record the light scattering, and the batch process function was used to analyze the sample-recordings.

3.4 FLOW CYTOMETRY

The surface expression of EVs was assessed in paper III by bead based multiplex flow cytometry analysis. The MACSPlex Exosome Kit^{TM} that was utilized has been stated to allow for qualitative and semiquantitative analysis of exosomal surface epitopes by flow cytometry [120]. This method utilizes fluorescently labelled antibody-coated capture beads that are incubated with isolated EVs. The used MACSPlex exosome Kit^{TM} includes 39 different capture beads targeting human EV surface epitopes (or control epitopes). The pan detection reagents with APC-conjugated anti-CD9, anti-CD63 and anti-CD81 antibodies were used to detect EVs captured by respective bead subsets. In addition, to detect the expression of the decoy receptors IL6ST and TNFR1 on respective EVs, APC-conjugated rat-anti-mouse gp130 (IL6ST) antibodies or AlexaFluor647-conjugated mouse-anti-human CD120a (TNFR1) antibodies, were used as detection antibodies. Median fluorescence intensities for all bead populations were background-corrected by subtracting background/unspecific median. All samples were analyzed with a MACSQuant 10 instrument with at least 20,000 cells or 10,000 beads recorded per sample.

3.5 TRANSMISSION ELECTRON MICROSCOPY

TEM was utilized for EV characterization in paper I and III. EVs were added onto glow discharged EM grids, which were stained with 2% uranyl acetate to visualize the vesicles. In paper III, immuno-EM was performed by incubating the engineered EVs with blocking solution, followed by incubation with primary antibodies against respective decoy receptor displayed on the EVs. Gold nanoparticles conjugated to protein A or secondary antibody were then added and incubated with the mixture, which was then transferred to glow

discharged EM grids that were stained and dried before being visualized with a transmission electron microscope.

3.6 DIR LABELLING OF EVS

In paper I and II, EVs were labelled with the fluorescent lipophilic dye DiR (1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanineiodide). DiR was chosen due to its near-infrared fluorescence spectrum, which offers high optical tissue penetrance. DiR is furthermore suitable owing to its properties of low fluorescence when unbound and highly fluorescent when incorporated into membranes [230]. The EVs were labelled by incubating the filtered CM with 1 μM DiR prior to the UC-based isolation, including a washing step. The DiR staining of EVs was evaluated with sucrose gradient to confirm that no free dye was remaining after the isolation and that the DiR was associated with the EVs during density separation. In addition, unconditioned media was incubated with DiR, ultra-centrifuged with a washing step and re-suspended, in the same fashion as the labelled EVs were isolated, to serve as a control for injection and tracing of labelled EVs in mice.

3.7 BIOENGINEERING OF EVs

In all three papers, chimeric proteins were utilized to display a protein of interest on EVs. The fusion constructs were made to encode for an EV sorting domain (such as Lamp2b, CD63 or syntenin) and a protein of interest, such as the fluorescent protein eGFP, the brain targeting peptide RVG, or the different variants of the cytokine receptors IL-6 signal transducer (IL6ST) and the TNF-receptor (TNFR). The respective parental cells were either transiently transfected using polyethylenimine (PEI) or TransIT LT1 transfection reagent, or transduced with a lentiviral vector encoding the respective construct. To achieve stable expression of the vectors, infected cells were selected with puromycin. The engineered EVs were subsequently isolated as described above. The presence of the EV-displayed chimeric protein was evaluated in all papers by western blot, with the addition of bead based multiplex flow cytometry analysis in paper III as described above. The function of the EV-displayed chimeric protein was evaluated depending on its intended effect. GFP-displayed EVs were analyzed with NanoSight, with and without the long pass filter for fluorescent or light scatter measurements, respectively. Functional delivery of siRNA by RVG displayed EVs to neuroblastoma cells (SH-SY5Y) were assessed in vitro. The cytokine decoy potential of the displayed cytokine receptors was evaluated in reporter cells as described in paper III. Briefly, NF-κB reporter (Luc)-HEK293 cells were treated with TNFα and EVs displaying TNFR or control EVs, and the luminescence was measured after six hours. Similarly, HEK-Blue IL-6 cells were treated with either IL-6 or IL-6/IL-6-receptor complex and EVs displaying IL6ST or control EVs, and the SEAP levels were quantified after six hours. The in vivo evaluation of the surface expressed chimeric proteins is presented below.

3.8 IN VIVO TECHNIQUES AND MOUSE DISEASE MODELS

3.8.1 Parkinson's Disease Mouse Model

The potential of utilizing brain targeted EVs as a mean for siRNA-mediated decrease of α-Syn levels in the brain was examined as a therapeutic approach for Parkinson's disease. The siRNA loaded EVs were assessed in wildtype mice and in a transgenic (Tg) mouse model that demonstrates α-Syn expression throughout the brain. Briefly, α-Syn siRNA was electroporated into RVG-EVs that had previously been isolated from engineered DCs. The RVG-EVs containing siRNAs were pelleted by UC, re-suspended and injected intravenously into wildtype or Tg mice. Brains were dissected 3 and 7 days after injection and analyzed for α-Syn mRNA and protein levels by quantitative PCR and western blot, respectively. The Tg mouse model was established by cloning the human S129D α-Syn cDNA with a C-terminal HA tag into the pPrP vector containing the promoter and exons 1 and 2 of the mouse prion protein gene. The transgene fragment was isolated from the plasmid vector and microinjected into pronuclei of one-cell eggs obtained from C57BL/6 × CBA F1 donors. The presence of α-Syn in the brain of the Tg mice was evaluated by immunohistochemical detection of S129D α-Syn HA expression using an anti-HA antibody. Tissue extracts of different brain regions were further assessed by WB, with or without high salt (HS), Triton X-100, and urea to indicate the presence of α-Syn aggregates. In addition, brain sections were stained with the green fluorescent dye Thioflavin S (ThioS), which stains amyloid deposits, to further evaluate the effect of the injected siRNA loaded RVG-EVs in the Tg mouse model.

3.8.2 Tissue Distribution of injected EVs

In order to assess the tissue distribution, EVs from different cell types were isolated and labelled with DiR as described above. Mice were intravenously, intraperitoneally, or subcutaneously injected with DiR-labelled EVs. The biodistribution of the EVs was analyzed by fluorescent measurements of the whole mouse as well as harvested organs, at different time points, using the In Vivo Imaging System (IVIS). Perfusion of the blood vessels with PBS was conducted to confirm that accumulation of labelled EVs in the different organs. Initially, a dose comparison study was conducted with different EV doses, based on NTA quantification. The dose of 1.0×10^{10} particles per gram body weight was found to be suitable and used in the subsequent experiments. In addition, immunohistochemistry of organs from mice injected with CD63-eGFP positive EVs was conducted to analyze the presence of EVs without the use of DiR, to validate the experimental set-up.

3.8.3 LPS Induced Systemic Inflammation

The engineered EVs used in paper III (termed decoy EVs), display cytokine receptors targeting the inflammatory TNFα and IL-6 pathways. To evaluate the anti-inflammatory potential of these EVs in vivo, a mouse model of systemic inflammation was used. Inflammation in the mice is induced by an intraperitoneal injection of lipopolysaccharide

(LPS) [340]. The EVs were subsequently injected via the tail vein and animals were observed and weighed daily after induction.

3.8.4 Experimental Autoimmune Encephalitis

To evaluate the decoy EVs potential to treat neuroinflammation, a multiple sclerosis mouse model, known as experimental autoimmune encephalitis (EAE), was utilized. EAE is induced by immunization with an emulsion of MOG35-55 in complete Freund's adjuvant (CFA), followed by administration of pertussis toxin. Mice were subcutaneously injected with EVs and the disease progression was assessed by daily weight measurements and scored using the typical EAE-scoring system, which rates the degree of paralysis [341].

4 RESULT AND DISCUSSION

The papers in this thesis share the general objective of investigating the potential of EVs to be employed for therapeutic purposes. The respective paper explores different aspects of this in order to, at least partially, increase our understanding of the possibilities of EVs.

4.1 PAPER I

Based on previous findings showing that EVs can be loaded with siRNA and be targeted to the brain using the brain targeting peptide RVG [9], it was hypothesized that siRNA against α-Syn could be delivered via brain targeted EVs to reduce the α-Syn pathological conditions associated with Parkinson's disease (PD). First, three different siRNAs against α-Syn were evaluated in neuroblastoma SH-SY5Y cells over-expressing mouse α-Syn. The siRNA was either introduced to the cells by Hiperfect transfection reagent or via EVs that had been engineered to express RVG (Lamp2b-RVG), which had been electroporated with the different siRNAs. The results indicated greater reduction in α-Syn mRNA and protein levels when the siRNA was delivered via EVs with an 80% mRNA and 85% protein reduction of α-Syn for the best siRNA candidate. Hence, clearly indicating that targeted EVs efficiently deliver functional siRNA to the receiving cells and that the approach is feasible. Following this, the brain distribution of intravenously injected RVG-coated EVs were assessed in wildtype animals using the fluorescent near infrared DiR, as discussed above, displaying a widespread distribution in the brain. Next, wildtype animals were injected with siRNA loaded RVG-coated EVs and the brains were analyzed 3 or 7 days post injection, with significant decrease of α-Syn mRNA and protein levels.

To further validate the therapeutic potential of RVG-coated EV delivery of siRNA to treat α-Syn aggregations, which are believed to be associated with PD, a transgenic (Tg) mouse model expressing the phosphomimic human S129D α-Syn was generated. The presence of α-Syn in the brain of the Tg mice was evaluated by immunohistochemical detection of S129D α-Syn HA expression using an anti-HA antibody, displaying α-Syn expression throughout the brain. Tissue extracts of different brain regions were further assessed by WB, with or without high salt (HS), Triton X-100, and urea to indicate the presence of α-Syn aggregates. All analyzed brain regions stained positive for Triton X-100 insoluble a α-Syn. However, it was more prominent in the cortex, cerebellum, and striatum, which is in line with previously demonstrated increased aggregation associated with S129 phosphorylated α-Syn in Lewy bodies in PD [342]. Phenotypic changes in activity and motor skills were not observed. However, Triton X-100 insoluble α-Syn was detected in the brain of mice from 3 months and increased with age until the endpoint of 24 months, indicating characteristic pathological changes associated with PD and the Tg mouse model was thus believed to be suitable for further evaluation of the siRNA loaded RVG-EVs.

Next, siRNA loaded RVG-displayed EVs were assessed in SH-SY5Y cells expressing human S129D α-Syn-HA, displaying a dose-response correlated reduction of α-Syn by western blot.

Following this, siRNA against α-Syn or control siRNA were loaded into RVG-EVs, which were subsequently injected intravenously to 5 months old Tg mice. Again, a significant decrease of α-Syn RNA and protein levels was seen in the mice treated with RVG-EVs carrying siRNA against α-Syn, whereas no effect was seen with the control siRNA. In addition, brain sections were stained with ThioS, which stains amyloid deposits. Tg mice treated with RVG-EVs carrying siRNA against α-Syn displayed an 84% decreased staining in the midbrain seven days after treatment, as compared to untreated Tg mice, further supporting the therapeutic effect of the EV delivered siRNA. Furthermore, immunohistochemistry of S129D α-Syn-HA in dopaminergic neurons in the substantia nigra, which is pathologically relevant in PD, demonstrated reduced α-Syn-HA puncta in the Tg mice treated with RVG-EVs carrying siRNA against α-Syn, compared to control.

In conclusion, the findings demonstrate that brain targeted EVs can be employed as efficient vectors for siRNA therapy against α-Syn pathological conditions, such as PD.

4.2 PAPER II

In pursuance of using EVs for therapeutic purposes, the fate of the injected EVs must be understood. We thus set out to elucidate the biodistribution of exogenous EVs and the potential impact of administration route, cell source, dosing and targeting, on the tissue distribution of EVs in vivo.

Following the findings from paper I and based on reports by others [213], the fluorescent lipophilic dye DiR was evaluated as an EV labeling tool. The rational was based on the properties of DiR, including exhibiting high fluorescence when incorporated into membranes, the near-infrared fluorescence spectrum, which offers high optical tissue penetrance, and the ability to homogeneously label the EVs [230]. There are however also potential limitations of these lipophilic dyes, including the risk of having excess or unbound dye, which may form micelles that can be mistaken as EVs. Risk of microenvironment contamination, i.e. the dye being transferred to a neighboring cell membrane, has also been reported [231]. Thorough assessment of the labelling technique was thus conducted. EVs were labelled and isolated by UC, as explained above. The stained EVs or free DiR were then loaded onto a sucrose gradient for density based separation. DiR stained EVs were visible and fluoresced at the expected density of small EVs. Western blot analysis further confirmed the presence of Alix in the same fraction, indicating that DiR thus binds to EVs. As expected, free DiR displayed a lower buoyant density and lower fluorescent values, indicating that it was not bound to any membrane. To further rule out the risk of monitoring free dye, unconditioned medium without EVs was incubated with DiR and subjected to UC in the same fashion as when labelling EVs. Following injection of the UC isolated free dye, ex vivo assessment of the organs displayed negligible fluorescence, thus indicating that any excess dye would be lost during the EV isolation. DiR labelled EVs on the other hand, displayed significant fluorescence, with the highest levels originating from liver and spleen. To further confirm

that EVs, and not merely the dye, were being traced, CD63-eGFP positive EVs were injected in mice. Immunohistochemistry against eGFP on organs harvested 24 hours after injection revealed positive staining in the parenchyma of the liver and spleen, which was in line with the findings of DiR labelled EVs. Taken together, this thus affirmed that DiR labeling was a suitable method for in vivo tracing of EVs.

EVs were further characterized by NTA, western blot and TEM, which displayed presence of EV markers and morphological properties of EVs. Next, a dose comparison study was conducted with escalading doses of DiR labelled EVs, which indicated a difference in tissue distribution with a relative decrease in the liver uptake with increasing doses. This was interpreted as a possible saturation of the MPS. In order to avoid the risk of saturating the MPS, the intermediate dose of 1.0×10^{10} particles per gram body weight was used in the subsequent experiments.

Next, a time laps study was undertaken to investigate the kinetics of the EV distribution. The overall EV biodistribution profile remained largely unchanged during the first 24 hours, although an initial increased pulmonary accumulation was noted at 5 minutes after injection, which was then stabilized at relatively lower levels. This might be due to the lung capillaries being the first capillary bed being subject to EV exposure following intravenous injection, where potentially aggregated EVs are trapped immediately, whereas the slower accumulation in the other tissues may reflect the EV uptake from the circulation. In fact, the absolute fluorescence levels increased in the organs over time, indicating a continues uptake from the circulation. At the later 48-hour time point, greater changes in the relative tissue distribution were detected. This may reflect redistribution or a later uptake phase of remaining EVs, or an artefact due to the long half-life of the dye. Due to the risk of unspecific signals after 24 hours, organs were analyzed at 24 hours in the subsequent experiments.

EVs are commonly injected systemically to assess the effect in vivo. However, different routes of injections can be employed for this. To assess whether the route of injection influenced the distribution pattern, mice were injected intravenously (i.v), intraperitoneally (i.p.) or subcutaneously (s.c). Interestingly, i.p. and s.c. injections showed a significantly lower EV accumulation in liver and spleen compared to i.v. injections. In addition, total fluorescence was significantly lower in s.c. treated mice, indicating a retention of the EVs around the adipose tissue and a possible slow release mechanism. These results show that the route of injection influences tissue distribution of EVs and suggest that the injection site employed could be adopted to reflect the most preferred biodistribution and/or pharmacokinetic profile of a given therapeutic approach.

To investigate potential intrinsic tropisms of EVs, three different mouse cell sources; a muscle cell line (C2C12), a melanoma cell line (B16F10), and primary immature bone marrow-derived DCs, two human cell sources (HEK293T and primary human MSCs) and rat-derived oligodendrocytes (OLN-93) were utilized as EV sources. The EVs were DiR labelled, i.v. injected and the tissue accumulation was evaluated at 24 hours after injection. A general distribution pattern, with highest accumulation of EVs in liver, followed by spleen

and lungs, was displayed independently of the cell source. However, significant differences of the relative EV distribution to liver, spleen and lung were seen between the different cell sources. The most significant difference in biodistribution was observed from DC-derived EVs that accumulated to a higher extent in the spleen and less in the liver, as compared to the other cell sources. This difference might be explained by a natural tropism that could be speculated to be governed by cell source dependent repertoire of surface receptors and extracellular matrix binding proteins, which is in line with the findings of homing mechanisms of EVs related to the display of integrins, as discussed above. This highlights the importance of studies on the intercellular communication of EVs, since further investigation of EV-tropisms may allow for researchers to hijack these mechanisms and employ them for targeted EV therapies.

Furthermore, based on the reports and clinical trials of utilizing EVs as an antitumor treatment, we set out to investigate the distribution of untargeted EVs in tumor-bearing mice. Interestingly, 3% of the total tissue fluorescence originated from the tumor of melanoma bearing mice. The accumulation of untargeted EVs in the tumor is most probably related to the leaky vasculature, which allows entry of EVs. The tumor penetrance can probably be further optimized, by e.g tumor targeting moieties. These findings do however support the notion that EVs can be utilized as delivery modalities in antitumor therapies.

Finally, the impact of surface displayed brain targeting moieties on the tissue distribution was investigated. Based on the findings in paper I, and by others [9] utilizing RVG for brain targeting of EVs, DCs were engineered to generate Lamp2b-RVG positive EVs. Interestingly, the general distribution pattern was not affected by the addition of RVG. DC-derived EVs, with or without RVG, displayed a similar distribution with the greatest accumulation in liver and spleen. However, the accumulation in brain was 2-fold higher for the targeted EVs. The small, yet significant, changes in the biodistribution of RVG-EVs highlights both the sensitivity of the DiR labelling method in detecting subtle differences and that RVG as a targeting moiety does not affect the overall tissue distribution of EVs, but still increases the accumulation in the targeted organ.

To conclude, DiR labelling of EV is a suitable method for biodistribution studies and allows for in vivo EV tracing with high sensitivity. EVs display a general distribution pattern in vivo with high accumulation in liver and spleen. This biodistribution profile of EVs is however, to a certain degree, influenced by the administration route, cell source, dosing and targeting.

4.3 PAPER III

In this project, the bioengineering technology utilized in paper I and II was adapted to produce EVs that display therapeutic proteins. A fusion construct, with an EV sorting domain and a non-signalling cytokine receptor, was introduced to the parental cell to produce EVs that can sequester cytokines, termed decoy EVs. The objective was to exploit the capacity of

EVs to convey the immunomodulatory effect of MSCs, to utilize the efficiency of EVs as delivery vector and combine this with receptors, devoid of intracellular signalling domains, against inflammatory cytokines. Hence, generating an anti-inflammatory therapy that combines the beneficial effects of stem cell therapy, EVs as delivery agents and cytokine targeted biologics. The central inflammatory pathways of TNFα and IL-6 trans-signalling was chosen as targets for the therapy by equipping the EVs with either TNF-receptor 1 (TNFR1) that binds TNFα and IL-6 signal transducers (IL6ST) that binds the IL-6/IL-6-receptor (IL6R) complex, both made to be signal-incompetent. First, several fusion protein constructs, with different EV sorting domains combined with either TNFR1 or IL6ST were created. The constructs were transiently transfected into HEK293T cells and the derived EVs were assessed in regard to expression level of respective decoy (TNFR and IL6ST) (by western blot, data not shown) and their efficiency to inhibit cytokine dependent reactions in reporter cells. For TNFR1 decoy EVs the activity of the downstream transcription factor NF-κB was measured in HEK293T cells stably expressing NF-κB-luciferase reporter gene, which was stimulated by TNFα. For IL6ST decoy EVs the activity of the downstream transcription factor STAT3 was monitored using HEK293T cells stably expressing a STAT3-inducible secreted alkaline phosphatase (SEAP) reporter gene, stimulated by IL-6 or IL-6/IL-6R complex. The results indicated that decoy EVs expressing TNFR1 or IL6ST were most efficient when fused to the EV sorting domain syntentin, which is one of the main components involved in the biogenesis of exosomes [30]. The potency of the TNFR-1 decoy EVs was increased 10-fold by the identification and subsequent removal of a matrix metalloprotease (MMP) cleavage site. The decoy EVs were further optimized by introducing a multimerization domain; dimerization domain for IL6ST (IL6ST-di) and trimerization domain for TNFR1 (TNFR-tr), mimicking the natural state of the respective receptor. In order to evaluate the effect of the decoy EVs in vivo they were assessed in an LPS-induced systemic inflammation mouse model. Mice injected with HEK293T-derived decoy EVs, following LPS challenge, displayed a significantly improved survival with 100% survival up to 60 hours compared to 0% survival of mock treated mice.

In contrast to HEK293T, MSCs constitute a more attractive and clinically relevant cell source for therapeutic EVs, as discussed above. Thus, MSCs were transduced with the optimized decoy construct for stable production of MSC-derived decoy EVs. Characterization of isolated EVs confirmed the surface expression of common EV markers as well as the respective decoy proteins by western blot and bead-based flow cytometry, as described above. The EVs further demonstrated a characteristic size range of small EVs, with a peak of approximately 100 nm as measured by NTA. Immuno-EM further validated the presence of EVs and the respective decoy receptor. The functional assessment of the decoy EVs demonstrated a dose-dependent inhibition of respective cytokine pathway in the reporter cells of NF-κB and STAT3 activity. LPS-induced mice, treated with MSC-derived decoy EVs, furthermore displayed a significantly reduced weight loss, which was further improved by combinational treatment with both decoy EVs, as compared to unmodified MSC-EVs.

Leaning on the findings of EVs ability to overcome biological barriers and penetrate into CNS, we hypothesized that decoy EVs could have a therapeutic effect in neuroinflammation. To explore this, the mouse model experimental autoimmune encephalitis (EAE), which mimics multiple sclerosis in humans, was employed. The fact that EAE is a progressive model with symptoms evolving over time and based on the biodistribution studies in paper II, indicating a slow release mechanism of EVs injected s.c., this route of administration was chosen. Surprisingly, treatment with unmodified MSC EVs did not affect the disease progression compared to mock treatment, which is in contrast to previous findings. This might be due to non-favourable culturing conditions or that the specific MSCs employed for EV production were not optimal for immunomodulation. [134]. In contrast, the MSC-decoy EVs (IL6ST-di, TNFR1-tr and combinational treatment) as well as the positive control (anti-IL6 antibody, Tocilizumab), significantly ameliorates the EAE symptoms at the end point. TNFR1-tr and combinational decoy EV treatment furthermore displayed a significantly improved bodyweight compared to mock treatment. In addition, the levels of pro-inflammatory cytokines (TNFα, IL-6 & IL-17a) and chemokines (Cxcl1) in cerebrospinal fluid were significantly reduced by combinational decoy EV treatment (TNFR1-tr and IL6ST-di) compared to mock treated EAE mice at the end point.

In conclusion, by utilizing the sorting mechanisms in the biogenesis of exosomes, parental cell sources can be engineered to produce EVs that display functional protein therapeutics. Here, EVs are engineered to express signal incompetent receptors that sequester the pro-inflammatory TNFα or IL-6/IL-6R complex. These decoy EVs significantly ameliorate systemic inflammation and neuroinflammation in vivo. In addition, this novel platform has the potential to be implemented in several disorders where detrimental signal molecules could be sequestered. By combining the beneficial effects of stem cell therapies and protein therapeutics with a natural delivery vehicle that can overcome biological barriers, engineered EVs have great potential to be next generation therapeutics.

5 FUTURE PERSPECTIVES

The intense research within the field of EVs over the last decades has led to an increased understanding of EVs' biogenesis, content and biological function. There are, however, still hurdles that need to be managed in many aspects of EV research for optimizing and utilizing EVs as therapies, which is illustrated in Figure 4.

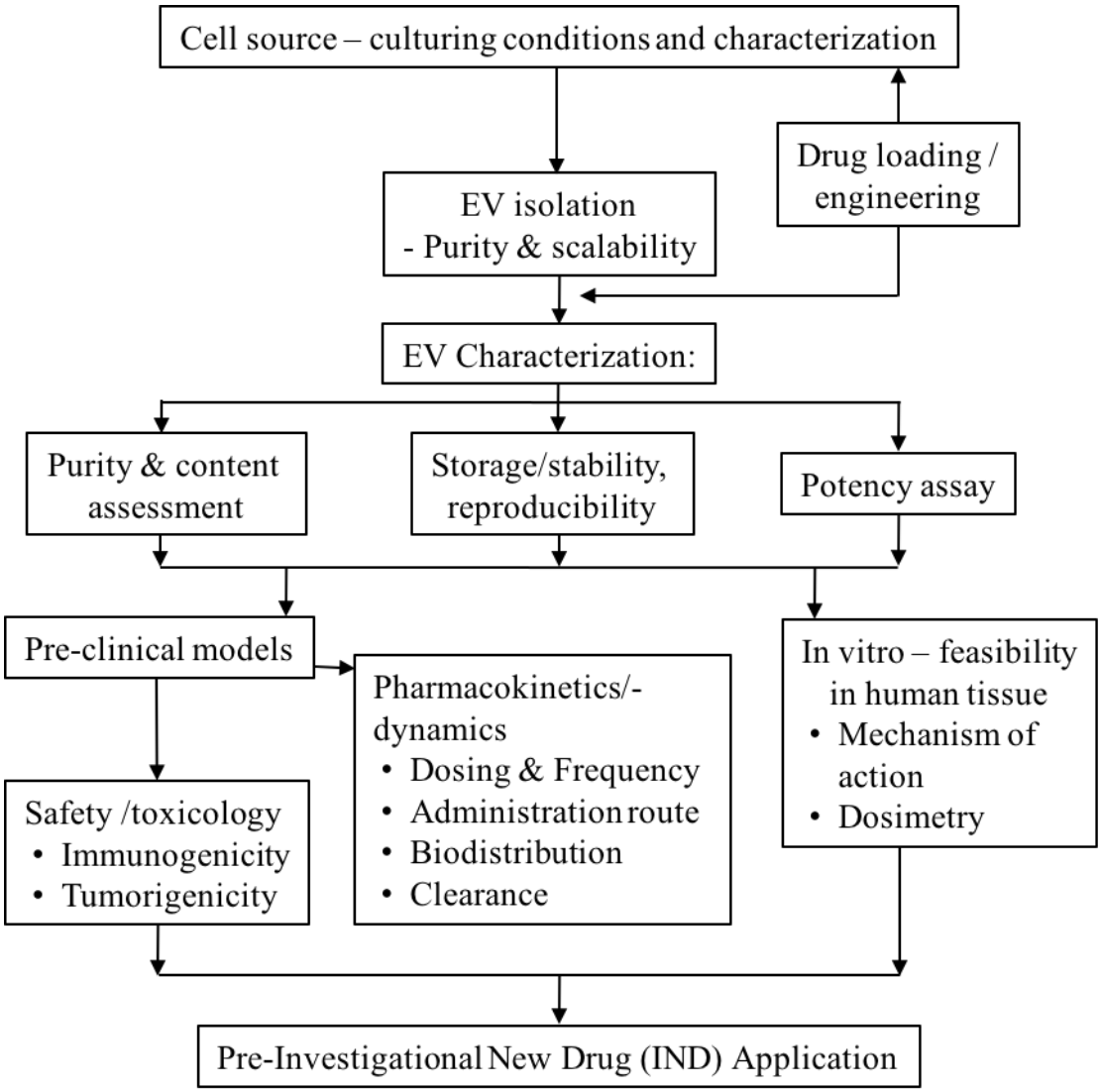

Figure 4 – Flowchart illustrating the preclinical testing of EVs, adapted from [343].

Choosing and characterizing an appropriate cell source for EV production, depending on the attended therapeutic approach, is of outmost importance. The well-studied MSCs and DCs are likely to be utilized, at least in certain disease settings, owing to their immunomodulatory properties and previous observations indicating their tolerability and safety. A variety of loading procedures and scalable isolation methods are currently being developed and optimized. In order to utilize EVs as off-the-shelf drugs, stability and storage must be further examined. In addition, the purity and potency of the isolated EVs must be assessed. The

therapeutic EVs must further be characterized in relevant pre-clinical models to assess safety as well as the pharmacokinetic and pharmacodynamics profile.

In conclusion, EVs are emerging as highly potent therapeutic entities and this thesis highlights the therapeutic potential of the innate properties of EVs and how EVs can be utilized for delivery of RNA species and therapeutic proteins in combination with targeting moieties. The field is still in its youth, but intense research is currently ongoing to optimize isolation and characterization techniques and to dissect the complex EV biology, content and function. To me, it thus seems very likely that EVs will become a future platform of highly potent multifaceted biopharmaceuticals.

6 CONFLICT OF INTEREST

I am a shareholder of and consultant for Evox Therapeutics Ltd.

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8 REFERENCES

1. Wolf, P., *The nature and significance of platelet products in human plasma.* Br J Haematol, 1967. **13**(3): p. 269-88.
2. Pan, B.T. and R.M. Johnstone, *Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor.* Cell, 1983. **33**(3): p. 967-78.
3. Harding, C. and P. Stahl, *Transferrin recycling in reticulocytes: pH and iron are important determinants of ligand binding and processing.* Biochem Biophys Res Commun, 1983. **113**(2): p. 650-8.
4. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles.* J Exp Med, 1996. **183**(3): p. 1161-72.
5. Zitvogel, L., et al., *Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes.* Nat Med, 1998. **4**(5): p. 594-600.
6. Escudier, B., et al., *Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial.* J Transl Med, 2005. **3**(1): p. 10.
7. Morse, M.A., et al., *A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer.* J Transl Med, 2005. **3**(1): p. 9.
8. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nat Cell Biol, 2007. **9**(6): p. 654-9.
9. Alvarez-Erviti, L., et al., *Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes.* Nat Biotechnol, 2011. **29**(4): p. 341-5.
10. S, E.L.A., et al., *Extracellular vesicles: biology and emerging therapeutic opportunities.* Nat Rev Drug Discov, 2013. **12**(5): p. 347-57.
11. Stegmayr, B. and G. Ronquist, *Promotive effect on human sperm progressive motility by prostasomes.* Urol Res, 1982. **10**(5): p. 253-7.
12. Gould, S.J. and G. Raposo, *As we wait: coping with an imperfect nomenclature for extracellular vesicles.* J Extracell Vesicles, 2013. **2**.
13. van der Pol, E., et al., *Recent developments in the nomenclature, presence, isolation, detection and clinical impact of extracellular vesicles.* J Thromb Haemost, 2016. **14**(1): p. 48-56.
14. Witwer, K.W., et al., *Standardization of sample collection, isolation and analysis methods in extracellular vesicle research.* J Extracell Vesicles, 2013. **2**.
15. Willms, E., et al., *Cells release subpopulations of exosomes with distinct molecular and biological properties.* Sci Rep, 2016. **6**: p. 22519.
16. Lotvall, J., et al., *Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles.* J Extracell Vesicles, 2014. **3**: p. 26913.
17. Lee, Y., S. El Andaloussi, and M.J. Wood, *Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy.* Hum Mol Genet, 2012. **21**(R1): p. R125-34.

18. Raposo, G. and W. Stoorvogel, *Extracellular vesicles: exosomes, microvesicles, and friends.* J Cell Biol, 2013. **200**(4): p. 373-83.

19. Grant, B.D. and J.G. Donaldson, *Pathways and mechanisms of endocytic recycling.* Nat Rev Mol Cell Biol, 2009. **10**(9): p. 597-608.

20. Akers, J.C., et al., *Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies.* J Neurooncol, 2013. **113**(1): p. 1-11.

21. Abels, E.R. and X.O. Breakefield, *Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake.* Cell Mol Neurobiol, 2016. **36**(3): p. 301-12.

22. Denzer, K., et al., *Exosome: from internal vesicle of the multivesicular body to intercellular signaling device.* J Cell Sci, 2000. **113 Pt 19**: p. 3365-74.

23. Thery, C., M. Ostrowski, and E. Segura, *Membrane vesicles as conveyors of immune responses.* Nat Rev Immunol, 2009. **9**(8): p. 581-93.

24. Babst, M., et al., *Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting.* Dev Cell, 2002. **3**(2): p. 271-82.

25. Wollert, T. and J.H. Hurley, *Molecular mechanism of multivesicular body biogenesis by ESCRT complexes.* Nature, 2010. **464**(7290): p. 864-9.

26. Hurley, J.H. and P.I. Hanson, *Membrane budding and scission by the ESCRT machinery: it's all in the neck.* Nat Rev Mol Cell Biol, 2010. **11**(8): p. 556-66.

27. Babst, M., *MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between.* Curr Opin Cell Biol, 2011. **23**(4): p. 452-7.

28. McCullough, J., et al., *ALIX-CHMP4 interactions in the human ESCRT pathway.* Proc Natl Acad Sci U S A, 2008. **105**(22): p. 7687-91.

29. Katoh, K., et al., *The ALG-2-interacting protein Alix associates with CHMP4b, a human homologue of yeast Snf7 that is involved in multivesicular body sorting.* J Biol Chem, 2003. **278**(40): p. 39104-13.

30. Baietti, M.F., et al., *Syndecan-syntenin-ALIX regulates the biogenesis of exosomes.* Nat Cell Biol, 2012. **14**(7): p. 677-85.

31. Roucourt, B., et al., *Heparanase activates the syndecan-syntenin-ALIX exosome pathway.* Cell Res, 2015. **25**(4): p. 412-28.

32. Ghossoub, R., et al., *Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2.* Nat Commun, 2014. **5**: p. 3477.

33. Stuffers, S., et al., *Multivesicular endosome biogenesis in the absence of ESCRTs.* Traffic, 2009. **10**(7): p. 925-37.

34. Trajkovic, K., et al., *Ceramide triggers budding of exosome vesicles into multivesicular endosomes.* Science, 2008. **319**(5867): p. 1244-7.

35. Stenmark, H., *Rab GTPases as coordinators of vesicle traffic.* Nat Rev Mol Cell Biol, 2009. **10**(8): p. 513-25.

36. Pereira-Leal, J.B. and M.C. Seabra, *Evolution of the Rab family of small GTP-binding proteins.* J Mol Biol, 2001. **313**(4): p. 889-901.

37. Zerial, M. and H. McBride, *Rab proteins as membrane organizers.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 107-17.

38. Schwartz, S.L., et al., *Rab GTPases at a glance.* J Cell Sci, 2007. **120**(Pt 22): p. 3905-10.

39. Zhen, Y. and H. Stenmark, *Cellular functions of Rab GTPases at a glance.* J Cell Sci, 2015. **128**(17): p. 3171-6.

40. Jordens, I., et al., *The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors.* Curr Biol, 2001. **11**(21): p. 1680-5.

41. Carroll, K.S., et al., *Role of Rab9 GTPase in facilitating receptor recruitment by TIP47.* Science, 2001. **292**(5520): p. 1373-6.

42. Barbero, P., L. Bittova, and S.R. Pfeffer, *Visualization of Rab9-mediated vesicle transport from endosomes to the trans-Golgi in living cells.* J Cell Biol, 2002. **156**(3): p. 511-8.

43. Aivazian, D., R.L. Serrano, and S. Pfeffer, *TIP47 is a key effector for Rab9 localization.* J Cell Biol, 2006. **173**(6): p. 917-26.

44. Mobius, W., et al., *Immunoelectron microscopic localization of cholesterol using biotinylated and non-cytolytic perfringolysin O.* J Histochem Cytochem, 2002. **50**(1): p. 43-55.

45. Savina, A., et al., *Exosome release is regulated by a calcium-dependent mechanism in K562 cells.* J Biol Chem, 2003. **278**(22): p. 20083-90.

46. Ostrowski, M., et al., *Rab27a and Rab27b control different steps of the exosome secretion pathway.* Nat Cell Biol, 2010. **12**(1): p. 19-30; sup pp 1-13.

47. Hsu, C., et al., *Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C.* J Cell Biol, 2010. **189**(2): p. 223-32.

48. Ohya, T., et al., *Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes.* Nature, 2009. **459**(7250): p. 1091-7.

49. Tsuboi, T. and M. Fukuda, *The C2B domain of rabphilin directly interacts with SNAP-25 and regulates the docking step of dense core vesicle exocytosis in PC12 cells.* J Biol Chem, 2005. **280**(47): p. 39253-9.

50. Tsuboi, T. and M. Fukuda, *The Slp4-a linker domain controls exocytosis through interaction with Munc18-1.syntaxin-1a complex.* Mol Biol Cell, 2006. **17**(5): p. 2101-12.

51. Fukuda, M., *Versatile role of Rab27 in membrane trafficking: focus on the Rab27 effector families.* J Biochem, 2005. **137**(1): p. 9-16.

52. Chen, Y.A. and R.H. Scheller, *SNARE-mediated membrane fusion.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 98-106.

53. Baker, R.W. and F.M. Hughson, *Chaperoning SNARE assembly and disassembly.* Nat Rev Mol Cell Biol, 2016. **17**(8): p. 465-79.

54. Fader, C.M., et al., *TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways.* Biochim Biophys Acta, 2009. **1793**(12): p. 1901-16.

55. Bevers, E.M., et al., *Lipid translocation across the plasma membrane of mammalian cells.* Biochim Biophys Acta, 1999. **1439**(3): p. 317-30.

56. Leventis, P.A. and S. Grinstein, *The distribution and function of phosphatidylserine in cellular membranes.* Annu Rev Biophys, 2010. **39**: p. 407-27.

57. Hankins, H.M., et al., *Role of flippases, scramblases and transfer proteins in phosphatidylserine subcellular distribution.* Traffic, 2015. **16**(1): p. 35-47.

58. Hugel, B., et al., *Membrane microparticles: two sides of the coin.* Physiology (Bethesda), 2005. **20**: p. 22-7.

59. Tricarico, C., J. Clancy, and C. D'Souza-Schorey, *Biology and biogenesis of shed microvesicles.* Small GTPases, 2016: p. 1-13.

60. Yang, J.S., et al., *A role for phosphatidic acid in COPI vesicle fission yields insights into Golgi maintenance.* Nat Cell Biol, 2008. **10**(10): p. 1146-53.

61. Pasquet, J.M., J. Dachary-Prigent, and A.T. Nurden, *Calcium influx is a determining factor of calpain activation and microparticle formation in platelets.* Eur J Biochem, 1996. **239**(3): p. 647-54.

62. Bucki, R., et al., *Calcium induces phospholipid redistribution and microvesicle release in human erythrocyte membranes by independent pathways.* Biochemistry, 1998. **37**(44): p. 15383-91.

63. Muralidharan-Chari, V., et al., *ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles.* Curr Biol, 2009. **19**(22): p. 1875-85.

64. Nabhan, J.F., et al., *Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein.* Proc Natl Acad Sci U S A, 2012. **109**(11): p. 4146-51.

65. Tauro, B.J., et al., *Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes.* Methods, 2012. **56**(2): p. 293-304.

66. Wang, T., et al., *Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis.* Proc Natl Acad Sci U S A, 2014. **111**(31): p. E3234-42.

67. Johnstone, R.M., et al., *Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes).* J Biol Chem, 1987. **262**(19): p. 9412-20.

68. Thery, C., et al., *Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73.* J Cell Biol, 1999. **147**(3): p. 599-610.

69. Hawari, F.I., et al., *Release of full-length 55-kDa TNF receptor 1 in exosome-like vesicles: a mechanism for generation of soluble cytokine receptors.* Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1297-302.

70. Graner, M.W., et al., *Proteomic and immunologic analyses of brain tumor exosomes.* FASEB J, 2009. **23**(5): p. 1541-57.

71. Caby, M.P., et al., *Exosomal-like vesicles are present in human blood plasma.* Int Immunol, 2005. **17**(7): p. 879-87.

72. Muller, L., et al., *Isolation of biologically-active exosomes from human plasma.* J Immunol Methods, 2014. **411**: p. 55-65.

73. Pisitkun, T., R.F. Shen, and M.A. Knepper, *Identification and proteomic profiling of exosomes in human urine.* Proc Natl Acad Sci U S A, 2004. **101**(36): p. 13368-73.

74. Dimov, I., L. Jankovic Velickovic, and V. Stefanovic, *Urinary exosomes.* ScientificWorldJournal, 2009. **9**: p. 1107-18.

75. Ronquist, G. and I. Brody, *The prostasome: its secretion and function in man.* Biochim Biophys Acta, 1985. **822**(2): p. 203-18.

76. Park, K.H., et al., *Ca2+ signaling tools acquired from prostasomes are required for progesterone-induced sperm motility.* Sci Signal, 2011. **4**(173): p. ra31.

77. Admyre, C., et al., *Exosomes with immune modulatory features are present in human breast milk.* J Immunol, 2007. **179**(3): p. 1969-78.

78. Vella, L.J., et al., *Packaging of prions into exosomes is associated with a novel pathway of PrP processing.* J Pathol, 2007. **211**(5): p. 582-90.

79. Harrington, M.G., et al., *The morphology and biochemistry of nanostructures provide evidence for synthesis and signaling functions in human cerebrospinal fluid.* Cerebrospinal Fluid Res, 2009. **6**: p. 10.

80. Asea, A., et al., *Heat shock protein-containing exosomes in mid-trimester amniotic fluids.* J Reprod Immunol, 2008. **79**(1): p. 12-7.

81. Andre, F., et al., *Malignant effusions and immunogenic tumour-derived exosomes.* Lancet, 2002. **360**(9329): p. 295-305.

82. Runz, S., et al., *Malignant ascites-derived exosomes of ovarian carcinoma patients contain CD24 and EpCAM.* Gynecol Oncol, 2007. **107**(3): p. 563-71.

83. Masyuk, A.I., et al., *Biliary exosomes influence cholangiocyte regulatory mechanisms and proliferation through interaction with primary cilia.* Am J Physiol Gastrointest Liver Physiol, 2010. **299**(4): p. G990-9.

84. Ogawa, Y., et al., *Proteomic analysis of two types of exosomes in human whole saliva.* Biol Pharm Bull, 2011. **34**(1): p. 13-23.

85. Thery, C., et al., *Isolation and characterization of exosomes from cell culture supernatants and biological fluids.* Curr Protoc Cell Biol, 2006. **Chapter 3**: p. Unit 3 22.

86. Momen-Heravi, F., et al., *Current methods for the isolation of extracellular vesicles.* Biol Chem, 2013. **394**(10): p. 1253-62.

87. Nordin, J.Z., et al., *Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties.* Nanomedicine, 2015. **11**(4): p. 879-83.

88. Linares, R., et al., *High-speed centrifugation induces aggregation of extracellular vesicles.* J Extracell Vesicles, 2015. **4**: p. 29509.

89. Lener, T., et al., *Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper.* J Extracell Vesicles, 2015. **4**: p. 30087.

90. Lamparski, H.G., et al., *Production and characterization of clinical grade exosomes derived from dendritic cells.* J Immunol Methods, 2002. **270**(2): p. 211-26.

91. Cantin, R., et al., *Discrimination between exosomes and HIV-1: purification of both vesicles from cell-free supernatants.* J Immunol Methods, 2008. **338**(1-2): p. 21-30.

92. Boing, A.N., et al., *Single-step isolation of extracellular vesicles by size-exclusion chromatography.* J Extracell Vesicles, 2014. **3**.

93. Arslan, F., et al., *Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury.* Stem Cell Res, 2013. **10**(3): p. 301-12.

94. Alvarez, M.L., et al., *Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers.* Kidney Int, 2012. **82**(9): p. 1024-32.

95. Kordelas, L., et al., *MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease.* Leukemia, 2014. **28**(4): p. 970-3.

96. Clayton, A., et al., *Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry.* J Immunol Methods, 2001. **247**(1-2): p. 163-74.

97. Koga, K., et al., *Purification, characterization and biological significance of tumor-derived exosomes.* Anticancer Res, 2005. **25**(6A): p. 3703-7.

98. Chen, C., et al., *Microfluidic isolation and transcriptome analysis of serum microvesicles.* Lab Chip, 2010. **10**(4): p. 505-11.

99. Davies, R.T., et al., *Microfluidic filtration system to isolate extracellular vesicles from blood.* Lab Chip, 2012. **12**(24): p. 5202-10.

100. Cvjetkovic, A., J. Lotvall, and C. Lasser, *The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles.* J Extracell Vesicles, 2014. **3**.

101. Momen-Heravi, F., et al., *Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles.* Front Physiol, 2012. **3**: p. 162.

102. Webber, J. and A. Clayton, *How pure are your vesicles?* J Extracell Vesicles, 2013. **2**.

103. Yuana, Y., et al., *Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation.* J Extracell Vesicles, 2014. **3**.

104. Cheruvanky, A., et al., *Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator.* Am J Physiol Renal Physiol, 2007. **292**(5): p. F1657-61.

105. Heinemann, M.L., et al., *Benchtop isolation and characterization of functional exosomes by sequential filtration.* J Chromatogr A, 2014. **1371**: p. 125-35.

106. Corso, G., et al., *Reproducible and scalable purification of extracellular vesicles using combined bind-elute and size exclusion chromatography.* Sci Rep, 2017. **7**(1): p. 11561.

107. Van Deun, J., et al., *The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling.* J Extracell Vesicles, 2014. **3**.

108. Logozzi, M., et al., *High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients.* PLoS One, 2009. **4**(4): p. e5219.

109. Nakai, W., et al., *A novel affinity-based method for the isolation of highly purified extracellular vesicles.* Sci Rep, 2016. **6**: p. 33935.

110. Liga, A., et al., *Exosome isolation: a microfluidic road-map.* Lab Chip, 2015. **15**(11): p. 2388-94.

111. Dai, S., et al., *Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer.* Mol Ther, 2008. **16**(4): p. 782-90.

112. Besse, B., et al., *Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC.* Oncoimmunology, 2016. **5**(4): p. e1071008.

113. Heijnen, H.F., et al., *Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules.* Blood, 1999. **94**(11): p. 3791-9.

114. Yuana, Y., et al., *Atomic force microscopy: a novel approach to the detection of nanosized blood microparticles.* J Thromb Haemost, 2010. **8**(2): p. 315-23.

115. Maas, S.L., et al., *Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics.* J Control Release, 2015. **200**: p. 87-96.

116. Thery, C., L. Zitvogel, and S. Amigorena, *Exosomes: composition, biogenesis and function.* Nat Rev Immunol, 2002. **2**(8): p. 569-79.

117. Ettelaie, C., et al., *Characterization of physical properties of tissue factor-containing microvesicles and a comparison of ultracentrifuge-based recovery procedures.* J Extracell Vesicles, 2014. **3**.

118. Keller, S., et al., *Body fluid derived exosomes as a novel template for clinical diagnostics.* J Transl Med, 2011. **9**: p. 86.

119. Poliakov, A., et al., *Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen.* Prostate, 2009. **69**(2): p. 159-67.

120. Koliha, N., et al., *A novel multiplex bead-based platform highlights the diversity of extracellular vesicles.* J Extracell Vesicles, 2016. **5**: p. 29975.

121. Kowal, J., et al., *Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes.* Proc Natl Acad Sci U S A, 2016. **113**(8): p. E968-77.

122. Mateescu, B., et al., *Obstacles and opportunities in the functional analysis of extracellular vesicle RNA - an ISEV position paper.* J Extracell Vesicles, 2017. **6**(1): p. 1286095.

123. Arroyo, J.D., et al., *Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma.* Proc Natl Acad Sci U S A, 2011. **108**(12): p. 5003-8.

124. Wei, Z., et al., *Fetal Bovine Serum RNA Interferes with the Cell Culture derived Extracellular RNA.* Sci Rep, 2016. **6**: p. 31175.

125. Chevillet, J.R., et al., *Quantitative and stoichiometric analysis of the microRNA content of exosomes.* Proc Natl Acad Sci U S A, 2014. **111**(41): p. 14888-93.

126. Kreimer, S., et al., *Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics.* J Proteome Res, 2015. **14**(6): p. 2367-84.

127. Atay, S., C. Gercel-Taylor, and D.D. Taylor, *Human trophoblast-derived exosomal fibronectin induces pro-inflammatory IL-1beta production by macrophages.* Am J Reprod Immunol, 2011. **66**(4): p. 259-69.

128. Barres, C., et al., *Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages.* Blood, 2010. **115**(3): p. 696-705.

129. Fitzner, D., et al., *Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis.* J Cell Sci, 2011. **124**(Pt 3): p. 447-58.

130. Morelli, A.E., et al., *Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells.* Blood, 2004. **104**(10): p. 3257-66.

131. Zomer, A., et al., *In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior.* Cell, 2015. **161**(5): p. 1046-1057.

132. Zomer, A., et al., *Studying extracellular vesicle transfer by a Cre-loxP method.* Nat Protoc, 2016. **11**(1): p. 87-101.

133. Tkach, M., et al., *Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes.* EMBO J, 2017.

134. Heldring, N., et al., *Therapeutic Potential of Multipotent Mesenchymal Stromal Cells and Their Extracellular Vesicles.* Hum Gene Ther, 2015. **26**(8): p. 506-17.

135. Larson, M.C., et al., *Calcium-phosphate microprecipitates mimic microparticles when examined with flow cytometry.* Cytometry A, 2013. **83**(2): p. 242-50.

136. Heusermann, W., et al., *Exosomes surf on filopodia to enter cells at endocytic hot spots, traffic within endosomes, and are targeted to the ER.* J Cell Biol, 2016. **213**(2): p. 173-84.

137. Steen, H.B., *Flow cytometer for measurement of the light scattering of viral and other submicroscopic particles.* Cytometry A, 2004. **57**(2): p. 94-9.

138. Nolte-'t Hoen, E.N., et al., *Quantitative and qualitative flow cytometric analysis of nanosized cell-derived membrane vesicles.* Nanomedicine, 2012. **8**(5): p. 712-20.

139. Welsh, J.A., et al., *Extracellular Vesicle Flow Cytometry Analysis and Standardization.* Front Cell Dev Biol, 2017. **5**: p. 78.

140. Mathivanan, S. and R.J. Simpson, *ExoCarta: A compendium of exosomal proteins and RNA.* Proteomics, 2009. **9**(21): p. 4997-5000.

141. Kalra, H., et al., *Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation.* PLoS Biol, 2012. **10**(12): p. e1001450.

142. Kim, D.K., et al., *EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles.* J Extracell Vesicles, 2013. **2**.

143. Thery, C., et al., *Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles.* J Immunol, 2001. **166**(12): p. 7309-18.

144. Stenqvist, A.C., et al., *Exosomes secreted by human placenta carry functional Fas ligand and TRAIL molecules and convey apoptosis in activated immune cells,*

suggesting exosome-mediated immune privilege of the fetus. J Immunol, 2013. **191**(11): p. 5515-23.

145. Shimoda, M. and R. Khokha, *Proteolytic factors in exosomes.* Proteomics, 2013. **13**(10-11): p. 1624-36.

146. Christianson, H.C., et al., *Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity.* Proc Natl Acad Sci U S A, 2013. **110**(43): p. 17380-5.

147. Sinha, A., et al., *In-depth proteomic analyses of ovarian cancer cell line exosomes reveals differential enrichment of functional categories compared to the NCI 60 proteome.* Biochem Biophys Res Commun, 2014. **445**(4): p. 694-701.

148. Zaborowski, M.P., et al., *Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study.* Bioscience, 2015. **65**(8): p. 783-797.

149. Biro, E., et al., *The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions.* J Thromb Haemost, 2005. **3**(12): p. 2754-63.

150. Simons, K. and J.L. Sampaio, *Membrane organization and lipid rafts.* Cold Spring Harb Perspect Biol, 2011. **3**(10): p. a004697.

151. Record, M., et al., *Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies.* Biochim Biophys Acta, 2014. **1841**(1): p. 108-20.

152. Matsuo, H., et al., *Role of LBPA and Alix in multivesicular liposome formation and endosome organization.* Science, 2004. **303**(5657): p. 531-4.

153. Kirkegaard, T., et al., *Hsp70 stabilizes lysosomes and reverts Niemann-Pick disease-associated lysosomal pathology.* Nature, 2010. **463**(7280): p. 549-53.

154. Subra, C., et al., *Exosomes account for vesicle-mediated transcellular transport of activatable phospholipases and prostaglandins.* J Lipid Res, 2010. **51**(8): p. 2105-20.

155. Esser, J., et al., *Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration.* J Allergy Clin Immunol, 2010. **126**(5): p. 1032-40, 1040 e1-4.

156. Kim, C.W., et al., *Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin.* Cancer Res, 2002. **62**(21): p. 6312-7.

157. Guescini, M., et al., *Astrocytes and Glioblastoma cells release exosomes carrying mtDNA.* J Neural Transm (Vienna), 2010. **117**(1): p. 1-4.

158. Balaj, L., et al., *Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences.* Nat Commun, 2011. **2**: p. 180.

159. Waldenstrom, A., et al., *Cardiomyocyte microvesicles contain DNA/RNA and convey biological messages to target cells.* PLoS One, 2012. **7**(4): p. e34653.

160. Kahlert, C., et al., *Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer.* J Biol Chem, 2014. **289**(7): p. 3869-75.

161. Batagov, A.O. and I.V. Kurochkin, *Exosomes secreted by human cells transport largely mRNA fragments that are enriched in the 3'-untranslated regions.* Biol Direct, 2013. **8**: p. 12.

162. Huang, X., et al., *Characterization of human plasma-derived exosomal RNAs by deep sequencing.* BMC Genomics, 2013. **14**: p. 319.

163. Eirin, A., et al., *MicroRNA and mRNA cargo of extracellular vesicles from porcine adipose tissue-derived mesenchymal stem cells.* Gene, 2014. **551**(1): p. 55-64.

164. Tang, Y.T., et al., *Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum.* Int J Mol Med, 2017. **40**(3): p. 834-844.

165. Crescitelli, R., et al., *Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes.* J Extracell Vesicles, 2013. **2**.

166. Ferguson, S.W. and J. Nguyen, *Exosomes as therapeutics: The implications of molecular composition and exosomal heterogeneity.* J Control Release, 2016. **228**: p. 179-190.

167. Skog, J., et al., *Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers.* Nat Cell Biol, 2008. **10**(12): p. 1470-6.

168. Bolukbasi, M.F., et al., *miR-1289 and "Zipcode"-like Sequence Enrich mRNAs in Microvesicles.* Mol Ther Nucleic Acids, 2012. **1**: p. e10.

169. Batagov, A.O., V.A. Kuznetsov, and I.V. Kurochkin, *Identification of nucleotide patterns enriched in secreted RNAs as putative cis-acting elements targeting them to exosome nano-vesicles.* BMC Genomics, 2011. **12 Suppl 3**: p. S18.

170. Koppers-Lalic, D., et al., *Nontemplated nucleotide additions distinguish the small RNA composition in cells from exosomes.* Cell Rep, 2014. **8**(6): p. 1649-1658.

171. Villarroya-Beltri, C., et al., *Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs.* Nat Commun, 2013. **4**: p. 2980.

172. Villarroya-Beltri, C., et al., *Sorting it out: regulation of exosome loading.* Semin Cancer Biol, 2014. **28**: p. 3-13.

173. Guduric-Fuchs, J., et al., *Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types.* BMC Genomics, 2012. **13**: p. 357.

174. McKenzie, A.J., et al., *KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes.* Cell Rep, 2016. **15**(5): p. 978-987.

175. Melo, S.A., et al., *Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis.* Cancer Cell, 2014. **26**(5): p. 707-21.

176. Kosaka, N., et al., *Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis.* J Biol Chem, 2013. **288**(15): p. 10849-59.

177. Segura, E., S. Amigorena, and C. Thery, *Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses.* Blood Cells Mol Dis, 2005. **35**(2): p. 89-93.

178. Yin, W., et al., *Immature dendritic cell-derived exosomes: a promise subcellular vaccine for autoimmunity.* Inflammation, 2013. **36**(1): p. 232-40.

179. Clayton, A., et al., *Induction of heat shock proteins in B-cell exosomes.* J Cell Sci, 2005. **118**(Pt 16): p. 3631-8.

180. Eldh, M., et al., *Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA.* PLoS One, 2010. **5**(12): p. e15353.

181. Hedlund, M., et al., *Thermal- and oxidative stress causes enhanced release of NKG2D ligand-bearing immunosuppressive exosomes in leukemia/lymphoma T and B cells.* PLoS One, 2011. **6**(2): p. e16899.

182. Atienzar-Aroca, S., et al., *Oxidative stress in retinal pigment epithelium cells increases exosome secretion and promotes angiogenesis in endothelial cells.* J Cell Mol Med, 2016. **20**(8): p. 1457-66.

183. Parolini, I., et al., *Microenvironmental pH is a key factor for exosome traffic in tumor cells.* J Biol Chem, 2009. **284**(49): p. 34211-22.

184. Li, J., et al., *Serum-free culture alters the quantity and protein composition of neuroblastoma-derived extracellular vesicles.* J Extracell Vesicles, 2015. **4**: p. 26883.

185. Kucharzewska, P., et al., *Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development.* Proc Natl Acad Sci U S A, 2013. **110**(18): p. 7312-7.

186. Saunderson, S.C., et al., *Induction of exosome release in primary B cells stimulated via CD40 and the IL-4 receptor.* J Immunol, 2008. **180**(12): p. 8146-52.

187. Pusic, A.D., et al., *IFNgamma-stimulated dendritic cell exosomes as a potential therapeutic for remyelination.* J Neuroimmunol, 2014. **266**(1-2): p. 12-23.

188. Groth, E., et al., *Stimulated release and functional activity of surface expressed metalloproteinase ADAM17 in exosomes.* Biochim Biophys Acta, 2016. **1863**(11): p. 2795-2808.

189. Watson, D.C., et al., *Efficient production and enhanced tumor delivery of engineered extracellular vesicles.* Biomaterials, 2016. **105**: p. 195-205.

190. Madrigal, M., K.S. Rao, and N.H. Riordan, *A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods.* J Transl Med, 2014. **12**: p. 260.

191. Yuyama, K., et al., *Sphingolipid-modulated exosome secretion promotes clearance of amyloid-beta by microglia.* J Biol Chem, 2012. **287**(14): p. 10977-89.

192. Franzen, C.A., et al., *Characterization of uptake and internalization of exosomes by bladder cancer cells.* Biomed Res Int, 2014. **2014**: p. 619829.

193. Obregon, C., et al., *Exovesicles from human activated dendritic cells fuse with resting dendritic cells, allowing them to present alloantigens.* Am J Pathol, 2006. **169**(6): p. 2127-36.

194. Tian, T., et al., *Dynamics of exosome internalization and trafficking.* J Cell Physiol, 2013. **228**(7): p. 1487-95.

195. Tian, T., et al., *Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy.* J Cell Biochem, 2010. **111**(2): p. 488-96.

196. Villarroya-Beltri, C., et al., *ISGylation controls exosome secretion by promoting lysosomal degradation of MVB proteins.* Nat Commun, 2016. **7**: p. 13588.

197. Montecalvo, A., et al., *Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes.* Blood, 2012. **119**(3): p. 756-66.

198. Escrevente, C., et al., *Interaction and uptake of exosomes by ovarian cancer cells.* BMC Cancer, 2011. **11**: p. 108.

199. Costa Verdera, H., et al., *Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis.* J Control Release, 2017. **266**: p. 100-108.

200. Feng, D., et al., *Cellular internalization of exosomes occurs through phagocytosis.* Traffic, 2010. **11**(5): p. 675-87.

201. Tian, T., et al., *Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery.* J Biol Chem, 2014. **289**(32): p. 22258-67.

202. Nanbo, A., et al., *Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells.* J Virol, 2013. **87**(18): p. 10334-47.

203. Naslund, T.I., et al., *Exosomes from breast milk inhibit HIV-1 infection of dendritic cells and subsequent viral transfer to CD4+ T cells.* AIDS, 2014. **28**(2): p. 171-80.

204. Xie, Y., et al., *Dendritic cells recruit T cell exosomes via exosomal LFA-1 leading to inhibition of CD8+ CTL responses through downregulation of peptide/MHC class I and Fas ligand-mediated cytotoxicity.* J Immunol, 2010. **185**(9): p. 5268-78.

205. Hao, S., et al., *Mature dendritic cells pulsed with exosomes stimulate efficient cytotoxic T-lymphocyte responses and antitumour immunity.* Immunology, 2007. **120**(1): p. 90-102.

206. Johnstone, R.M., *Exosomes biological significance: A concise review.* Blood Cells Mol Dis, 2006. **36**(2): p. 315-21.

207. Mulcahy, L.A., R.C. Pink, and D.R. Carter, *Routes and mechanisms of extracellular vesicle uptake.* J Extracell Vesicles, 2014. **3**.

208. Hoshino, A., et al., *Tumour exosome integrins determine organotropic metastasis.* Nature, 2015. **527**(7578): p. 329-35.

209. Wei, X., et al., *Surface Phosphatidylserine Is Responsible for the Internalization on Microvesicles Derived from Hypoxia-Induced Human Bone Marrow Mesenchymal Stem Cells into Human Endothelial Cells.* PLoS One, 2016. **11**(1): p. e0147360.

210. Sun, D., et al., *A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes.* Mol Ther, 2010. **18**(9): p. 1606-14.

211. Takahashi, Y., et al., *Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection.* J Biotechnol, 2013. **165**(2): p. 77-84.

212. Smyth, T., et al., *Biodistribution and delivery efficiency of unmodified tumor-derived exosomes.* J Control Release, 2015. **199**: p. 145-55.

213. Ohno, S.-i., et al., *Systemically Injected Exosomes Targeted to EGFR Deliver Antitumor MicroRNA to Breast Cancer Cells.* Molecular Therapy, 2013. **21**(1): p. 185-191.

214. Lai, C.P., B.A. Tannous, and X.O. Breakefield, *Noninvasive in vivo monitoring of extracellular vesicles.* Methods Mol Biol, 2014. **1098**: p. 249-58.

215. Ostro, M.J. and P.R. Cullis, *Use of liposomes as injectable-drug delivery systems.* Am J Hosp Pharm, 1989. **46**(8): p. 1576-87.

216. Lopez-Berestein, G., et al., *Clinical pharmacology of 99mTc-labeled liposomes in patients with cancer.* Cancer Res, 1984. **44**(1): p. 375-8.

217. Liu, D., A. Mori, and L. Huang, *Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes.* Biochim Biophys Acta, 1992. **1104**(1): p. 95-101.

218. Willekens, F.L., et al., *Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors.* Blood, 2005. **105**(5): p. 2141-5.

219. Sarin, H., *Physiologic upper limits of pore size of different blood capillary types and another perspective on the dual pore theory of microvascular permeability.* J Angiogenes Res, 2010. **2**: p. 14.

220. Rank, A., et al., *Clearance of platelet microparticles in vivo.* Platelets, 2011. **22**(2): p. 111-6.

221. Yamashita, T., et al., *Effect of exosome isolation methods on physicochemical properties of exosomes and clearance of exosomes from the blood circulation.* Eur J Pharm Biopharm, 2016. **98**: p. 1-8.

222. Clayton, A., et al., *Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59.* Eur J Immunol, 2003. **33**(2): p. 522-31.

223. Dasgupta, S.K., et al., *Lactadherin and clearance of platelet-derived microvesicles.* Blood, 2009. **113**(6): p. 1332-9.

224. Saunderson, S.C., et al., *CD169 mediates the capture of exosomes in spleen and lymph node.* Blood, 2014. **123**(2): p. 208-16.

225. Charoenviriyakul, C., et al., *Cell type-specific and common characteristics of exosomes derived from mouse cell lines: Yield, physicochemical properties, and pharmacokinetics.* Eur J Pharm Sci, 2017. **96**: p. 316-322.

226. Tannous, B.A., *Gaussia luciferase reporter assay for monitoring biological processes in culture and in vivo.* Nat Protoc, 2009. **4**(4): p. 582-91.

227. Imai, T., et al., *Macrophage-dependent clearance of systemically administered B16BL6-derived exosomes from the blood circulation in mice.* J Extracell Vesicles, 2015. **4**: p. 26238.

228. Lai, C.P., et al., *Dynamic biodistribution of extracellular vesicles in vivo using a multimodal imaging reporter.* ACS Nano, 2014. **8**(1): p. 483-494.

229. Hu, L., et al., *Exosomes derived from human adipose mensenchymal stem cells accelerates cutaneous wound healing via optimizing the characteristics of fibroblasts.* Sci Rep, 2016. **6**: p. 32993.

230. Texier, I., et al., *Cyanine-loaded lipid nanoparticles for improved in vivo fluorescence imaging.* J Biomed Opt, 2009. **14**(5): p. 054005.

231. Lassailly, F., E. Griessinger, and D. Bonnet, *"Microenvironmental contaminations" induced by fluorescent lipophilic dyes used for noninvasive in vitro and in vivo cell tracking.* Blood, 2010. **115**(26): p. 5347-54.

232. Bala, S., et al., *Biodistribution and function of extracellular miRNA-155 in mice.* Sci Rep, 2015. **5**: p. 10721.

233. Zomer, A., et al., *In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior.* Cell, 2015. **161**(5): p. 1046-57.

234. Ridder, K., et al., *Extracellular vesicle-mediated transfer of genetic information between the hematopoietic system and the brain in response to inflammation.* PLoS Biol, 2014. **12**(6): p. e1001874.

235. Muntasell, A., A.C. Berger, and P.A. Roche, *T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes.* EMBO J, 2007. **26**(19): p. 4263-72.

236. Admyre, C., et al., *Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid.* Eur Respir J, 2003. **22**(4): p. 578-83.

237. Andre, F., et al., *Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells.* J Immunol, 2004. **172**(4): p. 2126-36.

238. Nolte-'t Hoen, E.N., et al., *Activated T cells recruit exosomes secreted by dendritic cells via LFA-1.* Blood, 2009. **113**(9): p. 1977-81.

239. Admyre, C., et al., *Direct exosome stimulation of peripheral human T cells detected by ELISPOT.* Eur J Immunol, 2006. **36**(7): p. 1772-81.

240. Thery, C., et al., *Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes.* Nat Immunol, 2002. **3**(12): p. 1156-62.

241. Giri, P.K. and J.S. Schorey, *Exosomes derived from M. Bovis BCG infected macrophages activate antigen-specific CD4+ and CD8+ T cells in vitro and in vivo.* PLoS One, 2008. **3**(6): p. e2461.

242. Bhatnagar, S., et al., *Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo.* Blood, 2007. **110**(9): p. 3234-44.

243. Skokos, D., et al., *Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses in vivo.* J Immunol, 2003. **170**(6): p. 3037-45.

244. Skokos, D., et al., *Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes.* J Immunol, 2001. **166**(2): p. 868-76.

245. Bruno, S., et al., *Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury.* PLoS One, 2012. **7**(3): p. e33115.

246. Peche, H., et al., *Presentation of donor major histocompatibility complex antigens by bone marrow dendritic cell-derived exosomes modulates allograft rejection.* Transplantation, 2003. **76**(10): p. 1503-10.

247. Kim, S.H., et al., *Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis.* J Immunol, 2005. **174**(10): p. 6440-8.

248. MacKenzie, A., et al., *Rapid secretion of interleukin-1beta by microvesicle shedding.* Immunity, 2001. **15**(5): p. 825-35.

249. Kandere-Grzybowska, K., et al., *IL-1 induces vesicular secretion of IL-6 without degranulation from human mast cells.* J Immunol, 2003. **171**(9): p. 4830-6.

250. Baj-Krzyworzeka, M., et al., *Tumour-derived microvesicles contain interleukin-8 and modulate production of chemokines by human monocytes.* Anticancer Res, 2011. **31**(4): p. 1329-35.

251. Zhang, H.G., et al., *A membrane form of TNF-alpha presented by exosomes delays T cell activation-induced cell death.* J Immunol, 2006. **176**(12): p. 7385-93.

252. Munich, S., et al., *Dendritic cell exosomes directly kill tumor cells and activate natural killer cells via TNF superfamily ligands.* Oncoimmunology, 2012. **1**(7): p. 1074-1083.

253. Sprague, D.L., et al., *Platelet-mediated modulation of adaptive immunity: unique delivery of CD154 signal by platelet-derived membrane vesicles.* Blood, 2008. **111**(10): p. 5028-36.

254. Zuccato, E., et al., *Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation.* J Cell Sci, 2007. **120**(Pt 1): p. 191-9.

255. Boilard, E., et al., *Platelets amplify inflammation in arthritis via collagen-dependent microparticle production.* Science, 2010. **327**(5965): p. 580-3.

256. Cloutier, N., et al., *The exposure of autoantigens by microparticles underlies the formation of potent inflammatory components: the microparticle-associated immune complexes.* EMBO Mol Med, 2013. **5**(2): p. 235-49.

257. Hoyer, F.F., et al., *Monocytic microparticles promote atherogenesis by modulating inflammatory cells in mice.* J Cell Mol Med, 2012. **16**(11): p. 2777-88.

258. Prakash, P.S., et al., *Human microparticles generated during sepsis in patients with critical illness are neutrophil-derived and modulate the immune response.* J Trauma Acute Care Surg, 2012. **73**(2): p. 401-6; discussion 406-7.

259. Aswad, H., et al., *Exosomes participate in the alteration of muscle homeostasis during lipid-induced insulin resistance in mice.* Diabetologia, 2014. **57**(10): p. 2155-64.

260. Ying, W., et al., *Adipose Tissue Macrophage-Derived Exosomal miRNAs Can Modulate In Vivo and In Vitro Insulin Sensitivity.* Cell, 2017. **171**(2): p. 372-384 e12.

261. Holder, B.S., et al., *Heightened pro-inflammatory effect of preeclamptic placental microvesicles on peripheral blood immune cells in humans.* Biol Reprod, 2012. **86**(4): p. 103.

262. Kshirsagar, S.K., et al., *Immunomodulatory molecules are released from the first trimester and term placenta via exosomes.* Placenta, 2012. **33**(12): p. 982-90.

263. Sabapatha, A., C. Gercel-Taylor, and D.D. Taylor, *Specific isolation of placenta-derived exosomes from the circulation of pregnant women and their immunoregulatory consequences.* Am J Reprod Immunol, 2006. **56**(5-6): p. 345-55.

264. Hedlund, M., et al., *Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function.* J Immunol, 2009. **183**(1): p. 340-51.

265. Wolfers, J., et al., *Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming.* Nat Med, 2001. **7**(3): p. 297-303.

266. Elsner, L., et al., *The heat shock protein HSP70 promotes mouse NK cell activity against tumors that express inducible NKG2D ligands.* J Immunol, 2007. **179**(8): p. 5523-33.

267. Pucci, F., et al., *SCS macrophages suppress melanoma by restricting tumor-derived vesicle-B cell interactions.* Science, 2016. **352**(6282): p. 242-6.

268. Andreola, G., et al., *Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles.* J Exp Med, 2002. **195**(10): p. 1303-16.

269. Huber, V., et al., *Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape.* Gastroenterology, 2005. **128**(7): p. 1796-804.

270. Taylor, D.D., et al., *T-cell apoptosis and suppression of T-cell receptor/CD3-zeta by Fas ligand-containing membrane vesicles shed from ovarian tumors.* Clin Cancer Res, 2003. **9**(14): p. 5113-9.

271. Valenti, R., et al., *Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes.* Cancer Res, 2006. **66**(18): p. 9290-8.

272. Yu, S., et al., *Tumor exosomes inhibit differentiation of bone marrow dendritic cells.* J Immunol, 2007. **178**(11): p. 6867-75.

273. Hood, J.L., R.S. San, and S.A. Wickline, *Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis.* Cancer Res, 2011. **71**(11): p. 3792-801.

274. Grange, C., et al., *Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche.* Cancer Res, 2011. **71**(15): p. 5346-56.

275. Peinado, H., et al., *Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET.* Nat Med, 2012. **18**(6): p. 883-91.

276. Chivet, M., et al., *Exosomes as a novel way of interneuronal communication.* Biochem Soc Trans, 2013. **41**(1): p. 241-4.

277. Lachenal, G., et al., *Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity.* Mol Cell Neurosci, 2011. **46**(2): p. 409-18.

278. Antonucci, F., et al., *Microvesicles released from microglia stimulate synaptic activity via enhanced sphingolipid metabolism.* EMBO J, 2012. **31**(5): p. 1231-40.

279. Harrison, E.B., et al., *Traumatic brain injury increases levels of miR-21 in extracellular vesicles: implications for neuroinflammation.* FEBS Open Bio, 2016. **6**(8): p. 835-46.

280. Saenz-Cuesta, M., I. Osorio-Querejeta, and D. Otaegui, *Extracellular Vesicles in Multiple Sclerosis: What are They Telling Us?* Front Cell Neurosci, 2014. **8**: p. 100.

281. Zondler, L., et al., *Impaired activation of ALS monocytes by exosomes.* Immunol Cell Biol, 2017. **95**(2): p. 207-214.

282. Loov, C., et al., *alpha-Synuclein in Extracellular Vesicles: Functional Implications and Diagnostic Opportunities.* Cell Mol Neurobiol, 2016. **36**(3): p. 437-48.

283. Rajendran, L., et al., *Alzheimer's disease beta-amyloid peptides are released in association with exosomes.* Proc Natl Acad Sci U S A, 2006. **103**(30): p. 11172-7.

284. Joshi, P., et al., *Microglia convert aggregated amyloid-beta into neurotoxic forms through the shedding of microvesicles.* Cell Death Differ, 2014. **21**(4): p. 582-93.

285. Saman, S., et al., *Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease.* J Biol Chem, 2012. **287**(6): p. 3842-9.

286. Yuyama, K., et al., *Decreased amyloid-beta pathologies by intracerebral loading of glycosphingolipid-enriched exosomes in Alzheimer model mice.* J Biol Chem, 2014. **289**(35): p. 24488-98.

287. Katsuda, T., et al., *Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes.* Sci Rep, 2013. **3**: p. 1197.

288. Porto-Carreiro, I., et al., *Prions and exosomes: from PrPc trafficking to PrPsc propagation.* Blood Cells Mol Dis, 2005. **35**(2): p. 143-8.

289. Sampey, G.C., et al., *Exosomes and their role in CNS viral infections.* J Neurovirol, 2014. **20**(3): p. 199-208.

290. Mack, M., et al., *Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection.* Nat Med, 2000. **6**(7): p. 769-75.

291. Grapp, M., et al., *Choroid plexus transcytosis and exosome shuttling deliver folate into brain parenchyma.* Nat Commun, 2013. **4**: p. 2123.

292. Squillaro, T., G. Peluso, and U. Galderisi, *Clinical Trials With Mesenchymal Stem Cells: An Update.* Cell Transplant, 2016. **25**(5): p. 829-48.

293. Humphreys, B.D., et al., *Intrinsic epithelial cells repair the kidney after injury.* Cell Stem Cell, 2008. **2**(3): p. 284-91.

294. Pittenger, M., *Sleuthing the source of regeneration by MSCs.* Cell Stem Cell, 2009. **5**(1): p. 8-10.

295. Timmers, L., et al., *Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium.* Stem Cell Res, 2007. **1**(2): p. 129-37.

296. van Koppen, A., et al., *Human embryonic mesenchymal stem cell-derived conditioned medium rescues kidney function in rats with established chronic kidney disease.* PLoS One, 2012. **7**(6): p. e38746.

297. Xin, H., et al., *Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats.* J Cereb Blood Flow Metab, 2013. **33**(11): p. 1711-5.

298. Reis, L.A., et al., *Bone marrow-derived mesenchymal stem cells repaired but did not prevent gentamicin-induced acute kidney injury through paracrine effects in rats.* PLoS One, 2012. **7**(9): p. e44092.

299. Zhu, Y.G., et al., *Human mesenchymal stem cell microvesicles for treatment of Escherichia coli endotoxin-induced acute lung injury in mice.* Stem Cells, 2014. **32**(1): p. 116-25.

300. Lai, R.C., et al., *Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury.* Stem Cell Res, 2010. **4**(3): p. 214-22.

301. Bian, S., et al., *Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model.* J Mol Med (Berl), 2014. **92**(4): p. 387-97.

302. Wang, X., et al., *Exosomal miR-223 Contributes to Mesenchymal Stem Cell-Elicited Cardioprotection in Polymicrobial Sepsis.* Sci Rep, 2015. **5**: p. 13721.

303. Tan, C.Y., et al., *Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models.* Stem Cell Res Ther, 2014. **5**(3): p. 76.

304. Li, T., et al., *Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis.* Stem Cells Dev, 2013. **22**(6): p. 845-54.

305. Xin, H., et al., *Exosome-mediated transfer of miR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth.* Stem Cells, 2012. **30**(7): p. 1556-64.

306. Kim, H.S., et al., *Proteomic analysis of microvesicles derived from human mesenchymal stem cells.* J Proteome Res, 2012. **11**(2): p. 839-49.

307. Bai, L., et al., *Effects of Mesenchymal Stem Cell-Derived Exosomes on Experimental Autoimmune Uveitis.* Sci Rep, 2017. **7**(1): p. 4323.

308. Sheng, H., et al., *Insulinoma-released exosomes or microparticles are immunostimulatory and can activate autoreactive T cells spontaneously developed in nonobese diabetic mice.* J Immunol, 2011. **187**(4): p. 1591-600.

309. Rahman, M.J., et al., *Exosomes released by islet-derived mesenchymal stem cells trigger autoimmune responses in NOD mice.* Diabetes, 2014. **63**(3): p. 1008-20.

310. Robbins, P.D. and A.E. Morelli, *Regulation of immune responses by extracellular vesicles.* Nat Rev Immunol, 2014. **14**(3): p. 195-208.

311. Tian, H. and W. Li, *Dendritic cell-derived exosomes for cancer immunotherapy: hope and challenges.* Ann Transl Med, 2017. **5**(10): p. 221.

312. Kim, S.H., et al., *Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive.* Mol Ther, 2006. **13**(2): p. 289-300.

313. Thomson, A.W. and P.D. Robbins, *Tolerogenic dendritic cells for autoimmune disease and transplantation.* Ann Rheum Dis, 2008. **67 Suppl 3**: p. iii90-6.

314. Haneberg, B., et al., *Intranasal administration of a meningococcal outer membrane vesicle vaccine induces persistent local mucosal antibodies and serum antibodies with strong bactericidal activity in humans.* Infect Immun, 1998. **66**(4): p. 1334-41.

315. Aline, F., et al., *Toxoplasma gondii antigen-pulsed-dendritic cell-derived exosomes induce a protective immune response against T. gondii infection.* Infect Immun, 2004. **72**(7): p. 4127-37.

316. Roy, K., et al., *Outer membrane vesicles induce immune responses to virulence proteins and protect against colonization by enterotoxigenic Escherichia coli.* Clin Vaccine Immunol, 2011. **18**(11): p. 1803-8.

317. Singh, P.P., et al., *Exosomes isolated from mycobacteria-infected mice or cultured macrophages can recruit and activate immune cells in vitro and in vivo.* J Immunol, 2012. **189**(2): p. 777-85.

318. Kaparakis-Liaskos, M. and R.L. Ferrero, *Immune modulation by bacterial outer membrane vesicles.* Nat Rev Immunol, 2015. **15**(6): p. 375-87.

319. Al-Nedawi, K., et al., *Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells.* Nat Cell Biol, 2008. **10**(5): p. 619-24.

320. Batrakova, E.V. and M.S. Kim, *Development and regulation of exosome-based therapy products.* Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2016. **8**(5): p. 744-57.

321. Bryniarski, K., et al., *Antigen-specific, antibody-coated, exosome-like nanovesicles deliver suppressor T-cell microRNA-150 to effector T cells to inhibit contact sensitivity.* J Allergy Clin Immunol, 2013. **132**(1): p. 170-81.

322. Didiot, M.C., et al., *Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing.* Mol Ther, 2016. **24**(10): p. 1836-1847.

323. Tian, Y., et al., *A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy.* Biomaterials, 2014. **35**(7): p. 2383-90.

324. Haney, M.J., et al., *Exosomes as drug delivery vehicles for Parkinson's disease therapy.* J Control Release, 2015. **207**: p. 18-30.

325. Wahlgren, J., et al., *Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes.* Nucleic Acids Res, 2012. **40**(17): p. e130.

326. Kooijmans, S.A., et al., *Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles.* J Control Release, 2013. **172**(1): p. 229-38.

327. Pascucci, L., et al., *Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit in vitro tumor growth: a new approach for drug delivery.* J Control Release, 2014. **192**: p. 262-70.

328. Akao, Y., et al., *Microvesicle-mediated RNA molecule delivery system using monocytes/macrophages.* Mol Ther, 2011. **19**(2): p. 395-9.

329. Zhang, Y., et al., *Secreted monocytic miR-150 enhances targeted endothelial cell migration.* Mol Cell, 2010. **39**(1): p. 133-44.

330. Sugahara, K.N., et al., *Tissue-penetrating delivery of compounds and nanoparticles into tumors.* Cancer Cell, 2009. **16**(6): p. 510-20.

331. Kooijmans, S.A., et al., *Display of GPI-anchored anti-EGFR nanobodies on extracellular vesicles promotes tumour cell targeting.* J Extracell Vesicles, 2016. **5**: p. 31053.

332. Sterzenbach, U., et al., *Engineered Exosomes as Vehicles for Biologically Active Proteins.* Mol Ther, 2017. **25**(6): p. 1269-1278.

333. Maguire, C.A., et al., *Microvesicle-associated AAV vector as a novel gene delivery system.* Mol Ther, 2012. **20**(5): p. 960-71.

334. Sato, Y.T., et al., *Engineering hybrid exosomes by membrane fusion with liposomes.* Sci Rep, 2016. **6**: p. 21933.

335. Votteler, J., et al., *Designed proteins induce the formation of nanocage-containing extracellular vesicles.* Nature, 2016. **540**(7632): p. 292-295.

336. Yeo, R.W., et al., *Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery.* Adv Drug Deliv Rev, 2013. **65**(3): p. 336-41.

337. Kassis, I., et al., *Mesenchymal stem cells (MSC) derived from mice with experimental autoimmune encephalomyelitis (EAE) suppress EAE and have similar biological properties with MSC from healthy donors.* Immunol Lett, 2013. **154**(1-2): p. 70-6.

338. Dragovic, R.A., et al., *Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis.* Nanomedicine, 2011. **7**(6): p. 780-8.

339. Sokolova, V., et al., *Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy.* Colloids Surf B Biointerfaces, 2011. **87**(1): p. 146-50.

340. Copeland, S., et al., *Acute inflammatory response to endotoxin in mice and humans.* Clin Diagn Lab Immunol, 2005. **12**(1): p. 60-7.

341. Miller, S.D., W.J. Karpus, and T.S. Davidson, *Experimental autoimmune encephalomyelitis in the mouse.* Curr Protoc Immunol, 2010. **Chapter 15**: p. Unit 15 1.

342. Anderson, J.P., et al., *Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease.* J Biol Chem, 2006. **281**(40): p. 29739-52.

343. Willis, G.R., S. Kourembanas, and S.A. Mitsialis, *Therapeutic Applications of Extracellular Vesicles: Perspectives from Newborn Medicine.* Methods Mol Biol, 2017. **1660**: p. 409-432.