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Review

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Exosomes: potential model for complement-stealth delivery systems

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Abstract: Exosomes are nature's nanocarriers that transport biological information in humans. Their structural properties, origin and functions are making them interesting objects for the diagnosis of diseases, such as cancer, and also, as innovative tools for drug delivery. The interaction of exosomes with the immune system has been one of the focal points of interest; nevertheless their "stealth" properties helping to avoid adverse immune reactions are still not fully understood. In this review, after giving an overview of recent findings on the role of exosomes in disease pathogenesis and physiological functions, we focused on their interaction with the immune system and possibilities for clinical applications. The potential of exosomes of creating stealth nanoparticles that are better tolerated by the immune system than the presently available synthetic drug delivery systems represent a promising new approach in nanomedicine.

Keywords: complement; drug delivery; exosome; liposome; nanocarrier.

Introduction: nanocarriers of nature

In the field of bio-engineering scientific efforts often mimic nature (1–4), this can be said of liposomes. When Bangham and Horn first visualized phospholipid bilayers under the electron microscope 50 years ago, they thought they reproduced the bilayer membrane of an archetypical cell (5). They could hardly have suspected that two decades later a more elaborate natural design for liposomes would be discovered, in the form of exosomes. Exosomes were first described in the 1980s by Johnstone et al. (5) and were defined as vesicles formed in the endosomal compartments (multivesicular endosomes) which then get secreted into the extracellular space (Figure 1) to serve as nano-rafts carrying biological information between cells. Hence, they play a central role in intercellular communication (5). Exosomes have been found to originate from various types of cells in the body, including stem cells and fully differentiated cells. Their most important feature as compared to the endosomes is that the extracellular leaflet of the plasma membrane is fully preserved as an extracellular part of the exosomes.

Exosomes are defined by a size ranging from 30 to 100 nm (6). Their exact size differs according to their origin. The structure of exosomes depends to the cell type they originate from, as well as on the function they play in intercellular communication (7). Another name which is used interchangeably is microvesicles, which describe 100–1000 nm vesicular structures. But microvesicles are less precisely defined and cover both intra- and extracellular vesicles while exosomes are only the extracellular vesicles (8). In the following sections we will focus on the structure and function of exosomes and on their interplay with the immune system as well as what role their special properties may play in future medical applications.

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Structure of exosomes

It follows from the mechanism of exosome formation (Figure 1) that the content of exosomes contains mainly cytosol derived molecules, such as miRNA, mRNA, proteins, peptides, enzymes (9–13) and, as confirmed recently, also dsDNA (14, 15). Details about the content and the composition of exosomes can be found on the web

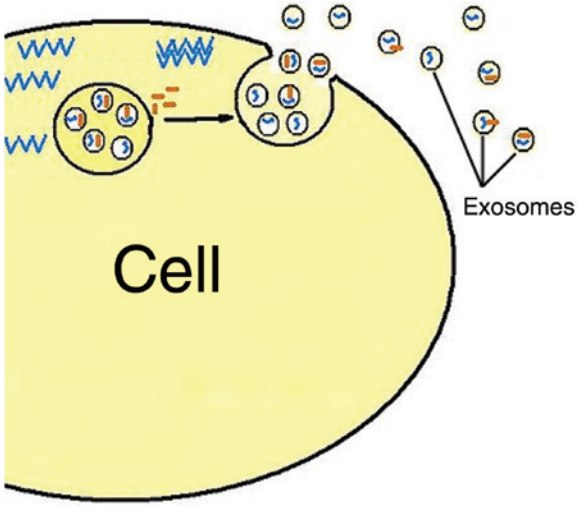

Figure 1: Mechanism of exosome release.

(http://www.exocarta.org/) provided by Mathivanan and Simpson (16). Extensive lists of molecules detected and identified in exosomes and microvesicles can be found in some excellent recently published reviews and research papers (8, 11, 12, 17–19). Figure 2 shows electron-microscopic images of exosomes and microvesicles that differ in content, shape and membrane structure.

The proteins attached to the lipid bilayer of exosomes (arrows) originate from the plasma membrane which is preserved its original orientation. They cover a broad spectrum of immune-modulating and cell recognizing molecules that are either common, ubiquitous proteins or cell-type specific proteins. The former group includes cytoskeletal proteins, such as actin and tubulin, membrane transport and fusion proteins (annexins and Rab proteins), integrins and proteins belonging to the heat-shock family (immune activators such as Hsp70, Hsc70 and Hsp90). The cell-type specific proteins include MHC class-I and class-II proteins, which present antigens, tetraspanins (CD63, CD81, CD82, CD9 and CD86) which are involved in cell-cell contacts and in selective binding to certain target cells (20). As discussed later in detail, the surface exposed proteins have different roles including the targeting of exosomes to specific cells and modulating the immune response via activation or suppression. Additionally it has to be mentioned that the fact that endogenous exosomes are made from fragments of the plasma membrane in the preserved original orientation means that they also inherit the glycome, the glycocalyx from the originating cells with its innate immune tolerance.

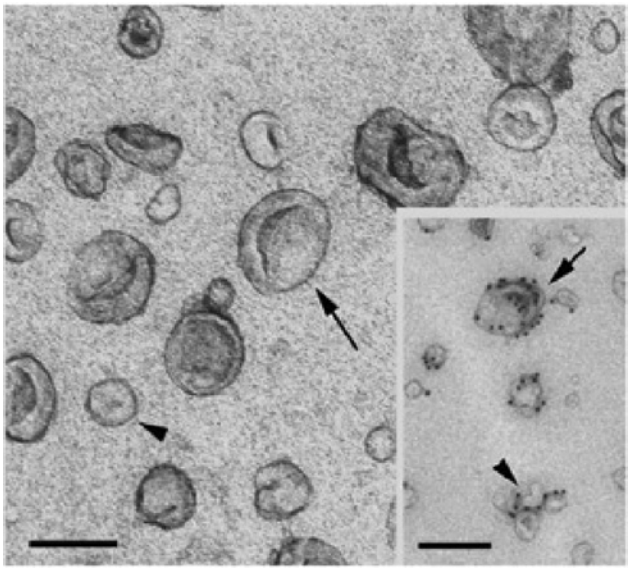

Figure 2: Electron micrographs of dendritic cell derived exosomes and microvesicles. The size bars in the EM images indicate 100 nm. The images were reproduced with permission from (19).

Physiological functions of exosomes

The first exosomes that were documented originated from circulating blood cells, particularly reticulocytes (5). Since then the importance of this phenomenon has been confirmed, further adding to the understanding of red blood cell differentiation (21). Platelets also shed exosomes, some of which may inhibit aggregation and act against thrombosis (22). White blood cell derived exosomes have several functions, the most complex one is the modulation of the immune system. These cell derived nano-vesicles mediate antigen presentation, which is one of the basic mechanisms of adaptive immunity and as such, has been a focal point of interest in immunology research (23). Recent publications revealed very complex roles of exosomes in immune modulation (24). Tracking of exosome release from immune cells was achieved by Soo et al., using antibody connected magnetic beads for visualizing the dynamics of this process (25). Exosome-mediated immune responses in tumor patients (26) represents a special new field that will be discussed below in detail.

Role in disease pathogenesis

Exosomes play a central role in the manifestation and progression of several diseases, as well as in drug resistance,

therefore mapping their functions in intercellular communication is essential in understanding the pathomechanism of these medical problems.

Several recent publications discuss the function of exosomes in oncogenesis, tumor cell exchanges and metastatic activities of tumors. Exosomes can transfer oncogenic materials which affect organization of tumor cells and the progression of a tumor. Intercellular communication can happen through the delivery of genetic information in the form of microRNA that is delivered from tumor cells to normal or pathological cells (27). Exosomes are pivotal in shielding tumor cells from the immune system, and at the same time induce inflammatory and angiogenetic responses helping tumor cell adhesion and metastatic growth (28).

In infections by different pathogens (viral, bacterial, fungal and parasite) exosomes play a key role in several areas. The pathomechanism of oncogenesis after specific viral infections, such as Epstein-Barr virus, has been recently connected to viral RNA carried by exosomes, creating an intercellular pathway for genetic information passage, leading to tumor formation (29). In HIV infection replication of the virus is helped by the presence of exosomes derived from HIV expressing cells (30). Exosomal delivery of virus components and proteins are essential for disease progression in human T-lymphotropic virus type 1 infections (31). The pathomechanism of parasite infections in some cases involves exosomes, where the vesicles can induce adhesion of the pathogen (32).

In neurologic disorders exosomes can be important factors. Exosomal communication between microglia and neurons of the brain is a way for the central nervous system to modulate the pathology of amyotrophic lateral sclerosis, which is the most common and most aggressive form of adult motor neuron degeneration (33). Exosomes can be a reason behind drug resistance in neurological diseases and cancer, an example of this can be found in the treatment of multiple myeloma (34). Exosomes have been identified as mediators of neuroinflammation after injuries of the central nervous system and have been implemented as potential therapeutic agents (35).

In the field of internal medicine the investigations of exosomal pathways present a new perspective on the pathomechanism of several diseases. A recent study found that exosomes may affect glycemic control in diabetes via the adiponectin pathway (36). It has also been observed that exosomes released by pancreatic cancer cells may play a role in the induction of diabetes associated with this type of malignancy (37). Exosomes coming from adipocytes can induce liver pathology as they deregulate hepatocytes, leading to obesity-related liver disease (38).

Exosomes and the immune system

Exosomes are frequently exposed to the immune system both in health and disease and developed or expose mechanisms to avoid recognition, or they modulate the immune system to induce immune tolerance. The interaction of exosomes with the immune system depends on their origin; on antigens derived from diseased cell (e.g., tumor or virus-infected cell), and on immune modulating molecules enclosed in the vesicular container. As to how much exosomes are able to interfere with the immune system was described in detail in a review by Thery (8).

Evidence for the appearance of microvesicular structures and exosomes in the early fetal development was provided for exosomes containing FasL ligand (39). This molecule is involved in many immune modulating activities, such as self-tolerance in T cells towards fetal tissue during gestation, progression of autoimmunity, clonal deletion of activated T cells, B-cell regulation and the establishment of immune privilege in certain organs, such as brain, ovary, testis, pregnant uterus, placenta and eye (40). Two mechanism are proposed as to how the FasL ligand induces immune tolerance: i) apoptosis in the relevant antigen-specific lymphocytes that respond to the administered antigen (41, 42) and ii) the uptake of FasL induced apoptotic cells by antigen-presenting cells (such as dendritic cells) and, in consequence, modulation of the activation of regulatory cells (43). The same mechanism was shown to be hijacked by tumors to induce immune tolerance (39, 44, 45).

Additionally, immune modulation can be gained by release of small (40 nm) exosome-like vesicular structures presenting MHC class II with antigens called "tolerosomes" (46). Another mechanisms for exosomes to modulate the immune system is by delivering microRNA enclosed in the exosomes into the cytosol of other cells.

Despite being effective modulators of immune response, exosomes are not recognized by the immune system as being foreign. Thus, they are not immunogenic and are not known to induce nonspecific innate immune responses, such as complement (C) activation or C activation-related pseudoallergy (CARPA), which is an immune barrier to the therapeutic use of liposomes and many other nanoparticle based i.v. drugs (47). Amazingly, there is no visible immune attack against exosomes when they get into the circulation. The explanation probably lies in the fact that exosomes are derived from the membranes of self-cells, against which the body develops specific and nonspecific tolerance.

The immunity of exosomes from immune destruction is due to an "inheritance" of surface molecules from their original cell that protects them from recognition. As to the

Table 1: Molecules on exosomes allowing escape of immune recognition.

Molecules	Physiologic function	Literature
Complement modulators		
CD46	Membrane cofactor protein; control of C3	41
CD55	Decay-accelerating factor control of C3	42, 43
CD59	Control of MAC (membrane attack complex inducing pore formation) via C9	41, 42
CK2	Phosphorylates C9 and prevents lysis	44
Specific tolerance inducers		
Human leucocyte antigen (HLA)-G class I molecule	Induces immune tolerance to tumors	45
MHC class I and II +antigens	Induce immune tolerance to tumors	46
FasL	Induce apoptosis in activated T cells	44

question, which surface molecules might provide this property, Table 1 may give the answer, that is further visualized by Figure 3. It shows the molecules present on the surface of exosomes, which include 4 C inhibitors, each of which is known to be effective inhibitor of C activation. In addition, the surface of exosomes contain three molecule types that effectively induce specific tolerance.

As mentioned before, another immune interacting aspect of exosomes that should not be underestimated is the fact that the exosome lipid membrane derives from endogenous cell membranes and the outer leaflet of the plasma membrane is also the outer leaflet of the exosomes. With this orientation they inherit the immune tolerance induced by the glycocalix of the cells. In several works the importance of the glycome for innate immunity (48, 49) and the recognition of different or foreign glycan patterns by the immune system (49–51) were emphasized. That the disguise of the immune system is most effective if the glycome is similar is supported by the observation about the immune evasion of HIV-1 and their glycome similarity with microvesicles. HIV-1 and microvesicles from T cells share a common glycome, arguing for a common origin (48).

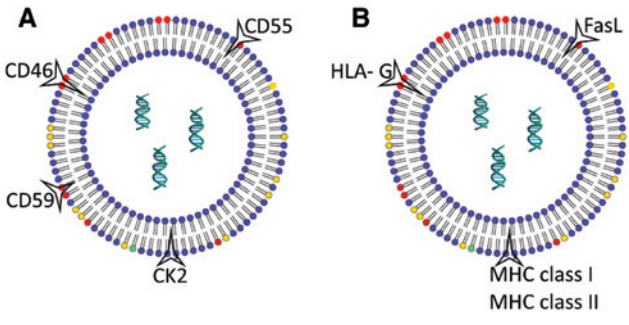

Figure 3: Exosomes with complement system modulator (A) and other immune-tolerance promoting molecules (B).

Potential of exosomes for clinical applications

There are several approaches that make use of the specificity and function of exosomes in the intercellular communication of tumors. An area of increasing interest in the field of oncology is cancer immunotherapy, where exosomes have been shown to have a therapeutic potential (52, 53). Exosomes containing antigens of specific tumors can induce an immune response against cancer cells containing their antigen spectrum. These acellular structures can activate cytotoxic T cells which leads to apoptosis of the tumor cells they are directed against. Creating vaccines based on specificity of tumor-derived exosomes which contain the biomarkers necessary to target tumor cells can be a realistic option for immunotherapy (54–58). One possibility was outlined in a recently published article using exosomes derived from dendritic cells to induce antitumor immunity (59). The tumor derived exosomes present a first therapeutic vaccine with antigen-presenting dendritic cell derived exosomes approved in 2010 by the name of Provenge® for the personalized treatment of metastatic prostate cancer (60). So far it is the only FDA approved exosomes based cancer immune therapy.

The discussion about the medical benefit of Provenge® was controversial. The best benefit for the patients was found for low PSA (prostate specific antigen) levels. In a 512 patients double-blind, placebo-controlled, multicenter phase 3 trial study a general improved survival in the treated patients was found to be 4.1 months with no effect on the tumour progression or decrease PSA levels (61). This improved survival was the reason for approval by the FDA and in 2013 also by EMA (European Medical Agency) and seems to be closely related to an activated immune system. There is still a critical discussion about the usefulness of PSA levels as prognostic markers in prostate cancer and if the high price of the treatment is justified but the approach to use dendritic cell derived exosomes was followed up in other clinical trials for the standalone treatment or combination therapy for other types of cancer (62).

Another option for a cancer therapy involves the general removal of exosomes from the circulation (6). This idea stems from the fact that exosomes play an important role in the pathomechanism of tumor progression and can also interfere with anti-tumor chemotherapy and lead to drug resistance. But one of the drawbacks is that the approaches used so far remove all exosomes, also the ones released from healthy cells and involved in cell-cell communications leading to adverse effects. A possible solution for this problem might be the selective removal of tumor-derived exosomes. With increasing knowledge about the mechanisms of immune escape and immune modulation of cancer derived exosomes it may be possible to identify molecules only present on the surface of these exosomes.

Exosomes for diagnosis and follow-up of medical conditions

Understanding the function of exosomes in the pathomechanism of diseases is an important step towards creating diagnostic strategies based on detecting disease-specific exosomes. Structural properties of these nanocarriers can be harnessed as biomarkers for various diseases, which can be detected in several ways. This strategy has been recently implemented in the following cases.

Diagnosis, risk assessments and follow-up examinations of laryngeal squamous cell carcinoma can be achieved by using exosomal biomarkers specific to the tumor cells (63). In patients with colorectal carcinoma, exosomes that contain tumor-specific antigens can be extracted from ascetic fluid or the plasma. These entities may be used as a fast and effective diagnostic tool (64, 65). In pancreatic cancer, the protein and microRNA content of tumor-specific exosomes can be analyzed in order to increase the effectiveness of diagnosis and complement available diagnostic strategies (66). Exosomes that contain different cargos and receptors, like epidermal growth factor receptor, in their structure can be used in the diagnosis of lung cancer (67, 68).

Exosomes and their microRNA contents are important factors in the pathomechanism of *asthma bronchiale* and other pulmonary conditions, where exhaled breath can be a source of exosome extraction. This has a potential to create an easy way of quantifying disease specific biomarkers for the follow-up controls of pulmonary diseases and also a possible diagnostic tool (69).

A recent review gives a concise overview of the clinical applications of exosomes (70). Another appealing application of exosomes is their use as a stealth drug delivery system which we will discuss in the next paragraph.

Exosomes as immune-tolerated delivery systems

Mapping tumor-specific exosomes in the body is important for diagnosis, but using their structural and "stealth" properties is a promising solution for creating novel highly specific immune-tolerated therapeutic systems. As mentioned before this approach failed if using synthetic liposomal delivery systems which can cause immune activation to an extent that can be fatal for the patient (71, 72). Creating a stable formulation that mimics the structure and function of exosomes is a complex task and has to be the result of careful consideration of previous trials and errors in the field of nanomedicine (73, 74).

Tumor cell derived exosomes that are synthesized based on the tumor cell's antibody spectrum also present a novel delivery solution. Hereby exosomes loaded with different agents (such as diagnostic particles or drugs) specifically bind to the target cells creating a therapeutic accumulation in a specific area. Modeling of the exosomal structure has been already proven to work on a large-scale basis (75). In a recent publication the potential use of exosomes combined with staphylococcal enterotoxin B in the therapy of pancreatic cancer was described (76). Exosomes were extracted from the tumor and based on their specific antigen biomarker could reach tumor sites, where the enterotoxin was unloaded and induced apoptosis of tumor cells.

There is an important feature of tumor-derived exosomes that needs to be kept in mind and needs close monitoring during their use for treatment and that is their involvement in preparing a niche for circulating cancer cells in the proccess of metastasis formation in distant tissues (77–80). While this property is of minor importance for cancer immune therapy it can be important for drug delivery as the equipment of molecules on the exosomes surface can affect auto-immunity and binding to unknown distant organs (81, 82). But Jung et al. also showed that for the pre-metastatic niche and cancer formation some soluble factors and circulating cancer cells are required (78).

There are also several medical fields, other than oncology, where the potential therapeutic use of exosomes may be possible. A recently published article in the field of cardiology pinpoints microRNA containing exosomes as the mediators of cardiac muscle regeneration after ischemic stress. This phenomenon can be used in direct therapy,

if these particles can be engineered ex vivo before being given to the patients directly (83).

Another important field were the immune modulating properties of exosomes were explored for therapeutic applications are dendritic cell-derived Fas L contain and/or antigen presenting exosomes for inflammatory diseases such as arthritis (84).

There are several ongoing preclinical studies on the therapeutic uses of exosomes, which show the potential for clinical use in the treatment of several medical conditions (85). While dendritic cell-derived exosomes were mainly used to induce immune response other cell type released exosomes were used for their potential as a stealth drug delivery system. Lai et al. reviewed e.g. the utility of mesenchymal stem cell-derived exosomes as a vehicle for drug delivery (86). In this review Lai and his co-worker identified the loading of the exosomes without disturbing their unique stealth properties as the major obstacle for the use as drug delivery system mainly for interfering RNA. The loading can be done during exosomes biogenesis in the cells or afterwards by electroporation or incubation with lipofectamine. Both techniques are likely to change the membrane of the exosomes as lipofectamine® is known to decrease cell viability as a positively charged polymer by nanoporation of the plasma membrane and depolarization of the mitochondrial membrane (for a review see e.g. the effect of surface properties on nanoparticle-cell interactions (87). As the interaction between membrane and polycation is electrostatic it is unlikely that the effect is reversible and a resealing will take place.

On the other hand electroporation was successfully investigated for some types of siRNA while for other type of RNA such as mRNA, miRNA or shRNA the approach was unsuccessful. A third strategy which was tested was the overexpression of RNA by the exosomes producing cells which allows even the incorporation of fully functional miRNA and mRNA (88).

That exosome loading with drug molecules or therapeutic proteins, peptides, and hormones is feasible is clearly indicated by the negative example of the

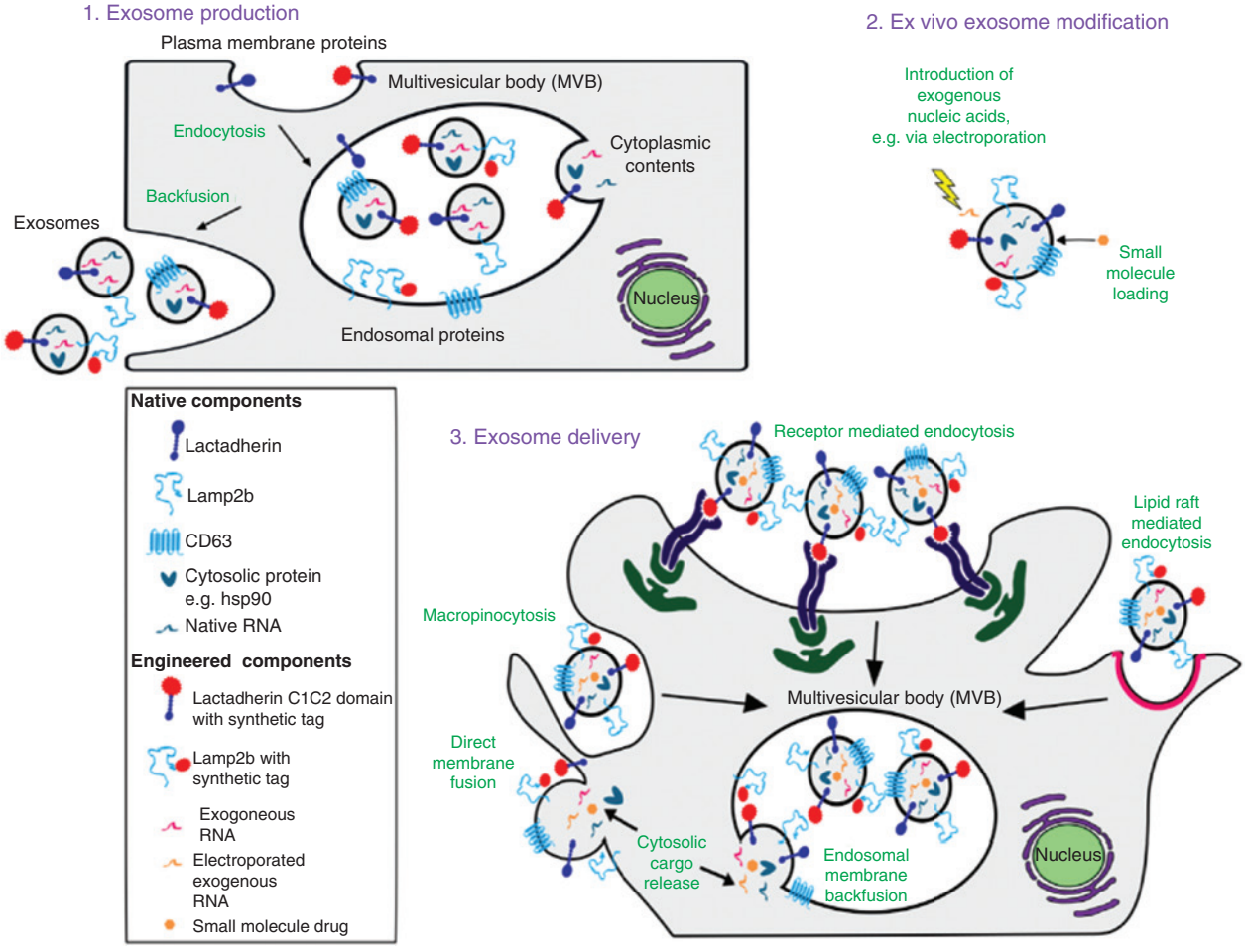

Figure 4: Exosomes based drug delivery (reproduced from the work of Marcus and Leonard) (88) (Permission has been granted by the authors).

Table 2: Exosomes based drug delivery [reproduced from Marcus and Leonard 2013 (88)] (Permission has been granted by the authors).

Exosome source (H=human cell)	Recipient cell type	Cargo delivered	Functional consequences
Immunosuppressive effects			
(H) EBV transformed B cells	Monocyte-derived DC	Viral miRNA	Down-regulation of antiviral response
Serum of pregnant human patients	(H) Jurkat T cells	FasL	Suppression of CD3 signaling and IL-2 production
Murine BMDC overexpressing IL-10	Murine T cells	Antigen, presented on MHCII	Suppression T cell proliferation
Immunostimulatory effects			
Murine BMDC	Murine CD8 and CD4 T cells (in vitro and in vivo)	Antigen, presented on MHC	Induction of T cell proliferation
CD28 stimulated CD3 T cells (H)	Unstimulated CD3 T cells (H)	Unidentified	T cell activation, induction of proliferation and cytokine production when co-delivered with IL-2
Murine BMDC	Murine BMDC (allogeneic)	Antigen	Transfer of foreign antigen, followed by foreign antigen presentation to and activation of T cells
PMBC derived DC incubated with HIV	Jurkat T cell line expressing CCR5	HIV viral particles	Delivery of functional HIV viral particles encapsulated in exosomes, leading to HIV infection of recipient cells

pathogenic exosome-loading of prion (89), or other disease-related proteins (90). An overview summarizing the progress made so far for exosomes as a drug delivery system mainly for therapeutic RNA in cancer therapy was published by Johnsen et al. (91) and even for a broader application by Marcus and Leonard, which is visualized in Figure 4 (88) and Table 2 (88).

But the first step in using the exosomes, both for diagnosis and for therapeutic applications is the isolation of the intact exosomes from body fluids such as urine (92), blood, semen (93) and others (9, 11, 94) which will be described with detection and characterization methods in the next paragraph.

Exosome isolation, detection, and characterization

In order to be able to explore not only the diagnostic value of exosomes derived from blood but also to produce and load exosomes from exogenous sources the process of isolating exosomes from surrounding biomaterial (such as plasma or interstitial fluid) is essential in being able to coherently investigate their structure and functions.

The extraction process can be achieved using several methods. Ultracentrifugation onto a sucrose cushion is a simple way of separating the vesicles. Ultracentrifugation can be followed by filtering the exosomes through a porous membrane. A recently published report shows another easy and efficient way of separating exosome populations from a sample that is called ExoQuick™ (95–98). Reproducibility of isolation methods is an important factor in exosome research and recent approaches, such as an effective semi-automated nanoparticle tracking analysis, have shown developments in this field (99).

Dynamic light scatter measurements, flow cytometric analysis, microspectroscopy and electron microscopy examination of exosome containing samples is an option for the characterization of specific exosomes. Other imaging technologies that have also been implemented for such use include micro nuclear magnetic resonance, and X-ray scattering (99–102). Finding the origin of a population of exosomes extracted from a sample can be achieved by analyzing the specific marker antigens on the surface of the exosome. A recent approach has been to use a micro-fluidic exosome analysis platform that enables identification using disease-specific markers (103).

Summary and outlook

In the past 10 years exosomes raised high expectations for a better understanding of long distance cell-cell

communication, development and progress of several severe diseases such as cancer and neurodegeneration due to protein oligomers and aggregates. Now it is commonly accepted that exosomes are involved in intercellular cell transport of proteins, peptides, RNAs and dsDNA. The molecules which allow the exosomes to evade immune recognition are identified and they are either immune suppressive or immune modulating by interaction with different complements.

In order to adapt the immune-stealth properties for beneficial use such as drug or gene delivery several techniques were explored either to produce autologous exosomes for cancer immune therapy but more interestingly to produce in large scale drug- or RNA-loaded exosomes from stem cells. While the main focus has been on identifying the immune modulators so far little attention has been given to the glycome. If exosomes are produced by exogenous cells, the glycome may not be similar and immune recognition may occur. The fact that synthetic liposomes cause immune response and CARPA can also be related to the mismatch of the glycome or the absence of it which induces the strong immune stimulation.

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Three fields stand out in his career where he has been most active: liposomes, artificial blood and the complement system. Among others, he described the protective role of cholesterol against heme distribution into biomembranes and he has been a main promoter of the CARPA (complement activation-related pseudoallergy) concept highlighting the causal role of complement activation in numerous drug-induced hypersensitivity (anaphylactic) reactions, including those caused by liposomal and micellar drugs. Along with numerous social commitments in Hungary and abroad, he is a founder and scientific director of an immune toxicity CRO in Hungary (SeroScience Ltd).

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Since 2011 Silke Krol has been with the 2009 funded Center of Nanotechnology@Fondazione I.R.C.C.S. Istituto Neurologico "Carlo Besta" in Milan, Italy. She is now studying the transport mechanisms for differently functionalized gold nanoparticles across the blood-brain barrier and how this is influenced by blood derived proteins. Moreover different novel metallic and non-metallic delivery systems for various other diseases (cardiovascular, prion disease, epilepsy, glioma, lymphomas, viral diseases) were designed for projects funded by Italian and European foundations. Her group develops multifunctional polymer/nanogold based drug or drug delivery systems as well as diagnostic tools for medical applications. Moreover, the multilayer-nanocoating is used for encapsulation and immune protection of living cells like, e.g. pancreatic islets. She has several pending patents for possible future drugs for prion disease and cancer treatment, viral diseases, and cancer diagnostics.

She occasionally lectures as a contract professor for "Nanomedicine" at the University of Udine and Trieste since 2008 and as guest lecturer for "Nanotoxicology". In 2009 she worked as an expert consultant for the United Nations and serves as external expert reviewer for National projects in France, Italy, Georgia and Greece. Recently she was announced as project technical advisor for three EU-FP7 projects. She is member of the advisory board of the CLINAM-Foundation of the journal *Euro-Nanotox-Letters* and the international advisory committee of the International scientific spring conference in Islamabad, Pakistan. She is an associate editor of **Frontiers in Nanobiotechnology** and an adjunct faculty member at the Pakistan Institute of Engineering and Applied Science. Recently she became a consultant and Member of General Scientific Advisory Board at *Midatech* Pharma PLC. She serves as an external expert reviewer for national projects in France, Italy, and Greece. She frequently reviews for **Nanoscale, Nanomedicine, Nanoletters**, and others.