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# The Application of Membrane Vesicles for Cancer Therapy

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## 1. Introduction

Cancer, a leading cause of death globally, is projected to become more prevalent in coming years due to increasing life expectancy. Among possible treatment options, the use of biomarkers especially has shown great potential as a gateway to personalized medicine, and in turn, great promise for improved diagnosis, prognosis and treatment of cancer and other diseases. However, the use of biomarkers in cancer therapeutics has thus far exhibited mild success, at best. The most common tool used for biomarker discovery is plasma proteomics due to the accessibility of blood samples and the rapid development of highly sensitive proteomics technologies. However, other body fluids such as urine may also be a reservoir for important marker proteins.

Extracellular membrane vesicles ranging in diameter of 30-1000 nm and originating from various cellular origins have been increasingly recognized for their participation in a variety of both normal and pathological cellular processes. Regardless of their cell type of origin these membrane bound vesicles or exosomes provide a protected and controlled internal microenvironment outside the cell for metabolic objectives of the host cell to be carried out at a distance from the host cell. They are also believed to be instrumental in cell-cell and cell-extracellular communication. Moreover, while knowledge of exosome biogenesis and physiological relevance remains limited, accumulating evidence suggests that their bioactivity may be clinically applicable in cancer therapeutics. One recent work suggests that the use of exosomes in immunotherapy may prime the immune system to recognize and kill cancer cells and thus could form a viable basis for the development of novel cancer vaccines.

This chapter will review current knowledge pertaining to exosomes, describe possible uses of exosomes in immunotherapy, and address challenges and future directions in bringing exosome-based cancer vaccines or immunotherapies closer to clinical reality.

## 2. Exosome biogenesis, secretion and physiologic considerations

Since their description in the process of reticulocyte maturation almost a quarter century ago (Johnstone *et al.*, 1987), exosomes; the intraluminal vesicles (ILV's) of multivesicular bodies (MVB's), have gained significant notoriety as evidenced by the nearly 10-15 fold rise in the

amount of publications devoted to the subject just within the past decade (Raimondo *et al.*, 2011).

Many types of vesicles have been described in the literature having quite heterogeneous size, protein content, RNA content and origin. As a result there have been many names given to these different vesicle types (**Table 1**) (Ronquist & Brody, 1985; Ronquist & Frithz, 1986; Rooney *et al.*, 1993; Arienti *et al.*, 1997; Thery *et al.*, 2006; Lehmann *et al.*, 2008; Schiller *et al.*, 2008; Simpson *et al.*, 2008; Skog *et al.*, 2008; Dashevsky *et al.*, 2009; Di Vizio *et al.*, 2009; Haubold *et al.*, 2009; Jansen *et al.*, 2009; Nilsson *et al.*, 2009; Duijvesz *et al.*, 2010). Unfortunately, the different names given to these vesicles lead to much confusion and it is still unclear if all of the different vesicles are unique in biological function or if they represent a sliding scale of one entity (Duijvesz *et al.*, 2010). Exosomes are nanometer sized lipid bound vesicles derived from late endosomes contained in MVB's. They are characterized by their size 40-100nm, density ranging from 1.13g/ml to 1.19g/ml on a sucrose gradient (Record *et al.*, 2011) and their specific protein content (heat shock proteins, tetraspanins, Rab proteins, etc) **Figure 1**. Exosomes are secreted by both hematopoietic (eg dendritic cells, lymphocytes, mast cells) and non-hematopoietic cells such as fibroblasts, intestinal epithelium, neurons and various tumor cells. Exosomes have been isolated from serum and other biological fluids such as urine, ascitic fluid, amniotic fluid and even cerebrospinal fluid (Vella *et al.*, 2008). Exosome biogenesis involves an inward budding of the limiting membrane of late endosomes, also known as multivesicular bodies (MVBs) (**Figure 2**), concurrent protein sorting into these budding exosomes, and subsequent splitting (scission) of these microvesicles (Keller *et al.*, 2006). As a consequence, the nascent exosome contains the inner leaflet of the limiting membrane, membrane associated proteins, both native and recruited, and some amount of cytosol and cytosolic proteins.

Vesicle	Size (nm)	Marker Proteins	Function	Reference
Exosomes	50-150	CD9, CD63, CD81, CD82, annexins, and RAB proteins	Antigen presentation, immune regulation, metastatic activity	Jansen FH, et al., 2009; Nilsson J, et al., 2009; Simpson RJ, et al., 2008; Thery C, et al., 2006
Prostasomes	50-500	CD13, CD46, CD55, CD59, annexins, and RAB proteins	Immunosuppressive and sperm cell motility improving	Ronquist G, et al., 1985; Arienti G, et al., 1997; Ronquist G, et al., 1986; Rooney IA, et al., 1993
Oncosomes	50-500	Signal transduction proteins	Function unknown	Di Vizio D, et al., 2009
Microvesicles	100-1000	Integrins, selectins, CD40 ligands	Procoagulation and anticoagulation	Skog J, et al., 2008; Lehmann BD, et al., 2008
Ectosomes (microparticles)	50-1000	CR1 and proteolytic enzymes, no CD63	Procoagulation and anticoagulation	Haubold K, et al., 2009; Dashevsky O, et al., 2009; Duijvesz D, et al., 2011
Membrane Particles	P4: 50-80 P2: 600	CD133, no CD63	Stem cell marker, possible role in tissue development and maintenance	Marzesco AM, et al., 2005
Exosome-like Vesicles	20-50	TNFR1	Constitutive production of soluble cytokine receptors may be regulated	Hawari FI, et al., 2004
Apoptotic Vesicles	50-500	Histones H1, H2A, H2B, H3 and H4 as well as DNA & RNA, no cytochrome c, p53 or Hsp70	Autoantigen seclusion/isolation	Thery C, et al., 2001; Schiller M et al., 2008

Table 1. Characteristics of different types of microvesicles.

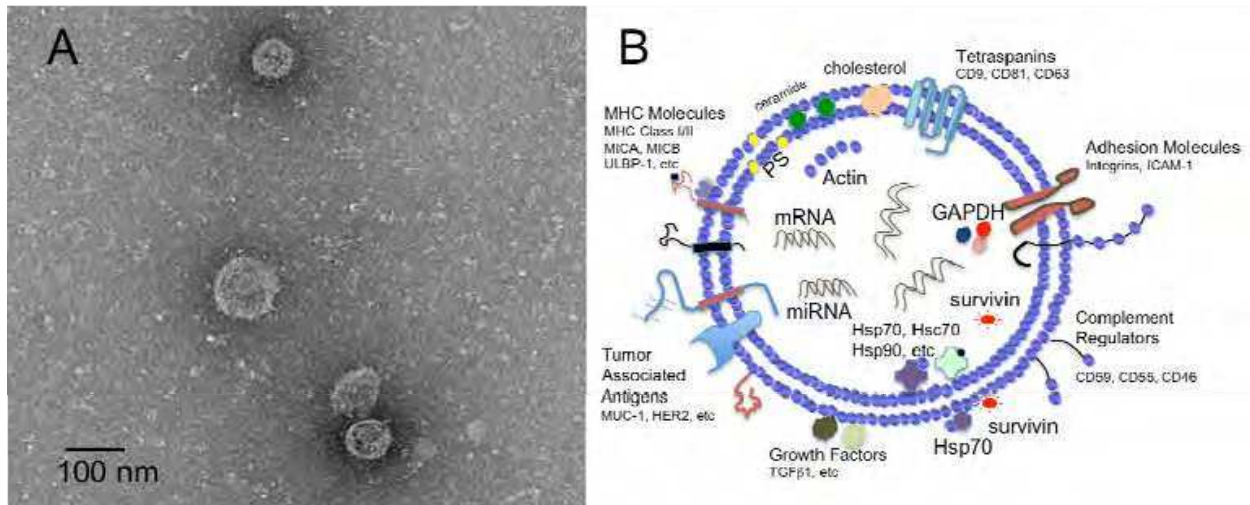


Fig. 1. Tumor derived exosomes (TEX). A. Electron microscopy revealed 50-100 nm sized microvesicles (Khan *et al.*, 2011). B. Diagram depicting a typical TEX (modified from JP Mitchel, exosomes in cancer immunology).

The presence of specific proteins within and on the vesicle suggest the existence of a protein sorting mechanism during its formation. Some of these proteins are shared by exosomes derived from different sources and used to identify these vesicles during proteomic analysis. In our laboratory, we have consistently used the lysosomal associated membrane protein LAMP1 (common to exosomes from a wide variety of cells) as a positive control for exosome presence in western blot analysis (Khan *et al.*, 2011).

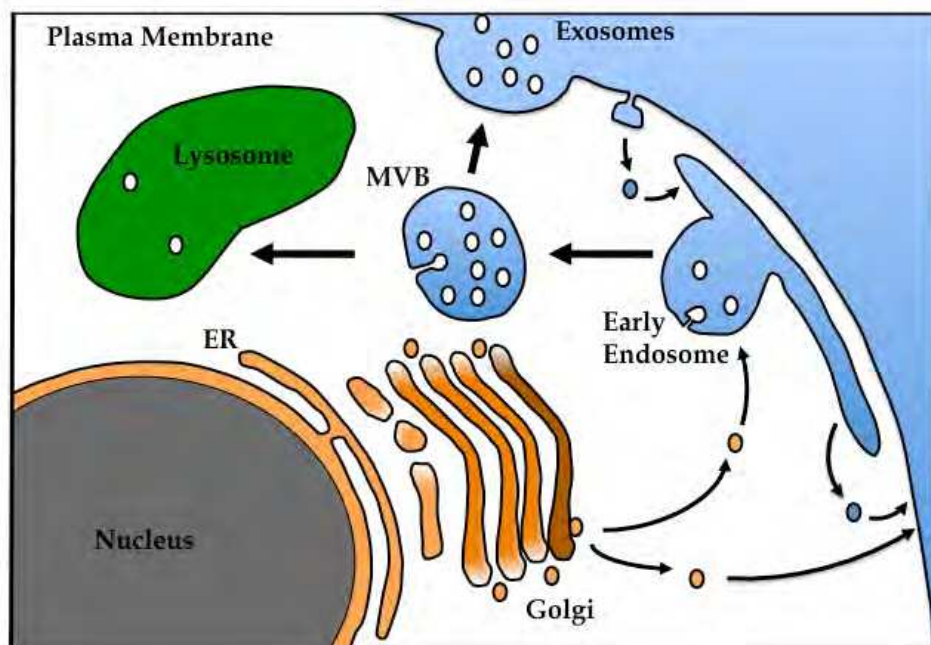


Fig. 2. Protein loading and release by exosomes. Endocytosed surface proteins as well as a subset of cytosolic proteins are taken by the endosomal system. Those destined for release as exosomes or for lysosomal degradation are sorted into luminal vesicles called multivesicular bodies (MVB).

### 2.1 ESCRT-dependent protein sorting

The specific protein content of exosomes can be exploited to identify these exosomes and the cell-types from which they are derived via proteomic analysis. The fact that the exosome's protein content can be used as an identifier suggests the existence of a protein sorting mechanism during its formation. Endosomal Sorting Complex Required for Transport (ESCRT), comprised of a series of three protein complexes: ESCRT I, ESCRT II, and ESCRT III (Figure 3), is suspected to play a critical role in sorting proteins into exosomes at the endosomal limiting membrane.

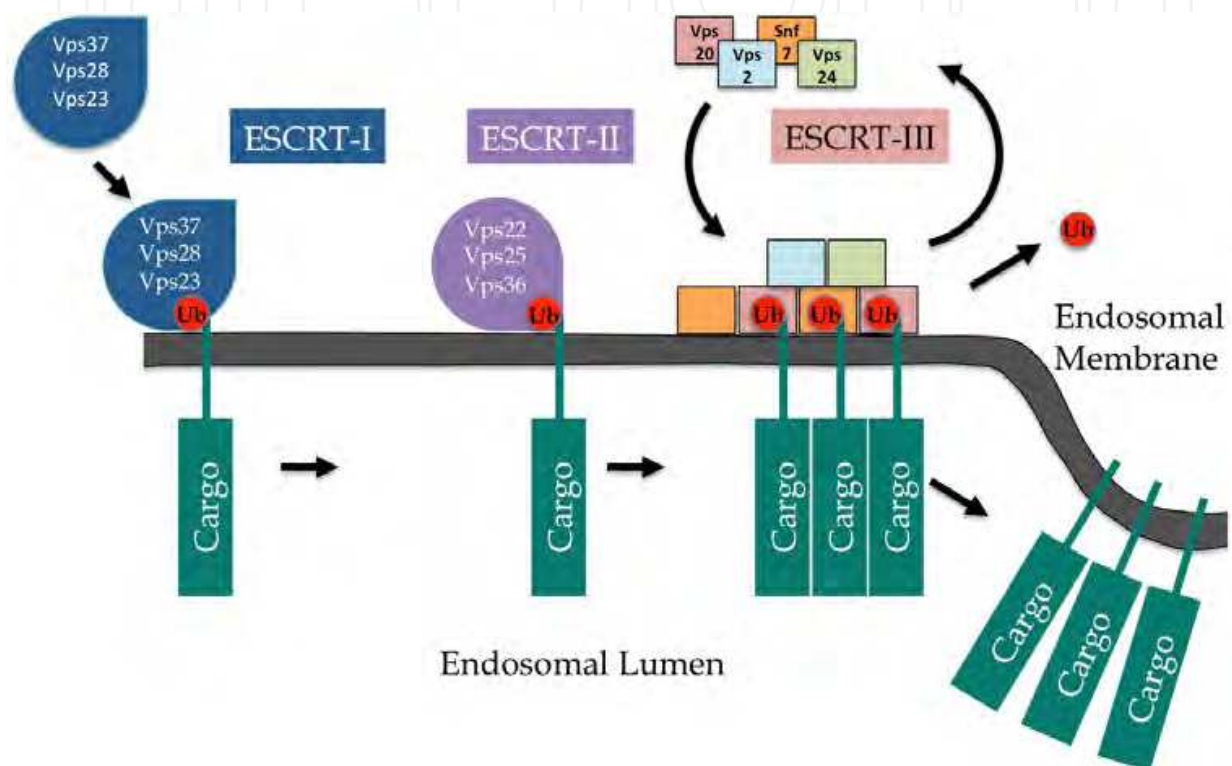


Fig. 3. Ubiquitin marks cargo proteins for endosomal sorting.

Monoubiquitination appears to mark cargo proteins for protein sorting within the MVBs. In yeast, recruitment of ubiquitinated cargo proteins depends on a class of proteins known as class E Vps (Vacuolar Protein Sorting) proteins that have been highly conserved from yeast to mammals—at least one mammalian homolog has been identified for each yeast class member (Babst, 2005). In the yeast model, Vps 27, is thought to complex on the endosomal membrane's outer leaflet with clathrin and ubiquitin-binding proteins, such as Golgi-associated,  $\gamma$ -adaptin homologs, Arf-binding (GGA) proteins, to form an endosomal clathrin coat that recruits monoubiquitinated cargo to the inner leaflet of the endosomal membrane. This complex recruits the ESCRT I complex from the cytoplasm and transfers to it the ubiquitinated cargo. ESCRT I then activates ESCRT II, which in turn occasions the oligomerization of Vps 2, Vps 20, Vps 24, and Snf 7 to form the ESCRT III complex and transfers to ESCRT III the ubiquitinated cargo. ESCRT III continues to accumulate and concentrates the cargo into still-budding exosomes of the MVB (Babst, 2005). The deubiquitinating protein Doa4 is then recruited into the complex where it functions to remove the ubiquitin from the cargo protein (Luhtala & Odorizzi, 2004). The ESCRT III

complex subsequently dissociates from the cargo when the ATPase Vps 4 binds, thus releasing the cargo protein into the budding exosome.

## 2.2 Other modes of protein sorting

Other exosomal proteins, for which no ESCRT mechanisms have been described, may be incorporated into the exosome via varying levels of co-sorting. For example, tetraspanins may sort into exosomes due to their affinity for exosomal membrane components like sphingolipids and ceramides and may follow these phospholipids to further aggregate into lipid raft domains (de Gassart *et al.*, 2003). Protein-protein associations also contribute to co-sorting. MHC Class II molecules can associate with tetraspanins and be co-sorted into exosomal membranes (Blanc & Vidal, 2010). Some evidence also suggests lipid co-sorting into exosomes. The exosomal lipid LBPA (Lysobisphosphatidic Acid) can sort into exosomal membranes by interaction with the exosomal protein Alix. When present in media, LBPA was also shown to assist with the formation of vesicles in the MVBs (Matsuo *et al.*, 2004).

Hematopoietic cells including dendritic cells, lymphocytes and mast cells, and non-hematopoietic cells such as fibroblasts, intestinal epithelial cells, neurons and various tumor cells secrete exosomes. Exosome release from cells may follow a constitutive versus an inducible mode of secretion. The constitutive pathway utilizes the trans-golgi network, after which the vesicles travel through the cytoplasm via an intricate tubular network and eventually are released into the extracellular space (Ponnambalam & Baldwin, 2003). The inducible mode involves the release of preformed vesicles contained in MVBs. Such release may be dependent on known cellular triggers of vesicle release, such as increased intracellular calcium levels (Savina *et al.*, 2003). Other triggers, such as cellular depolarization, have also been described. These events culminate in the fusion of MVB membrane with the cell membrane and subsequent exosome release into the extracellular space.

Exosomes interact with target cells via specific receptors present on these target cells (Losche *et al.*, 2004). The exosome thus exerts its effect either via receptor-receptor interaction or internalization of the exosome with subsequent interaction of the exosome content with the recipient cellular machinery (Record *et al.*, 2011).

As will be described in other sections of this chapter, exosomes influence immune cells both in normal and abnormal states such as cancer. Furthermore RNA contained in exosomes could be translated within the recipient cell and as a result exert an epigenetic influence (Baj-Krzyworzeka *et al.*, 2006). In this regard, exosomes mimic viral particles by directing host cellular processes to its advantage. The role of exosomes in inflammatory conditions has recently been questioned after platelets and macrophage derived microparticles/exosomes were found in the lipid core of atherosclerotic plaques (Leroyer *et al.*, 2008). It is of interest to better understand the precise pro-inflammatory and thrombotic roles of exosomes and potentially use them as therapeutic targets in these conditions. Finally, the discovery of exosomes in urine has opened up the possibility of exosomal proteins being used as biomarkers for disease. It was recently shown that decreased levels of exosomal aquaporin-1 correlates with renal ischemia-reperfusion injury (Sonoda *et al.*, 2009). What makes this prospect exciting is the relative abundance of urine that can be obtained without an invasive procedure.

## 2.3 Summary

Exosomes are small membrane bound vesicles of endocytic origin that are released by most cells. The purpose for release seemed at first to be to discard membrane proteins and those

believed to be resistant to lysosomal degradation. Now, it is believed that they mediate intercellular communication without the need for direct cell-to-cell interaction.

### 3. Existence of secreted membrane vesicles in cancers

Tumors are known to shed membrane vesicles (Taylor & Gercel-Taylor, 2005). In particular, human and mouse tumor cells have been shown to secrete tumor cell-derived exosomes (TEX), constitutively into the extracellular space (Wolfers *et al.*, 2001). The morphology, density and certain membrane markers expressed, such as LAMP1, MHC class I, HSP70 and HSP80, on the released TEX are similar to the dendritic cell-derived exosomes (DEX) (Andre *et al.*, 2002b) which will be discussed later. Despite similarities to DEX, there are differences in the molecular profiles and biological roles of TEXs, both of which give an indication of the cell of origin (Wieckowski & Whiteside, 2006). The specific protein content found on and within exosomes not only reflects their origin, but in addition, establishes their functional role (Zitvogel *et al.*, 1998) (**Table 2**). TEX secreted from neoplastic cells express diverse tumor antigens, which signifies the type of tumor cells from where TEXs were released (Iero *et al.*, 2008). *In-vitro*, it has been shown that TEX released from breast carcinoma cells contain HER2, while carcinoembryonic antigen (CEA) was found in the exosomes secreted from colon carcinoma cells, and proteins MelanA/Mart-1 and gp100 that are expressed in melanoma cells are found on the released TEX (Andre *et al.*, 2002b; Andreola *et al.*, 2002). This phenomenon is also evident *in-vivo*, where plasma from cancer patients contain membrane vesicles that are characterized by the expression of tumor antigens which reflect the tumor of origin (Hegmans *et al.*, 2004; Mears *et al.*, 2004).

When immunocompetent and nude mice were pre-treated with murine mammary TEX, an accelerated growth of the tumor was observed (Liu *et al.*, 2006). This observation led to various studies to try to elucidate the role of secreted membrane vesicles in cancer. TEX can be described as “multi-purpose carriers” which have important roles in the communication, protection, as well as the exchange of genetic information with neighboring cells (Nieuwland & Sturk, 2010). The production and secretion of TEX is important for the tumor. They serve a protective function, have a supportive role in the survival and growth of the tumor cells, are involved in the promotion of host tissue invasion and subsequent metastasis, and facilitate evasion from the immune response (Valenti *et al.*, 2007; Anderson *et al.*, 2010). Acting in a paracrine fashion, the diverse function of TEX is speculated to be due to the various bioactive molecules found within and on the vesicles having a strong influence on the surrounding environment (Hegmans *et al.*, 2004; Mears *et al.*, 2004; van Niel *et al.*, 2006; Iero *et al.*, 2008).

The promotion of angiogenesis is due in part to the upregulation of vascular endothelial growth factor (VEGF) (Skog *et al.*, 2008) and release of matrix metalloproteinases (MMPs) in neighboring, even distant endothelial cells, which are brought by TEX containing tetraspanin family members (Gesierich *et al.*, 2006), epithelial growth factor receptor (EGFR) (Al-Nedawi *et al.*, 2009), platelet-derived tissue factor (TF) (Osterud, 2003) or developmental endothelial locus-1 protein (Hegmans *et al.*, 2004). TEX has also been implicated in the further growth of tumor by the exchange of genetic material. mRNA was detected within exosomes released from glioblastoma cells. Neighboring microvascular endothelial cells that take up the exosomes and translate the mRNA become liable for further tumor growth leading to the stimulation of angiogenesis (Skog *et al.*, 2008). In addition, tissue invasion and stromal remodeling can be facilitated by proteases and MMP transport and release via exosomes (Ginestra *et al.*, 1998; Graves *et al.*, 2004).

Cancer Type	Protein	References
<b>Breast adenocarcinoma</b> (BT-474 MDA-MB-231, TS/A, H-2, P815)	ERBB2*/HER2*	(Andre <i>et al.</i> , 2002b; Koga <i>et al.</i> , 2005)
<b>Cervical Cancer</b> (heLa)	Survivin, HSP 70, HSP 90	(Khan <i>et al.</i> , 2011)
<b>Colorectal Cancer</b> (LIM1215, HT29, SW403, 1869COL, AND CRC28462, LIM1215)	GPA 33*, CEACAM5*, EFNB1* annexins, ARFs*, Rabs*, ADAM10*, CD44*, NG2 ephrin-B1, MIF*, b- catenin, junction plakoglobin galectin-4, RACK1*, and tetraspanin- 8, FASL* AND TRAIL* FASLG* TNFSF10*	(Andreola <i>et al.</i> , 2002; Abusamra <i>et al.</i> , 2005b; Huber <i>et al.</i> , 2005; Taylor & Gercel-Taylor, 2005; Choi <i>et al.</i> , 2007; Simpson <i>et al.</i> , 2009)
<b>Glioma Cancer</b>	EGFR*	(Al-Nedawi <i>et al.</i> , 2009)
<b>Melanoma</b> (Fon and Mel-888)	TRP*GP100*MART-1* Mel-CAM*FASLG*TNFSF10* A33*, CEA, EGFR*, ADAM10*, dipeptidase 1 ephrin-B1, hsc70, tetraspanins, ESCRT proteins (integrins, annexins, Rabs, and GTPases)	(Andre <i>et al.</i> , 2002b; Mears <i>et al.</i> , 2004; Abusamra <i>et al.</i> , 2005b)
<b>Mesothelioma</b>	PLVAP*	(Hegmans <i>et al.</i> , 2004)
<b>Ovarian</b>	ERBB2*	(Andre <i>et al.</i> , 2002b; Andreola <i>et al.</i> , 2002; Koga <i>et al.</i> , 2005)
<b>Prostate</b>	FASLG* TNFSF10*	(Huber <i>et al.</i> , 2005; Kim <i>et al.</i> , 2005)
<b>Squamous cell cancer</b>	FASLG TNFSF10	(Taylor <i>et al.</i> , 2006; Martinez-Lostao <i>et al.</i> , 2010)

Table 2. Protein content on and within exosomes reflects their origin and establishes their functional role. Cell surface A33 antigen precursor (GPA 33); Carcinoembryonic antigen-related cell Adhesion molecule 5 (CEACAM5); Ephrin-B1 (EFNB1); ADP-ribolysing factor (ARF), Ras-Gprotein superfamily (Rabs), A disintegrin and metalloprotease (ADAM10), Macrophage Migration inhibitory factor (MIF), Receptor for activated C-kinase (RACK1), 5,6-Dihydroxyindole-2 carboxylic acid oxidase (TRP); Epidermal-growth factor receptor (EGFR); Receptor tyrosine-protein kinase erbB-2 (ERBB2); Plasmalemma vesicle associated protein (PLVAP); Melanocyte Protein Pmel 17 (GP100); Melanoma Antigen Recognized by T cells 1 (MART-1), Mel-CAM, TNF-ligand superfamily member 10 (TRAIL) TNF-ligand superfamily member 6 (FasL).



Recent studies have shown that TEX provide a protective role to the cancer cells, which can be manifested in different ways. Survivin, a member of the inhibitor of apoptosis (IAP) protein family, was found to be released from tumor cells via exosomes (Khan *et al.*, 2011). The protective role of TEX can be attained by the accumulation and packaging of chemotherapeutic drugs or its metabolites into the vesicles, thus decreasing cellular levels of the drug, a factor leading to drug resistance (Shedden *et al.*, 2003; Safaei *et al.*, 2005). This phenomenon has been observed in various cancer cells. Cisplatin enhanced the shedding of the vesicle from melanoma cells (Chen *et al.*, 2006a), while doxorubicin was found in the exosomes released from ovarian carcinoma cells (Shedden *et al.*, 2003). Despite the beneficial roles of TEX for the tumor cells and the tumor microenvironment, TEX can be a useful tool for detecting the malignant condition. Serum levels of exosomes taken from cancer patients are significantly increased. These vesicles taken from serum (Ginestra *et al.*, 1999), as well as from malignant tumor fluids, such as ascites fluids (Adams *et al.*, 2005), pleural effusions (Andre *et al.*, 2002b) and urine (Nilsson *et al.*, 2009), positively correlate with the tumor progression.

### 3.1 Constitutive and inducible vesicle secretion in cancer and cancer therapy

In the tumor microenvironment, various changes are taking place, which could have an effect on the release of vesicles, such as exosomes. Environmental changes, such as stress induced by chemo- and radio-therapy, can modulate TEX release and the biome they contain. This phenomenon may induce the tissues to adapt to changes taking place in the microenvironment (Thery *et al.*, 2009). Tumor cells that have undergone radiation or chemotherapy treatment have been shown to increase the release of TEX (Yu *et al.*, 2006; Lehmann *et al.*, 2008). Interestingly, when treated with chemotherapeutic agents, there is a significantly enhanced membrane vesicle secretion in chemoresistant cells compared to chemosensitive cells. This activity may be a factor leading to drug resistance (Shedden *et al.*, 2003; Safaei *et al.*, 2005). Chemotherapy and radiation (Chen *et al.*, 2006b) treatment lead to DNA-damaging conditions. In this state, the p53 pathway is activated, leading, among various other changes physiologically, to an induced expression of the transmembrane protein tumor suppressor-activated pathway 6 (TSAP6). TSAP6 is an important cellular component as it regulates the secretion of protein via the non-classical pathway or the ER/Golgi-independent protein secretion pathway needed for the enhanced release of exosomes (Nickel, 2003; Yu *et al.*, 2006; Lespagnol *et al.*, 2008). Normally, the secretion of exosomes in various cell types happens at a low rate. However, when p53 is activated, endosomal compartment activities are activated. Simultaneously, there is an increased expression of TSAP6, inducing the release of exosomes at a higher rate (Yu *et al.*, 2009) (**Figure 4**). It is suggested that following p53 activation, exosomal release may act as a 'detoxifier' to expel unwanted chemotherapeutic agents (Shedden *et al.*, 2003; Safaei *et al.*, 2005; Chen *et al.*, 2006b; Lespagnol *et al.*, 2008). Communication to the microenvironment is the other proposed role of TSAP6 and exosomal release after p53 activation, which may act as a warning signal to the neighboring cells, the immune system, and the extracellular matrix, that there are abnormal intracellular events happening (Lespagnol *et al.*, 2008; Yu *et al.*, 2009).

### 3.2 Summary

TEX can be used as an important biomarker for the disease, which will give information not only on the disease progression, but on the tumor type. As previously mentioned, TEX

express specific tumor antigens which reflect the protein content of the tumor, giving an indication of the tumor type. The content of these vesicles can also be useful as markers for the aggressiveness of the disease.

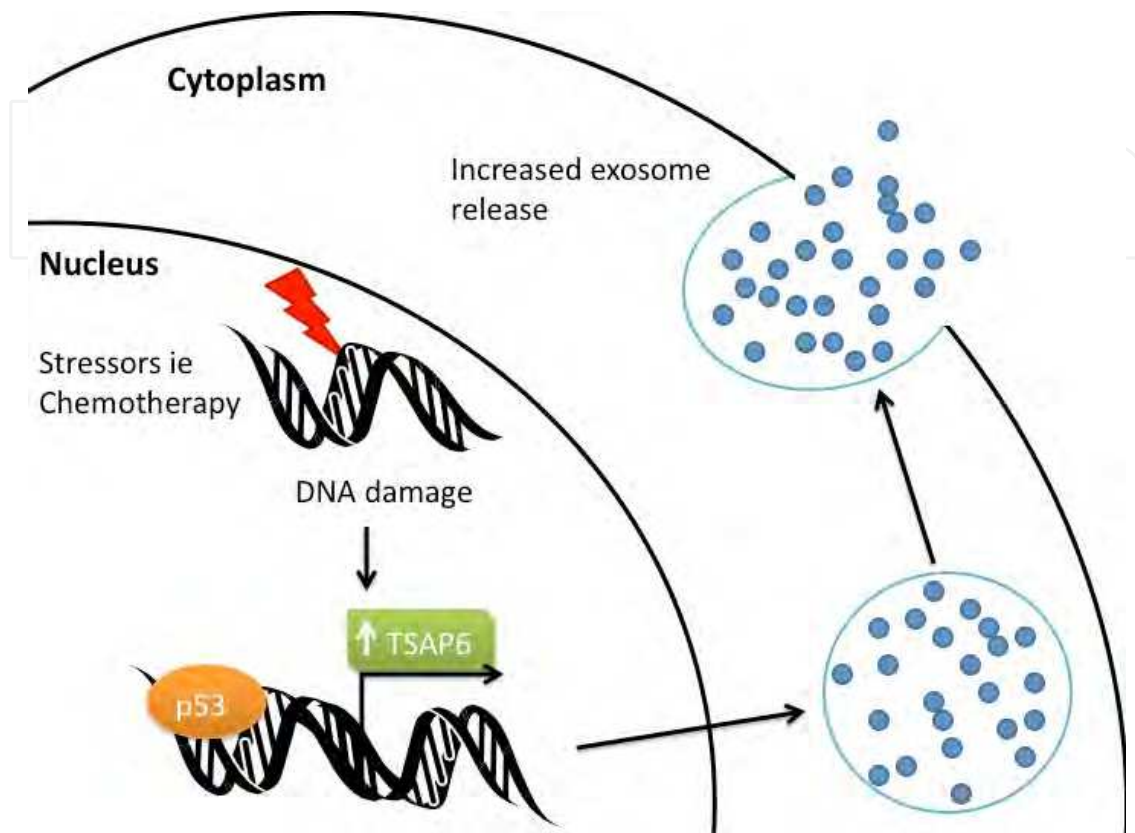


Fig. 4. Activation of p53 by DNA damage induces the expression of TSAP6 which enhances exosome-mediated secretion.

#### 4. Proteome of cancer membrane vesicles

Mass spectrometry-based proteomic tools coupled with advanced purification methods for exosomes, has allowed more in-depth proteome analyses, contributing immensely to our understanding of the molecular composition of exosomes. Proteomic analysis of exosomes from diverse cell types, including cancers has revealed a common set of membrane and cytosolic proteins, suggesting the evolutionary importance of these membrane particles. In addition, exosomes express an array of proteins that reflect the originating host cell. The excessive release of exosomes in tumor cells, as evidenced by their increased levels in body fluids during the late stage of a disease and their overexpression of certain tumor cell biomarkers, suggests an important role of exosomes in diagnosis and biomarker studies (Simpson *et al.*, 2009).

By proteomic analysis we can enrich low abundance membrane proteins from underrepresented conventional cell lysates and unfractionated biological fluids. Identification of a conserved set of common proteins that are essential for vesicle biogenesis, structure and trafficking mechanisms can be explored. We can also detect cell-specific biomarkers. These concepts suggest that analyzing the composition and abundance of such proteins in exosomes may be useful to reveal different cell behaviors.

#### 4.1 Proteomics of cell-type dependent exosomes

Exosomes have a unique protein composition that varies depending on cellular origin (**Table 2**). Analysis of exosomes from a wide variety of cells and body fluids have been identified. Exosomes from various cancer cells expose Fas ligand (FasL, CD95L), a ligand of the death receptor Fas (CD95), which induces T-cell apoptosis and diminishes the function of adaptive immune cells (Andreola *et al.*, 2002; Huber *et al.*, 2005). It was shown that a modest correlation exists between lymph node infiltration and tumor burden and the numbers of circulating FasL-exposing exosomes in blood from patients with oral squamous cell cancers (Kim *et al.*, 2005). Exosomes from lymphoblastoma cells exposed latent membrane protein-1 (LMP-1), another immune suppressing transmembrane protein, thereby inhibiting leukocyte proliferation. In addition, low numbers of circulating exosomes, in cancer patients, stained positive for MUC1, a cancer cell antigen, and glycoprotein IIIa (integrin  $\beta$ 3), which is mainly present on platelets and platelet-derived exosomes. Exosomes are released after fusion of exosomes from malignant epithelial cells with platelets (Tesselaar *et al.*, 2007). Alternatively, platelet-derived exosomes were shown to transfer integrins to breast and lung cancer cells (Janowska-Wieczorek *et al.*, 2001; Janowska-Wieczorek *et al.*, 2005). Thus, cancer cells can fuse with non-cancer cell-derived exosomes, thereby receiving lipids and membrane specific proteins which may help them escape from immune surveillance.

Degradation of the extracellular matrix (ECM) is essential for tumor growth (Hotary *et al.*, 2003). Exosomes expose and contain proteases, including matrix metalloproteinase 2 (MMP-2) and MMP-9 and its zymogens, and urokinase-type plasminogen activator (uPA). Neovascularization is also responsible for the increased entry of tumor cells into the circulation and metastasis. It is believed that tumor and stromal cells secrete angiogenic factors such as VEGF and basic fibroblast growth factor (bFGF) and that tumor-associated angiogenesis occurs by the action of these factors (Carmeliet, 2005). Furthermore, growing evidence suggests that exosomes derived from tumor cells and platelets also possess angiogenic activities. Vesicular components such as sphingomyelin, CD147, tetraspanin-8, VEGF, and bFGF are likely involved in exosome-mediated neovascularization (Kim *et al.*, 2002; Brill *et al.*, 2004; Gesierich *et al.*, 2006; Millimaggi *et al.*, 2007).

In a colorectal cancer cell line study, several exosomal proteins have been identified that are believed to be involved in tumor-associated angiogenesis: ADAM 10, CD44, NG2, ephrin-B1, macrophage migration inhibitory factor (MIF), RACK1, and tetraspanin-8 (Dong-Sic *et al.*, 2007). Clinical exosome analysis may also prove useful for solid cancers (Mathivanan *et al.*, 2010). Using exosomes from ovarian carcinoma cell lines, malignant ascites and sera from ovarian carcinoma patients, it was found that malignant ascites-derived exosomes cargo tumor progression related proteins (L1CAM, CD24, ADAM10 and EMMPRIN). It was also observed that exosomes move systemically via the blood stream (Keller *et al.*, 2009). Therefore, if some membrane proteins are typically and specifically expressed by a certain tumor, their detection on circulating exosomes, (which could be isolated from only 1mL of blood), may be exploited for diagnostic purposes as an early signal of cancer presence. Proteomic analysis of exosomes was also performed on human mesothelioma cell lines and malignant pleural effusions. Bard and colleagues, described exosomes which contained antigen presenting molecules, cytoskeletal proteins, and signal transduction-involved proteins were in mesothelioma, lung, breast, and ovarian cancers. In addition, SNx25, BTG1, PEDF, and Thrombospondin were also identified (Bard *et al.*, 2004).

Characterization of urinary exosomal composition may be proposed as a potential source of diagnostic markers in bladder cancer. Two different approaches (Smalley *et al.*, 2008; Welton *et al.*, 2010) were taken to characterize these exosomes. In the first, urine exosomes were isolated from a limited number of individuals with bladder cancer and their protein composition compared to that of healthy controls. Eight proteins were found elevated, among which five have been linked to the EGF receptor pathway (Smalley *et al.*, 2008). In the second study, extensive steps were taken to produce high purity and quality-assured exosome preparations prior to beginning proteomics workflows. Working with conditioned media from cultured bladder cancer cell lines, 350 proteins were identified. Eighteen were proven to be present in exosomes isolated from the urine of three bladder cancer patients (Welton *et al.*, 2010). This suggests that conditioned media from cultured cell lines could represent an interesting starting model to detect exosomal proteomic alterations, which must then be confirmed *in vivo*, using biological fluids from a wide cohort of patients, in order to supply a non-invasive source for biomarker discovery.

Exosomes prepared from urine of prostate cancer patients contain typical markers of such a tumor (PSA and PCA3) (Mitchell *et al.*, 2009; Nilsson *et al.*, 2009). Moreover,  $\delta$ -catenin immunoreactivity was identified in vesicles prepared from culture media of PC3 cells and found significantly increased in prostasomes of the urine of prostate cancer patients (Lu *et al.*, 2009).

#### 4.2 Summary

Studies confirm that urinary exosome protein profiling is an important topic and may be a valuable tool for biomarker discovery in the field of urinary tract pathology. Proteomic approaches to investigate membranous vesicles and exosomes are still immature. However, they show a great potential for future developments in the diagnostic and prognostic applications.

### 5. Targeting interactions of membrane vesicles with neighboring tumor microenvironment

Tumor cells release large quantities of exosomes containing procoagulant, growth regulatory, and oncogenic cargo, which can be transferred throughout the cancer cell population and to transformed stromal cells, endothelial cells and possibly to the inflammatory infiltrates. These events likely impact tumor invasion, angiogenesis, metastasis, drug resistance, and cancer stem cells. Instead of physical contact, the influence of exosomes on the target cell may also involve pericellular discharge/activation of the bioactive cargo (Dolo *et al.*, 2005; Hendrix *et al.*, 2010; Muralidharan-Chari *et al.*, 2010). For instance, this may involve proteolytic remodelling of the extracellular microenvironment, modulation of ligand-receptor interactions, and a variety of other effects that could change the behaviour of target cells and properties of their surroundings (Hendrix *et al.*, 2010) (**Figure 5**). In some instances, such interactions could be rather complex and multifactorial. The recently described Rab27B-regulated exosomal release of MMPs and HSP90a from metastatic cancer cells is believed to control invasive cellular behaviour by inducing changes in the extracellular matrix (ECM) as well as through modification of growth factor responses (Hendrix *et al.*, 2010). Likewise, procoagulant exosomes may facilitate tumor initiation, invasion, and dissemination by activating the clotting cascade extracellularly and coagulation-dependent signalling intracellularly (Milsom *et al.*, 2007). Exosome-mediated

emission of various factors including tetraspanins, chemoattractants, adhesion molecules and proteases from cancer cells, platelets, and other cellular sources contributes to metastatic regulation in several experimental systems (Janowska-Wieczorek *et al.*, 2005; Jung *et al.*, 2009). As mentioned earlier, exosomes may also act as important reservoirs of cytokines and mediators of inflammatory and immune responses (Bianco *et al.*, 2009; They *et al.*, 2009).

### 5.1 Mediators of intercellular communication

Contact with the cell death ligand (FasL) exposed on certain tumor cell-derived exosomes is lethal for Fas-expressing lymphoid cytotoxic effector cells, a process implicated in the induction of immunotolerance in colorectal cancer and possibly other malignancies (Albanese *et al.*, 1998). These influences may affect recipient cells via a random distribution of exosomes in tissue and body fluids, or more directional exosome homing/uptake mechanisms. For instance, an acidic pH commonly present in hypo-perfused areas of solid tumors may lead to localized disruption of exosomes and consequent discharge of their proangiogenic and pro-inflammatory cargo such as VEGF and other factors (Taraboletti *et al.*, 2006). Exosomes may also be directed to specific sites due to the molecular addresses they carry on their surfaces (Celi *et al.*, 2004; Zhou *et al.*, 2008; Xiao *et al.*, 2009). The nature, directionality, and efficiency of this molecular exchange depends on several factors. For instance, the physical properties of vesicular plasma membranes affect the fusion rate between exosomes and target cells, which may increase their exosome uptake under acidic pH (Parolini *et al.*, 2009). In some instances, exosome transfer could also be directed by specific molecular addresses, for example, a high concentration of phosphatidyl serines (PS) on the surface of certain exosomes (e.g. ectosomes or procoagulant microparticles) may enable their recognition by PS receptors (PSRs) on the surface of specific types of target cells. Many of such PSRs have been described within the context of phagocytosis of apoptotic cells by mononuclear cells; examples of such PSRs include Tim1, Tim4, stabilin 2 and BAI1 (Park *et al.*, 2007; Zhao *et al.*, 2010), at least some of which could be expressed more widely and may be involved in the uptake of exosomes. Indeed, blocking PSRs often obliterates exosome incorporation by endothelial cells, platelets and cancer cells (Del Conde *et al.*, 2005; Al-Nedawi *et al.*, 2008; Al-Nedawi *et al.*, 2009). A corollary to this point would be that phagocytes could be particularly susceptible to molecular influences of PS-positive exosomes, beyond their simple destruction. It has also been proposed that Tim1/4 receptors on two adjacent cells could allow formation of exosome bridges, thereby promoting additional indirect intercellular interactions (Xiao *et al.*, 2009). Similarly, the presence of PSGL-1 (P-selectin ligand) on the surface of procoagulant exosomes directs them to P-selectin-expressing platelets and endothelial cells (Thomas *et al.*, 2009).

### 5.2 Oncosomes: Oncogene-driven vesiculation

During malignant transformation, the action of mutant oncogenes, such as K-ras, EGFR, or its constitutively active mutant EGFR (variant III) (EGFRvIII), as well as several others, appear to stimulate the formation and release of exosomes (Yu *et al.*, 2005; Al-Nedawi *et al.*, 2008). Similarly, the activation or loss of specific tumor suppressor proteins appears to impact cellular vesiculation either positively or negatively (Yu *et al.*, 2005; Yu *et al.*, 2006). While the exact nature of the signalling pathways involved in oncogene-driven exosome biogenesis remains largely unknown, recent studies have begun to shed more light on the underlying processes. For instance, in cultures of prostate cancer cells, elevated exosome

production was detected in association with increased oncogenic activity of protein kinase B (PKB/Akt), or upon stimulation with growth factors (EGF), and depending on the status of the actin regulating protein known as diaphanous related formin 3 (DRF3) (Di Vizio *et al.*, 2009). In this case, inhibition of DRF3 expression through RNA interference enhanced the rate of exosome formation, and membrane blebbing activity, suggesting that DRF3 may be an inhibitor of ectosome release (Di Vizio *et al.*, 2009). Interestingly, DRF3 expression is lost during the progression of prostate cancer to metastatic disease, which suggests an intriguing link between oncogenesis, vesiculation and metastasis (Di Vizio *et al.*, 2009).

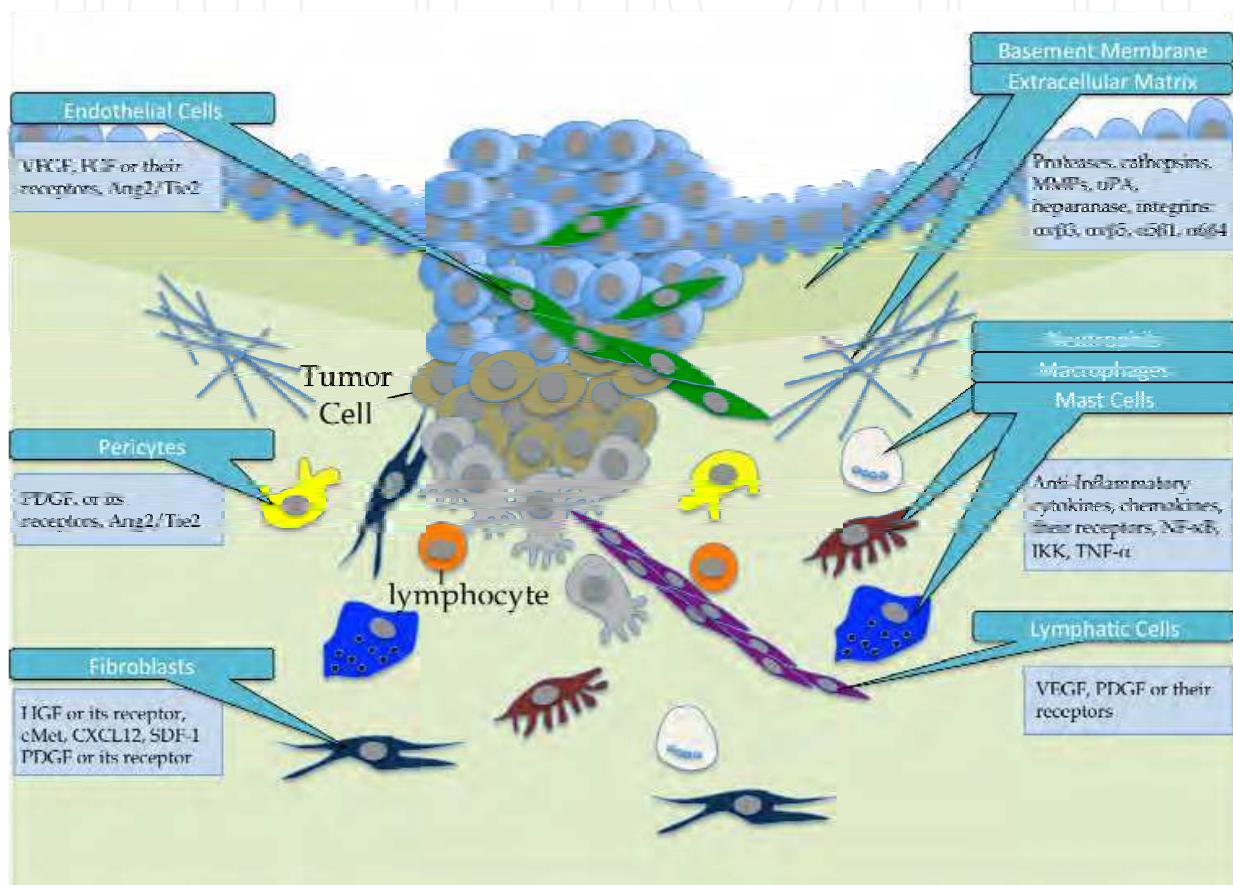


Fig. 5. Tumor microenvironment. The heterotypic interactions within the tumor microenvironment and their give and take of exosomes and their contents provide many targets for possible therapy. The goal of targeting these interactions will interrupt the heterotypic signaling that would thus deprive the cancer cells of the support they have within the tumor microenvironment.

### 5.3 Effect of TEX on tumor microenvironment

Exosome release by colorectal cancer cells is a function of K-ras and p53 status (Yu *et al.*, 2005). It is noteworthy that oncoproteins not only stimulate exosome formation but also become incorporated into their cargo (Al-Nedawi *et al.*, 2008; Al-Nedawi *et al.*, 2009). As a result, oncogene-containing exosomes (sometimes referred to as *oncosomes*) may serve as vehicles that carry oncogenic cargo and mediate its transfer between cells (Al-Nedawi *et al.*, 2009). At least four different modes of such oncogenic transfer have been described: (a) intercellular passage of active oncoproteins (Al-Nedawi *et al.*, 2008), (b) transfer of oncogenic mRNA transcripts

(Skog *et al.*, 2008), (c) exchange of oncogenic miR and/or (d) passage of genomic sequences containing oncogenic DNA (Bergsmedh *et al.*, 2001). In many instances, this horizontal transfer may have marked biological (transforming) consequences. Thus, oncosomes containing EGFRvIII may emanate from malignant tumor cells and be taken up by their indolent counterparts inducing their growth, survival, and clonogenic and angiogenic capacity (Al-Nedawi *et al.*, 2008). These exosomes may also act on endothelial cells and reprogram their responses such that they exhibit an increase in angiogenic activity (Skog *et al.*, 2008) or switch to an autocrine mode of secretory pathway, e.g. by turning on VEGF production (Al-Nedawi *et al.*, 2008). Indeed, blocking exosome uptake using the Annexin V analogue (Diannexin) is associated with a measurable anti-angiogenic effect *in vivo* (Al-Nedawi *et al.*, 2008). In chronic lymphoblastic leukaemia (CLL), exosomes containing AXL kinase conditioned the bone marrow stroma to support disease progression (Ghosh *et al.*, 2010). These and similar effects identify exosomes as possible effectors of oncogenic and proangiogenic *field effects*, long postulated to exist in cancer (Slaughter *et al.*, 1953; Al-Nedawi *et al.*, 2009) and viewed as a mechanism of cell recruitment to the malignant process.

#### 5.4 Intercellular exchange

Interactions between exosomes and their target cells may depend on the specific ligand-receptor recognition events. For instance, platelets take up procoagulant exosomes in a manner that depends on the expression of P-selectin and its ligand (PSGL-1) on the respective surfaces (Falati *et al.*, 2003). After the uptake, exosome-associated material was shown to penetrate into the cytoplasm of the acceptor cell (Skog *et al.*, 2008) or to remain on the cell surface, potentially in the immediately active form (Del Conde *et al.*, 2005; Al-Nedawi *et al.*, 2008). Interestingly, cloaking PS by exposure of exosomes to Annexin V often obliterates their uptake by target cells. Recent studies suggested that exosomal transfer would encompass multiple effectors at once (Skog *et al.*, 2008). Such exosomal exchange could contribute to tumor angiogenesis by mechanisms dependent on transfer of oncogenes (Rak, 2010) (e.g., EGFRvIII4 or mRNA), but possibly also through bidirectional trafficking of other molecules (e.g., VEGFRs, Tie, Notch), including ligands traditionally assumed to act almost exclusively in a cell-associated manner (Dll4, ephrins) (Kerbel, 2008). This prototype has been already validated in the case of exosomal emission of coagulation factors (e.g., tissue factor—TF21), chemokine receptors (Mack *et al.*, 2000) adhesion molecules (Falati *et al.*, 2003), immunomodulators (Valenti *et al.*, 2007), cell surface antigens (Dolo *et al.*, 2005), intact RNA species (Ratajczak *et al.*, 2006; Valadi *et al.*, 2007), and oncogenic proteins (Koga *et al.*, 2005; Al-Nedawi *et al.*, 2008; Sanderson *et al.*, 2008).

#### 5.5 Summary

So far in the literature, exosomal cargo associated proteins and their importance in angiogenesis, inflammation, proteolysis regulators, membrane receptors, soluble factors, oncoproteins and tumor suppressors, lipids, nucleic acids are described briefly. Therefore, exosomes represent an integral part of both physiological regulation and disease pathogenesis, which may influence new therapeutic and diagnostic modalities.

### 6. Membrane vesicles and antigen presentation

As described earlier in this chapter, various cell types release exosomes, which contain a proteomic sampling from the cell of origin. Proteins within exosomes can be presented as

antigens to the immune system and, when derived from certain cell types, are capable of presenting antigens to immune cells directly. Antigen presentation by antigen presenting cells (APCs) requires several important steps to elicit an immune response: 1. the APC internalizes and processes the antigen, 2. the processed antigen is inserted into Major Histocompatibility Complex (MHC) molecules and displayed on the cell surface, 3. MHC molecules interact with T cell receptors to start a signaling cascade to activate the T cell (Kim *et al.*, 2004). Dendritic cells are the primary APC type that stimulates T cells *in vivo*, which requires MHC molecules on the dendritic cell, as well as expression of co-stimulatory molecules that enhance T cell activation, such as CD40, CD80 and CD86 (Kim *et al.*, 2004). Like many other cell types, dendritic cells release membrane vesicles, particularly exosomes, which help modulate the immune response (Chaput *et al.*, 2006; Schorey & Bhatnagar, 2008; They *et al.*, 2009).

### 6.1 Antigen presentation by immune cell exosomes

Antigen presenting cells, such as dendritic cells and B cells, release exosomes equipped with MHC class I and class II molecules that allow direct presentation of antigens to cytotoxic and helper T cells, respectively (Kim *et al.*, 2004; Chaput *et al.*, 2006). Antigens processed by APCs are loaded into MHC molecules and the MHC-antigen complex is released into the extracellular space within exosomes. These exosomes then travel throughout the body and induce an immune response by stimulating antigen-specific T cells. In addition to MHC molecules, APC exosomes express surface co-stimulatory molecules CD40, CD80 and CD86 to enhance T cell activation (Raposo *et al.*, 1996). The ability of exosomes to induce a T cell response is dependent on the expression of these molecules, as introduction of antibodies against CD40, CD80 and CD86 inhibit antigen presentation and T cell activation by APC exosomes. Additionally, the tetraspanin molecule CD54 (ICAM1) plays a crucial role in this process by enhancing exosome-T cell contact through its interaction with CD11a (LFA-1) on the T cell (Kim *et al.*, 2004). This interaction allows MHC and co-stimulatory molecules to bind to their receptors on the T cells long enough to provide sufficient signaling. As observed with inhibition of the co-stimulatory molecules, antibody blockade of CD54 on exosomes dramatically reduces T cell activation by APC exosomes (Kim *et al.*, 2004).

### 6.2 Transfer of antigens by exosomes

In addition to their ability to present processed antigens, exosomes can also transfer antigens from one cell to another. Exosomes containing cellular antigens are taken up by APCs, where the antigens are processed and inserted into MHC molecules for presentation (Obregon *et al.*, 2006). This is a very important mechanism in disease states, where infected or malignant cells release exosomes into the bloodstream that can be processed and presented by APCs throughout the body to induce an immune response (Bhatnagar & Schorey, 2007). In cancer, TEXs are taken up by APCs and activate tumor-specific T cells (Andre *et al.*, 2002a). *In vitro* studies have shown that when T cells are incubated with TEX in the presence of naïve dendritic cells, both helper and cytotoxic T cells are activated in an antigen-specific manner (They *et al.*, 2009). Additionally, *in vivo* vaccination studies comparing TEX and irradiated tumor cells showed a stronger immune response in animals vaccinated with TEXs. These studies have led to two clinical trials, which will be discussed in detail later.



### 6.3 Summary

Antigen presentation to T cells is the first and most critical step in the adaptive immune response. The ability of exosomes to supply antigens to dendritic cells for presentation as well as present antigens directly via exosomal MHC molecules is an important mechanism for detection of infection and malignancy. Induction of anti-cancer responses via tumor-released exosome antigens is likely a key mechanism in immune surveillance to prevent tumor progression. Whether this can be utilized in tumor immunotherapies is under investigation and could prove useful in combination therapies.

## 7. Antigen-independent roles of membrane vesicles in immune responses

Antigen presentation by exosomes is primarily an immunostimulatory process, however exosomes have many antigen-independent functions that can both stimulate and inhibit the immune system (Schorey & Bhatnagar, 2008; They *et al.*, 2009). These roles are dependent on the type of cell the exosomes originate from and the molecules they express (Andre *et al.*, 2002a; Abusamra *et al.*, 2005a; Xiang *et al.*, 2009; Chalmin *et al.*, 2010; Szajnik *et al.*, 2010; Zhang *et al.*, 2011). In this way exosomes are important for proper immune function and immune regulation, but can also be hijacked by malignant cells to prevent detection by the immune system.

### 7.1 Immune cell exosomes induce immune responses

Dendritic cell exosomes (DEXs) are vital for stimulation of the adaptive immune response by their presentation of antigen to T cells (**Figure 6**), but induction of the humoral immune response is as important a function (Chaput *et al.*, 2006). DEXs have been shown to stimulate Natural Killer (NK) cell responses critical to the clearance of infection and malignancy (Viaud *et al.*, 2009). This function is mediated by the expression of interleukin-15 receptor alpha (IL-15R $\alpha$ ) and Natural Killer Group 2D (NKG2D) ligands on dendritic cell exosomes. Interleukin-15 (IL-15) is a cytokine that stimulates NK cell activation and proliferation. Exosomal IL-15R $\alpha$  can bind IL-15 in the extracellular space and deliver it to NK cells by exosomal binding to NKG2D on the surface of the NK cells (Viaud *et al.*, 2009). Delivery of IL-15 in this manner significantly increases NK cell responses *in vitro* and in mice injected with autologous DEXs (They *et al.*, 2009; Viaud *et al.*, 2009). Because of their critical importance to tumor clearance, NK cell stimulation by DEXs could provide therapeutic benefits by increasing NK cell numbers in cancer patients.

### 7.2 Tumor cell exosomes and the immune response

The role of TEX in the progression of cancer is multifaceted, as they can affect tumor growth, invasion and metastasis, and therapy resistance. In addition to influencing other malignant cells, TEXs have significant effects on the immune response, both positive and negative.

#### 7.2.1 Exosomes from heat-shocked tumor cells induce immune activation

The release of exosomes from normal and malignant cells is increased by heat shock and stress, with amplified proportions of Hsps within the exosome (Schorey & Bhatnagar, 2008). Culture of dendritic cells with exosomes from heat-shocked tumors resulted in higher expression of CD40, CD80 and CD86 than in dendritic cells cultured with control immune

cell exosomes or non-heat shocked tumor exosomes (Chen *et al.*, 2006c). Additionally, the dendritic cells treated with heat shocked TEXs exhibited increased production of pro-inflammatory cytokines tumor necrosis factor alpha (TNF $\alpha$ ), IL-1 $\beta$ , and IL-12 (Chen *et al.*, 2006c). This is thought to be mediated by the increased amount of Hsps in the heat-shocked TEXs, though the exact mechanism is still under investigation.

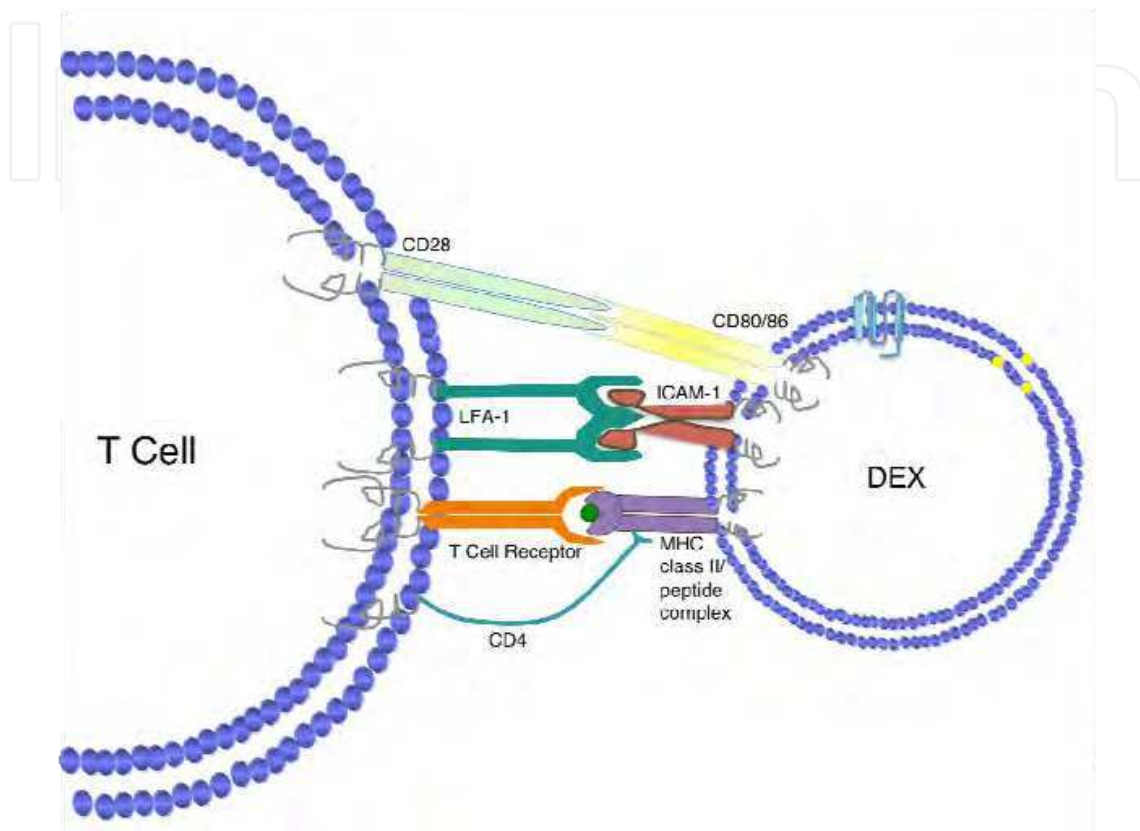


Fig. 6. Dendritic cell-derived exosomes interact with T cells through MHC class I, II, tetraspanins such as CD9, CD63, CD81 and through co-stimulatory molecules such as CD86.

### 7.2.2 Tumor cell exosomes inhibit natural killer cells via NKG2D

NK cells kill cancer cells by release of granules and perforins (Figure 7). NK cell activity is often lost in cancer patients, resulting in a reduced ability of the immune system to eliminate malignant cells (Clayton *et al.*, 2008; Viaud *et al.*, 2009; Ahiru *et al.*, 2010). The NK cell receptor NKG2D is important in regulation of NK cell function, with some ligands stimulating and others inhibiting cytotoxic function (Clayton *et al.*, 2008). As discussed earlier, DEXs expressing activating NKG2D ligands can enhance NK cell function and promote tumor clearance (Viaud *et al.*, 2009). Other NKG2D ligands, such as MHC class I-related chain A (MICA) can reduce NK cell function. Tumor cells abuse this normal ligand by upregulating MICA on the cell surface as well as on TEXs (Ahiru *et al.*, 2010). Ovarian cancer exosomes expressing high levels of MICA were shown to decrease NK cell function *in vitro* by reducing their NKG2D receptor expression and their responsiveness to activating NKG2D ligands (Ahiru *et al.*, 2010). This reduction in NK cell function is highly detrimental to the anti-tumor response and is one of many mechanisms by which tumors escape immune detection (Thery *et al.*, 2009).

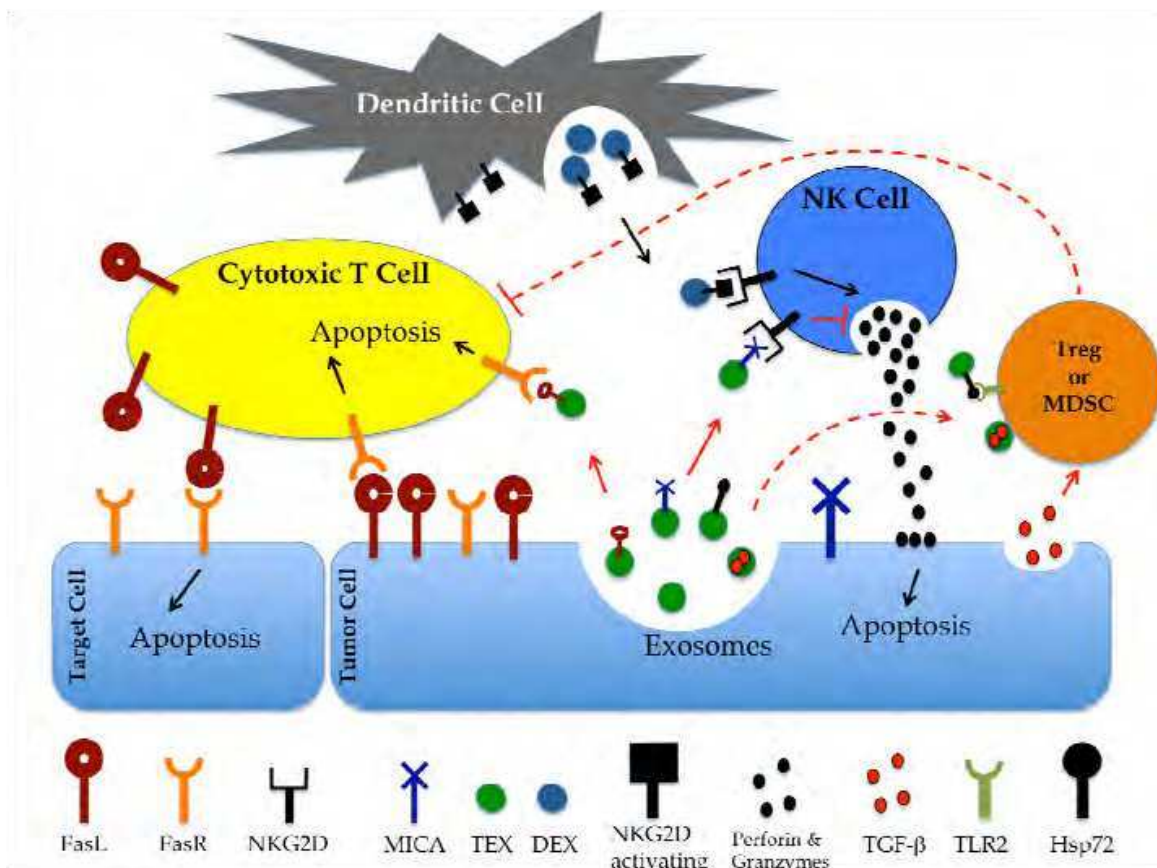


Fig. 7. DEX and TEX play opposing roles with regard to immune cell activation and prohibition.

### 7.2.3 Fas ligand expression on tumor cell exosomes induce immune cell apoptosis

Cytotoxic immune cells like cytotoxic T cells express Fas Receptor (FasR; CD95) and Fas ligand (FasL; CD95L) that allow them to induce apoptosis in target cells. Several cancer types including colon and ovarian carcinoma and melanoma cells express FasL to induce death of the very immune cells that attempt to kill them (Koyama *et al.*, 2001). This is a primary mechanism of tumor-immune escape that prevents the immune system from eliminating transformed cells, resulting in disease progression. To further prevent cytotoxic killing by T cells, TEXs have been found to express high levels of surface-bound FasL (Abusamra *et al.*, 2005a). Release of FasL via exosomes allows the tumor to kill FasR-expressing immune cells at distant sites, as well as in the tumor microenvironment, further preventing tumor clearance. Interestingly, although both helper and cytotoxic T cells express FasR, exosomes containing FasL preferentially induce apoptosis in cytotoxic T cells and not in helper T cells (Abusamra *et al.*, 2005a). The mechanism behind this is not fully understood, but may be due to favored interactions between exosomes and cytotoxic T cells due to other exosomal surface molecules (Figure 7).

### 7.2.4 Tumor exosomes induce suppressive immune cell phenotypes

Immune suppression within the tumor microenvironment prevents cytotoxic attack and promotes tumor progression. Like FasL and NKG2D inhibition, increasing immunosuppressive cell types can reduce cytotoxic immune responses. Two primary

inhibitory cell types have been implicated in cancer progression: regulatory T cells (Tregs) (Szajnik *et al.*, 2010) and myeloid derived suppressor cells (MDSCs) (Xiang *et al.*, 2009). These cell types inhibit both helper and cytotoxic T cell responses and reduces their production of inflammatory cytokines like interferon gamma (IFN- $\gamma$ ). Large numbers of immune cells are found within the tumor microenvironment, but Tregs and MDSCs are far more prominent in cancer patients than in healthy patients, leading to the hypothesis that immune suppression can be induced by the tumors (Xiang *et al.*, 2009; Szajnik *et al.*, 2010). Tumor cells can directly induce regulatory populations by secreting transforming growth factor beta (TGF- $\beta$ ), which promotes differentiation of naïve T cells and myeloid precursor cells into their respective suppressive phenotypes. A similar mechanism is utilized by tumors to induce Tregs and MDSCs within the tumor microenvironment and the periphery by secretion of TGF- $\beta$  in exosomes (Xiang *et al.*, 2009; Chalmin *et al.*, 2010; Szajnik *et al.*, 2010). Ovarian carcinoma derived exosomes were found to induce CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs from CD4<sup>+</sup>CD25<sup>-</sup> naïve T cells *in vitro* (Szajnik *et al.*, 2010). This was found to be dependent on the presence of TGF- $\beta$  and IL-10 in the exosomes and was inhibited by addition of neutralizing antibodies to both cytokines. In a similar study, mouse bone marrow myeloid precursor cells were shown to take up TEXs, inducing their differentiation into CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid derived suppressor cells. Exosomal expression of TGF- $\beta$  and prostaglandin E2 (PGE2) was shown to induce this differentiation, which was inhibited by antibody neutralization (Xiang *et al.*, 2009). The suppressive function of existing MDSCs is also increased by TEXs. Interaction of Hsp72 on the exosome with Toll-like receptor 2 (TLR2) on the MDSC induces Stat3 signaling to induce their immunosuppressive functions (Chalmin *et al.*, 2010). By increasing immunosuppressive cell types, TEXs can prevent attack by cytotoxic cells (Figure 7).

### 7.3 Summary

Exosomes can have important antigen-independent effects on immune cells that vary with the cell type they are derived from as well as the state of the cells upon release. DEXs and TEXs can stimulate immune responses, which could provide therapeutic benefits. In contrast, tumor derived exosomes from several cancer types have been shown to prevent cytotoxic attack on the tumor by FasL-induced T cell death, NKG2D ligand-mediated suppression of NK cells and through induction of immune suppressor cells like Tregs and MDSCs. How TEXs can be utilized or inhibited therapeutically remains to be seen, but currently is an active area of research.

## 8. Immune responses induced by *in vitro* purified membrane vesicles *in vivo*

Many studies described in the Sections 6 and 7 were performed using membrane vesicles and exosomes isolated and introduced to immune cell cultures *in vitro*. Before these findings can be utilized therapeutically, they must be confirmed in *in vivo* animal models. Results from these animal studies have demonstrated the importance of exosomes in immune modulation and are currently being implemented in clinical trials.

### 8.1 Vaccination with tumor exosomes prevent tumor growth in mice

Early studies in tumor vaccination utilized irradiation-killed tumor cells with limited success. With the discovery of TEXs, mouse vaccination studies with TEXs were shown to be more effective at generating tumor-specific T cell responses than irradiated tumor cells.

Additionally, vaccination of mice with exosomes containing ovalbumin resulted in delayed growth of ovalbumin-expressing tumors and induced pro-inflammatory Th1 T cell responses. As described in Section 7.2, exosomes isolated from heat-shocked cancer cells are more immunogenic *in vitro*. Using heat-shocked TEXs for immunization in mice showed similar immunogenicity, with 80% of mice remaining tumor free after challenge. While these studies have provided the groundwork for cancer vaccines, the immunosuppressive functions of cancer exosomes have caused many to be skeptical of their use as vaccine.

## 8.2 Dendritic cell exosomes containing tumor antigens induce anti-tumor immune responses

The use of DEXs as artificial antigen presenting cells can elicit strong antigen-specific T cell responses (Kim *et al.*, 2004). Mouse studies using DEXs pulsed with tumor peptides resulted in the activation of tumor-specific T cells, proliferation of NK cells and tumor regression (Kim *et al.*, 2004; Chaput *et al.*, 2006). Utilization of this technology with great success in murine studies has led to the development of several clinical trials using autologous DEXs as a treatment in cancer patients (Figure 8).

### 8.2.1 Melanoma phase I trial

A Phase I trial using dendritic cell exosomes to induce tumor regression was evaluated in 15 patients with metastatic melanoma (Escudier *et al.*, 2005). Autologous monocytes were isolated from patients by leukapheresis and differentiated *in vitro* to dendritic cells. These dendritic cells were cultured, exosomes isolated from the culture medium and pulsed with peptides from the tumor antigen MAGE3 (Escudier *et al.*, 2005). Patients received escalating doses of cryopreserved exosomes and their tumor progression and immune responses monitored. All 15 patients completed therapy but only 1 had specific T cell responses. Skin and lymph node mass reduction was observed in 5 of 15 patients and 7 of 15 patients showed increased NK cell activity (Escudier *et al.*, 2005; They *et al.*, 2009).

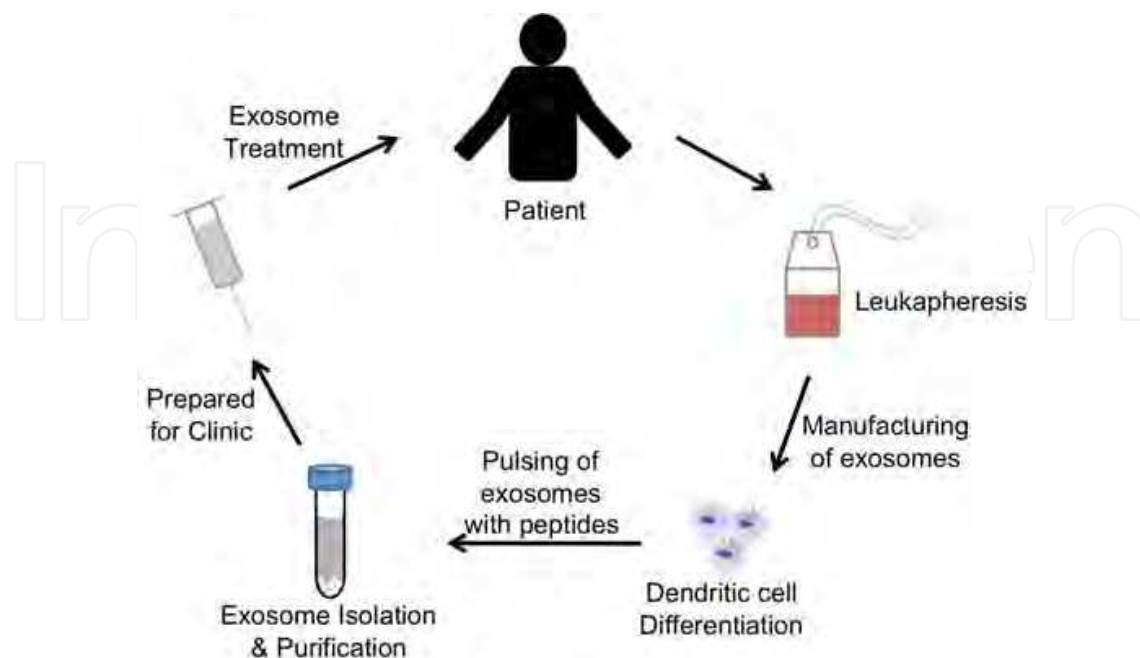


Fig. 8. Dendritic cell exosomes can be harvested and used in immunotherapy.

### 8.2.2 Non-small cell lung cancer phase I and II trials

A similar trial to the melanoma study was performed in Stage III and IV non-small cell lung cancer (NSCLC) (Morse *et al.*, 2005). Of the 13 patients enlisted in the trial, 9 finished the treatments and no toxicity was observed. Immune responses to MAGE3 was observed in 3 of 9 patients, with MAGE3-specific T cells only detected in 1 patient and while an increase in Treg cells was observed in 2 patients (Morse *et al.*, 2005). This trial concluded that dendritic cell exosomes are safe for use therapeutically, but that better responses may result from including Treg inhibitors in the treatment. A Phase II trial has begun adding IL-15R and NKG2D to the exosomes as well as Treg inhibitor therapy in NSCLC patients whose disease has been stabilized by chemotherapy (Thery *et al.*, 2009).

### 8.3 Summary

The use of exosomes as a cancer therapeutic has been demonstrated to be effective in murine studies and safe by Phase I clinical trials. Although some benefit was seen in patient trials, making these exosomal therapies more effective will likely require combination immunotherapies, chemotherapies and radiation. The use of exosomes to induce anti-cancer responses presents an exciting new field in cancer immunotherapy.

## 9. Membrane vesicles as therapeutic agents

Secreted microvesicles, known as exosomes, provide a form of cell to cell signaling in which the recipient cell can be modified by the contents of the delivered exosomes. The contents (cytosolic proteins, lipids, siRNA, miRNA, DNA, etc) (Tan *et al.*, 2010; Khan *et al.*, 2011) are protected and stably delivered unlike those secreted into the extracellular matrix (Pap *et al.*, 2010); thus securing the bioactivity of the delivered contents. Exosomes could be an attractive tool as an immunotherapeutic as they maintain much of the anti-tumor activity of a dendritic cell with the advantage of a cell free vehicle. Understanding that exosomes are involved in many levels of tumorigenesis, and potential strategies against this form of cellular communication has led to the creation of potential therapeutics against exosomes.

TEXs are a protective measure for tumor cells that aids in survival, growth, invasion, metastasis, and evasion of the immune system (Clayton *et al.*, 2007; Valenti *et al.*, 2007; Anderson *et al.*, 2010; Khan *et al.*, 2011). Taxol and vinca alkaloids are well known chemotherapeutic agents, but they also limit exosome release via the microtubule formation. Exosome formation has also been shown to be blocked by decreasing acidity of the microcellular environment (Iero *et al.*, 2008; Pap *et al.*, 2010). Prevention of exosome formation involves a broad variety of proteins and is essential for life in all cells. Therefore any inhibition of exosomes within the body could lead to unwarranted side effects (Pap *et al.*, 2010). It was thought that inhibition or treatment focused against TEXs and its ability to aid tumor cells could bear significant benefit to patients, but that was overshadowed by the realization that exosomes could potentially be employed as biomarkers and diagnostic tools of malignancy in blood (Anderson *et al.*, 2010) and urine (Nilsson *et al.*, 2009). One such study analyzed the correlation between serum PSA and urinary-exosomes, but none was observed. However, the data suggests that healthy donor urinary-exosomes were negative for PSA, PSMA, and 5T4 (Mitchell *et al.*, 2009).

Realizing that exosomes are secreted/released by a wide variety of cell types (Skokos *et al.*, 2001; Wolfers *et al.*, 2001; Chaput *et al.*, 2004b; Tan *et al.*, 2010; Khan *et al.*, 2011) including

immune cells and tumor cells, many studies set out to manipulate exosomes for cancer therapy, specifically immunotherapy. Ever since B lymphocytes were described to contain MHC class II molecules and stimulate CD4<sup>+</sup> T cells (Raposo *et al.*, 1996), studies have suggested exosomes may have an immunogenic role. Currently, DEXs from pulsed dendritic cells are under investigation for their ability to enhance and prime the immune system against tumor cells. Dendritic cells activate T cell responses through direct dendritic to T cell contacts. However, exosomes secreted by dendritic cells are also able to stimulate T cells (Zitvogel *et al.*, 1998).

### 9.1 Anti-cancer therapy

The importance of exosomes in cell to cell communication can be seen in their antigen presenting ability. Seminal work during the late 1990's showed DC's pulsed/loaded with cancer antigens or tumor peptides could yield DEXs which elicited better immune responses towards tumor cells (Zitvogel *et al.*, 1998; Wolfers *et al.*, 2001; Couzin, 2005; Chaput *et al.*, 2006; Tan *et al.*, 2010). The immune response was due to DEXs displaying the appropriate MHC class I molecules and tumor peptides. In fact, DC can be pulsed with both MHC class I and II, therefore effectively priming T cells against tumors (They *et al.*, 2002; Andre *et al.*, 2004; Chaput *et al.*, 2004a; Mignot *et al.*, 2006) as well as stimulating the activation and proliferation of NK cells (Clayton *et al.*, 2007; Viaud *et al.*, 2009). Current literature suggests that MHC class I and II containing exosomes are potential cell-free cancer vaccines (Zitvogel *et al.*, 1998; Andre *et al.*, 2004; Chaput *et al.*, 2004a; Tan *et al.*, 2010). Unlike current clinical cancer vaccines, exosomes are not technically vaccines as they are preventative and not therapeutic. The prophylactic vaccines are only effective against oncogenic viruses and do not treat the cancer directly, not to mention too expensive for the average patient (Tan *et al.*, 2010). Treatment of tumors with DEXs has had beneficial results such as initiation of immune response (Couzin, 2005), tumor growth suppression (Zitvogel *et al.*, 1998), tumor shrinking (Viaud *et al.*, 2009), and tumor rejection (Wolfers *et al.*, 2001; Mignot *et al.*, 2006). Exosomes are a better alternative than DC in terms of therapy because its composition can be identified, measured, is stable for storage, and has predictable behavior after administration (They *et al.*, 2002; Chaput *et al.*, 2004b).

The utilization of exosomes for cancer immunotherapy is a very new but very rapidly growing field. Most studies were conducted *in vitro*, but it is still unclear how oncologists will be able to translate the literature's data into the realm that will be most beneficial for the patient. One such complication is how the DEXs will be collected and implemented for patient treatment, and will the collected DEXs be sufficient enough? One potential strategy involves isolating DEXs through filtration of the patient's blood which will then be returned and employed to stimulate the immune system (Couzin, 2005). This would certainly allow for personalized therapy, but the question still remains: would there be enough DEXs and if not could adjuvant augmentation increase their numbers. Although the mode of exosome action *in vivo* is not clear yet, DEXs are a very interesting and potential substitute for dendritic cells in tumor vaccination therapy.

## 10. Conclusion

Various secreted extracellular vesicles have been found in blood, saliva, breast milk, bronchoalveolar lavage fluid, urine and amniotic fluid. Cellular uptake mechanisms have been shown to range from ligand/receptor interactions, integrin/cell adhesion molecule

attachment and fusion, or through transcytosis. Physiologically, these extracellular vesicles have been shown to play a role in inter-cellular communication, disposal of defective or weakened proteins, formation of morphogen gradients for tissue patterning during development and for antigen presentation to T cells. Pathologically, these vesicles have been shown to aid in the transmission of viruses, prions,  $\beta$ -amyloid in Alzheimer's disease, and specifically to this chapter, tumor pathogenesis. Although additional investigation is still needed to fully exploit these extracellular vesicles for therapy, there is increasing evidence that in better understanding them and the molecules that they transport, determinations of diagnosis and prognosis and the prediction of response to treatment may be possible. However, it is also the hope that these vesicles may also act at therapeutic targets and perhaps even replacement therapies.

Conventional treatments for cancer include the use of chemotherapeutic drugs, radiation therapy and interventional surgery if the tumors are operable. Recent data suggests that the application of dendritic cell-derived, tumor cell-derived and even ascitic cell-derived exosomes could be developed as novel treatments for cancer. In fact, it has already been established that exosomes can be safely administered to patients, though the number of patients in these trials have been small and thus argue for larger studies. Furthermore, the goal of exosome therapy is to increase the biological magnitude of the immune response and as such exosomal immunotherapy is heavily reliant on the immune system. It will therefore be important to address issues such as patient immunosuppression due to therapeutic treatment, as the efficacy of the immunotherapy will be reliant upon the capability of the immune system. In order to increase the biological magnitude of the immune response researchers are artificially coating and engineering exosomes with tumor antigens to make them more recognizable to the immune system. The effects of heat shocking exosomes is also being investigated as heat shocked tumor exosomes are more effective than those not receiving heat treatment. Heating of the exosome confers a greater immunogenicity and thus elicits a greater immune response. Another possible application for exosomes is in vaccine delivery as exosome-producing cells could be engineered to produce a miRNA or specific genetic component or toxin that could be loaded into an exosome with the proper surface molecules for a cell specific uptake and response. Finally, the application of exosomes as immunotherapeutics represents a new chapter in cancer treatment development. However, much more research must go into elucidation of mechanism and targeting in order to improve potency.

## 11. Acknowledgment

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The book "Advances in Cancer Therapy" is a new addition to the InTech collection of books and aims at providing scientists and clinicians with a comprehensive overview of the state of current knowledge and latest research findings in the area of cancer therapy. For this purpose research articles, clinical investigations and review papers that are thought to improve the readers' understanding of cancer therapy developments and/or to keep them up to date with the most recent advances in this field have been included in this book. With cancer being one of the most serious diseases of our times, I am confident that this book will meet the patients', physicians' and researchers' needs.

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