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Prospects for exosomes in immunotherapy of cancer

G. Mignot [a #], S. Roux [a #], Clotilde Thery [b], Elodie Ségura [b], L. Zitvogel [a *]

[a] U805 Institut National de la Santé et de la Recherche Médicale, Faculté de Médecine Paris Sud - Université Paris XI, Institut Gustave Roussy, Villejuif, France
[b] U653 INSERM, Institut Curie, Paris, France

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Abstract

Exosomes are nanometer sized membrane vesicles invaginating from multivesicular bodies and secreted from epithelial and hematopoietic cells. They were first described "*in vitro*" but vesicles with the hallmarks of exosomes are present *in vivo* in germinal centers and biological fluids. Their protein and lipid composition are unique and could account for their expanding functions such as eradication of obsolete proteins, antigen presentation or "Trojan horses" for viruses or prions. Exosome secretion could be a regulated process participating in the transfer of molecules inbetween immune cells. Despite numerous questions pertaining to their biological relevance, the potential of dendritic cell derived-exosomes as cell-free cancer vaccines is currently being assessed. This review will summarize the composition and formation of exosomes, pre-clinical data, Phase I trials and optimization protocols for improving their immunogenicity in tumor bearing patients.

Keywords: exosomes - immunity - immunotherapy

Introduction

Cancer treatment is an ever more challenging area. New strategies emerge such as targeted therapies by monoclonal antibodies (mAb) or anti-angiogenesis products. Some of the most promising emerging approaches involve the manipulation of the immune system [1]. Adoptive transfer (*i.e.* iv injection) of T cells, of antigen-loaded dendritic cells and mAb targeting FcgRIIIa brought up proofs of concept [2, 3]. As we learn more and more from cell therapies, approaches aimed at developing cell free vaccines are being developed [4]. Targeting dendritic cells (DC) *in vivo* using DEC205 Ab or DC-SIGN coupled to tumor antigens [5, 6] is in the current prospects. We have investigated an alternate approach of targeting *i.e.* the use of exosomes derived from DC which mediate immunogenicity

# These authors contributed equally to this work.
* Correspondence to: Laurence ZITVOGEL
U805 Institut National de la Santé Et de la Recherche Médicale, Institut Gustave Roussy, 32 rue Camille Desmoulins, 94805 Villejuif, France.
Tel.: +33 142 11 50 41
Fax: +33 142 11 60 94
E-mail: zitvogel@igr.fr

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by transfer of MHC/peptide complexes to naïve DC *in vitro*. This review presents the main biological features of exosomes, their physicochemical characteristics, discusses the potential mechanisms of exosome biogenesis, and underscores the rationale of their use for immunotherapy of cancer.

Exosome characterization

Exosome genesis

Originally, the demonstration of the exosome as a non-plasma membrane vesicle emerged with the description of the shedding of transferrin receptors during the maturation of reticulocytes into red blood cells. The release process appears to require a specific sorting into "internal vesicles" contained inside the endosomes leading to the formation of multivesicular bodies (*i.e.* MVBs) followed by the fusion of the limiting membrane of the MVBs with the plasma membrane. The latter fusion results in exosome release into the extracellular medium [7, 8]. Therefore, the "intraluminal vesicles" become "exosomes". The pioneering studies in reticulocytes (which lack lysosomes) demonstrated that MVBs and their contents, by fusing with the cell surface, eliminate obsolete proteins that do not follow an intracellular degradation pathway [9, 10].

Many cell types generate exosomes *in vitro*. Exosome secretion was first described in hematopoïetic cell types, such as reticulocytes [9, 10], B and T lymphocytes [11, 12], platelets [13], mast cells [14], and dendritic cells [4]. Other cell types also produce exosomes: tumor cells [15], intestinal epithelial cells [16], astrocytes and neurons [17].

Formation of MVBs requires protein sorting. This phenomenon involves ubiquitinylated protein-binding proteins working in sequence [18]. The endosomal sorting complex required for transport (ESCRT) 0 recognize the ubiquitinylated cargo *via* protein Hrs (mammalian Hepatocyte Receptor tyrosine kinase Substrate) also termed Vps (vacuolar protein sorting)-27. Hrs recruits ESCRT-1 and Tsg (Tumor susceptibility gene)-101, which recruits ESCRT-III via ESCRT-II or AIP-1/Alix. The last complex drives the cargo into the budding vesicle, while the ESCRT complexes are recycled *via* Vps4 activity [19, 20]. A passive mechanism could also be implicated in protein sorting into the MVBs, involving the presence of membrane microdomains, tetraspanin enriched [21] or cholesterol enriched (lipid rafts) [22]. Lipid rafts are highly hydrophobic regions, wich are enriched in cholesterol and glycosphingolipids. They can concentrate hydrophobic proteins, such as GPI anchored or acylated ones, and could play a role in exosome formation [23]. The budding itself seems to be dependent on the properties of a conically shaped, pro-fusogenic lipid, the lyso(bis)phosphatidic acid (LBPA), and the LBPA binding protein Alix [24]. However, Laulagnier *et al.* were unable to detect enrichment of LBPA in exosomes from mastocytes [23].

Exosome composition

The characterization of exosomes has been facilitated by their purification from culture supernatants using several procedures including differential ultracentrifugation and sucrose density flotation gradients [25]. Analyses of the purified exosome preparations have been performed using immunoelectronmicroscopy, Western Blotting, mass spectrometry (proteomics) and thin-layer chromatography (lipid composition) [26, 27].

The protein composition of exosomes analyzed by SDS-PAGE and Coomassie Blue staining differs from that of lysates from whole cells, cytosol and total membranes. Moreover, exosomes are distinct from microvesicles produced by apoptotic cells and are only secreted by living cells [26]. All the data obtained for exosomes derived from murine DC, human intestinal epithelial cells and Epstein-Barr virus (EBV) transformed B lymphocytes revealed common and cell-specific constituents. Such exosomes are highly enriched in MHC class II molecules, contained MHC class I, cytosolic chaperones (heat shock proteins), subunits of trimeric G proteins, annexins, rab proteins, integrins, adhesion molecules, enzymes, elongation factors, cytoskeleton proteins, raft-associated proteins and glycolipids. Tetraspanins are the most abundant protein family found in exosomes. Tetraspanins are membrane proteins with four transmembrane domains, involved in many different biological functions such as fusion, motility, immune stimulation, adhesion and protein sorting [28]. Several members of

Table 1a Identification of exosomal proteins by mass spectrometry.

Protein name	MW (kda)
Cytoskeleton	
Actin γ	41
Actinin α 4	104
Advillin	92
β cathenin	85
Catenin E α	100
Cap 1	51
Claudin 1	22
Cofilin 1	18
Coronin	50
Dynamin 2	97
Elongation factor 1α1	50
Ezrin	69
Fascin 1	54
Formin-related protein	122
IQGAP1	188
Moesin	66
Myosin heavy chain IX	226
Radixin	68
Rho GDI1	23
Syntaxin binding protein 3	67
Syntenin	32
Talin	270
TCTP	19
Tubulin β 5	50
Vimentin	51

Protein name	MW (kda)
Adhesion	
Cadherin 1	98
CD11a	128
CD11b	127
CD11c	129
CD13	109
CD166	65
CD18	85
CD9	25
Dectin 1	24
Dectin 2 β	20
Dectin 2 γ	19
Galectin 3	27
Neuropilin 1	103
PGRL	65
Pira7	75
Plexin a1	211
Plexin c1	176
Thrombospondin 1	129
Vinculin	116
Membrane fusion	
Annexin 1	38
Annexin 2	54
Annexin 11	38
Annexin 4	36
Annexin 5	35

Table 1a Identification of exosomal proteins by mass spectrometry.

Protein name	MW (kda)
Annexin 6	75
Annexin 7	49
Arp2/3 complex	20
Clathrin heavy chain	192
Rab10	22
Rab11b	24
Rab13	22
Rab14	24
Rab15	19
Rab2, rab4, rab5a, rab5b, rab6, rab7	23
Rab gdi α, rab gdi β	50
Rho a	23
Rho g	21
Snap 23	23
Syntaxin 7	29
Wd repeat containing protein 1	66
T cell stimulation	
CD86	80
MHC I	32
MHC II	28
Metabolic enzymes	
Transketolase	66
Aldehyde reductase	36
Aspartate aminotransferase	46
ATP citrate lyase	119
ATPase Na^+/K^+	115
Dipeptidylpeptidase 4	87
Fatty acid synthase	272
Glucose6phosphate isomerase	32
Glucose6phosphate deshydrogenase	35
M2 pyruvate kinase	57
Peroxiredoxin 1	22
Phosphogluconate deshydrogenase	53
Phosphoglycerate kinase 1	44
Sh2 phosphatase 1	67
Tyk 2	132
Mvb formation	
Alix	96
Tsg101	44
Ubiquitin c	100
Signaling	
14-3-3 (β, ε, γ, ζ)	28
Copine 1	49
C-src tyrosine kinase	50
Erk2	33
Fyn	60
Gi3α	40
Gi2α	38
Grb3-3	37
Gs α	82
H-ras	17
Phospholipase c α	58

Table 1a Identification of exosomal proteins by mass spectrometry.

Protein name	MW (kda)
Rap2b	20
Shps-1	56
Stat1	83
Chaperone	
Cyclophilin a	17
Cyclophilin b	22
H2-m	28
Hsc 70	70
Hsp84	84
Ia associated invariant chain	24
Tcp-1 (β, δ, ε and ζ)	58
Tcp-1 τ	59
Other	
CD98	58
Complement factor 3	186
EEF2	95
Ferritine light chain 1	20
Ferritine light chain 2	20
Histone 1	14
Histone 2	13
Histone 3	13
Mfge-8	51
Mvp	96
Nicastrin	78
Proteasome 26s subunit3	49
Ribosomal protein l18	21
Ribosomal protein l6	32
Ribosomal protein s18	17
Transferin receptor	85
AP-1	104
Integrin β1	88
Flotillin 1	47
Prohibitin	29
Stomatin	31

Table 1b Antibody detection of other exosomal proteins.

Protein name	Family
ULBP-1	NKG2D ligand
MICA/B	NKG2D ligand
ICAM-1 (t cell stimulation)	Ig superfamily
CD81	Tetraspan
CD82	Tetraspan
CD62	Tetraspan

this family, including CD9, CD37, CD53, CD63, CD81 and CD82 are highly enriched in exosomes from virtually any cell type. In **Table 1**, we show the list of the major proteins found in mouse DC derived-exosomes (DEX) [26, 27, 29, 50].

The specific role of many proteins found in exosomes or involved in exosome formation have not yet been defined. For some, however, the roles are becoming clearer. For example, exosomes contain enriched fractions of phospholipase D2, and PLD2 activity has been related to exosome release [30]. Likewise, the GPI-anchored proteins CD55 and CD59 which are associated with exosomes, appear to

protect them from complement attack. Another example includes the identification in exosomes of proteins that are part of the machinery involved in the biogenesis of MVBs. Thus, DEX contain Tsg101 and Alix which are components of the conserved machinery that selects ubiquinated cargo proteins for sorting to intraluminal vesicles [24]. The role of the most abundant proteins found on DEX, such as MHC class I, II, CD86 molecules, ICAM-1/CD54, a milk fat globule protein MFG-E8/Lactadherin, for eliciting antigen-specific T cell immune responses is discussed below.

The lipid composition of exosomes from DC and mast cells was reported in 2004 [23] and showed that their lipid composition differed from any other DC and mast cell membranes, in that they are enriched in some lipids (sphingomyelin, lysophosphatidylcholine LPC, saturated fatty acid), and relatively poor in some others (phosphatidylcholine, diacylglycerol). They are characterised by the distribution of phosphatidylethanolamine (PE) between the inner and outer leaflets. For most membranes, the PE is asymmetrically distributed, with an enrichment of PE in the inner leaflet. But the exosome membrane has a symmetrical PE repartition, and a rapid flip-flop activity between the two leaflets. Paradoxally, exosomes are very rigid and stable at pH=7, with a low lateral diffusion of lipids. However, this rigidity decreases with decreased pH. The presence of sphingomyelin and saturated fatty acids in PE and PC may account for the high rigidity. The presence of tetraspanins, which form microdomains that are not lipid rafts, may explain the pH-dependant loss of rigidity: rigidity of lipid-only vesicles vary only according to the temperature, while pH variations may induce protein plasticity. The physical properties of exosome membranes make them resistant to enzymatic degradation in the circulation. Their symmetrical PE distribution may facilitate their adsorption, but not their fusion with target cells, such as follicular dendritic cells [31]. The mechanisms of cell targeting by exosomes is still a matter of debate. Exosomes contain many adhesion molecules (integrins, ICAM-1, lactadherin) or lysophosphatidylcholine that could promote dendritic cell targeting *in vivo* [32].

Exosome purification

The biophysical properties of exosomes enable their purification/extraction from culture medium supernatant or physiological fluids (blood [33], ascites [34], malignant effusions [35]). A good manufacturing procedure (GMP) has been described [25]. The size and density of exosomes allow their purification by diafiltration (500KDa filter) and/or differential ultracentrifugation (100.000g) on D2O/sucrose density cushion or sucrose gradient (1,13 to 1,21 kg/L), which may vary with cell origin. Exosomes can be stored for at least two years at –80°C.

Exosomes purified from serum containing culture medium may be contaminated by serum derived-exosomes [33]. Therefore, clearance of the serum by ultra filtration or ultracentrifugation prior to use in cultures is necessary to remove any preexisting serum vesicles.

Exosomes and the immune system

Immunostimulatory properties of DEX

The first report demonstrating immunostimulatory properties of exosomes derived from APC was performed by Raposo *et al* who showed that exosomes secreted by EBV-transformed B cells stimulated human CD4+ T cell clones in an antigenic-specific manner [36]. Two years later, we showed that exosomes produced by mouse DC pulsed with tumor peptides induced the rejection of established tumors [4]. These antitumor effects were antigen specific, required T cells and have been associated with long-term survival.

Therefore, the molecular caracterisation and the immunologic properties of DEX were further investigated with *in vitro* and *in vivo* experimental models.

First, we developed conditions to obtain optimal peptide loading onto DEX. Exosomal MHC class I molecules could be loaded directly onto DEX with a high peptide concentration after an initial step of acid elution of the exosomal pellet [37]. Exosomal MHC class II molecules could be indirectly loaded by pulsing DC with synthetic peptides [38].

Second, the immunogenicity of exosomal MHC class II molecules was examined. Thery *et al*, found that H-Y peptide- bearing DEX promoted antigen driven-proliferation of naïve CD4+ T cells in a Marilyn transgenic mouse model [38]. However, H-Y DEX could not directly trigger CD4+ T cell pro-

liferation in vitro unless transferred onto naïve, immature DC. She showed that the I-Ab molecules required for efficient T cell proliferation were presented by DEX and not by the naïve DC. Therefore, it was the first demonstration of functional transfer of MHC molecules from cell to cell *in vitro*, suggesting that exosomes could be critical cellular components of the immune system.

Third, we confirmed this observation in humans using HLA-A2 DEX pulsed with $Mart1_{26-35}$ peptide. Indeed, human DEX derived from normal volunteers or melanoma patients could promote IFNγ production by Mart1 specific CTL clones only when pulsed onto naïve monocyte derived-DC *in vitro*. These DEX could also elicit CTL priming from naïve PBL or PBL from melanoma patients in the presence of LPS-activated DC [39]. In HLA-A2 transgenic mice (HHD2), HLA-A2 DEX loaded with Mart1 peptide could induce the differentiation of Tc1 cells in the draining lymph nodes, only if pulsed onto DC prior to the injection into footpad of the mice [39]. Importantly, however, in the MHC class I system, DEX-mediated T cell activation occurred only in the presence of maturing DC or TLR3 and 9 ligands [39, 40]. In this model, T cell priming was as efficient with DEX as with 3.10^5 mature DC (a saturating condition of priming with footpad DC).

Thus, these observations showed that DEX could elicit both CD4 and CD8 specific T cell priming in tumor free mice by transferring functional MHC class I and II/peptide complexes onto recipient maturing DC.

Next, we investigated the immunizing capacities of exosome- based vaccines against established tumors in HHD2 mice. We demonstrated that exosomes mixed with the ligands of TLR3 or TLR9 such as double stranded RNA (Ampligen) or CpG oligodeoxynucleotides (CpG-ODN), were efficient for tumor growth retardation. Using B16F10 co-expressing human HLA-A2.1 and gp100 tumor antigen, we established that DEX mixed with CpG-ODN mediated tumor rejection with an equal or greater efficacy than 3.10^5 A2/gp100 mature DC or 50 µg of gp100 peptide mixed with CpG-ODN respectively [40].

Since breaking tumor induced- tolerance is an important goal to improve current vaccines, we investigated the role of regulatory T cells in preventing DEX efficacy. CD4+CD25+ regulatory T lymphocytes (Tregs) are able to prevent the elicitation of T and NK cell-mediated antitumor immune responses. Tumors induce the expansion of Tregs in humans and mice and prevent the eradication of immunogenic tumors [41, 42]. Conversely, the elimination of Tregs is able to trigger antitumor responses leading to tumor rejection [43–45]. Cyclophosphamide (CTX) has been shown to inhibit Treg functions [46, 47]. Therefore, we studied the role played by Tregs in the inhibition of CD8 response induced by DEX in HHD2 mice bearing a B16A2gp100 tumor [48]. We demonstrated that pre-treatment of tumor bearing-mice with CTX prior to DEX/CpG-ODN vaccines promoted long lasting complete tumor eradication, up to 30%, compared to 10% with DEX/CpG-ODN or CTX alone. Furthermore CpG-ODN were not required for the antitumor effects mediated by DEX in combination with CTX. Moreover, the adoptive transfer of Tregs curtailed the synergistic effects of CTX/DEX. In naïve HHD2 mice, CTX could not promote the capacity of DEX to prime naïve CD8+ T cells, but in Mart1 peptide pre-immunized mice, DEX vaccination after CTX pre-treatment was able to boost the Mart1-induced specific response. Hence, in the presence of CTX, DEX can boost effector T cell functions. Tumor growth was able to promote the expansion of tumor specific T cells since splenic T cells from B16A2/gp100 bearing HHD2 mice produced specific IFNγ in mixed tumor lymphocytes cultures when mice were treated with CTX. Thus, DEX vaccines in association with CTX were able to boost a pre-exhisting immune response induced by tumor growth or peptides.

Another strategy tested to boost DEX-mediated antitumor activity was to modulate exosome composition to improve intrinsic immunogenicity. Segura *et al.* showed that the protein composition of exosomes and their biological activity depends on the maturation state of DC [49]. They demonstrated that exosomes secreted by LPS -treated DC ("mature exosomes") exhibited superior priming abilities than exosomes from untreated DC ("immature exosomes"). Indeed, mature exosomes induced strong antigen specific T cell proliferation both *in vitro* and *in vivo* compared to immature exosomes. In male skin graft- bearing female mice, the injection of H-Y peptide loaded mature exosomes triggered effector T cell responses leading to accelerated skin graft rejection. The comparison of mature and immature exosomes [50] revealed their similar

morphology, density and overall protein composition, but they presented a quantitative differences in specific protein composition. MHC class II, CD86 and ICAM-1 molecules were greatly elevated in mature exosomes whereas MFG-E8 and MHC class I molecules were less abundant. The production of exosomes secreted by DC from different KO mice led them to conclude, that expression of ICAM-1 but neither CD86 nor MFG-E8 is necessary on mature exosomes for their increased immunogenicity. CD86 molecules were required on recipient DC to promote T cell activation efficiently. The absence of MFG-E8 on mature exosomes, a molecule that ensures phagocytosis of apoptotic cells by macrophages and DC, did not impair T cell activation [51]. Thus, MFG-E8 is not required for targeting exosomes to recipient DC in mice. However, MFG-E8 can interact with αVβ3 and αVβ5 which are expressed by human DC but not by murine BM-DC. So it is still possible that MFG-E8 might be useful to target exosomes to recipient DC in the human system. In conclusion, exosomes from "*in vitro*" cultured, mature DC, armed with peptide MHC II complexes and ICAM-1, are efficient in CD4 T cell activation "*in vivo*".

The use of tumor-derived exosomes as tumor antigen bearing vehicles

The initiation of a T cell antitumor immune response requires the uptake and processing of tumor antigens by DC and their presentation on MHC I molecules. To load DC with tumor antigens, several "*ex vivo*" approaches have been tested using tumor lysates, tumor RNA, apoptotic or necrotic debris. Our group has demonstrated by electron microscopy that tumor cells harboured MVBs which release tumor cell-derived exosomes (TEX) to the extracellular medium [15]. We showed that both human and mouse tumor cells constitutively secreted these membrane vesicles, similar to DEX in their morphology, density and expression of certain membrane markers (MHC I, LAMP1, tetraspanins, HSP70-80). Moreover, we reported that TEX contain whole native tumor antigens and could trigger a MHC class I restricted T cell clone reactivity *in vitro*. TEX represent a source of tumor rejection antigens since they promote T cell-dependent cross-protection against syngenic and allogenic tumors in mice. In cancer patients, malignant effusions like ascitic or pleural effusions accumulate membrane vesicles bearing MHC I, heat shock protein, tetraspanins and tumor antigens (Her2/neu, Mart1, TRP, gp100) indicating similarities with exosomes [35]. Exosomes from melanoma patients carry Mart1 tumor antigen to monocyte-derived dendritic cells (MD-DC) for cross-presentation to Mart1 specific CTL clones. In 7/9 cancer patients, tumor specific lymphocytes could be efficiently expanded from peripheral blood cells using autologous MD-DC pulsed with autologous ascites purified exosomes (Exas). Thus, Exas represent a natural and new source of tumor rejection antigens, opening up a novel immunization strategy for cancer patients. While these findings are encouraging for the development of a future therapy, some data suggest that tumor -derived exosomes have inhibitory effects on the host's immune system. TEX harbour functional molecules such as FAS-L [52-54], NKG2D ligands [55] or HLA-G [56] involved in the inhibition of effector T cell response. Recently, Liu *et al.* found that pre-treatment of mice with TEX promoted an accelerated growth of tumors in both immunocompetent and nude mice. The protective effect of TEX pulsed DC on tumors was reduced when mice were pre-treated with TEX. Their results were attributed to a potent inhibition of the cytotoxic activity of NK cells induced by tumor exosomes [57]. In contrast, Dai *et al.* demonstrated that exosomes secreted by heat-stressed carcinoembryonic antigen-tumor cells exhibited a more potent stimulatory activity onto carcinoembryonic antigen specific CTL than exosomes from non stressed cells *in vitro* and *in vivo* [58]. Therefore, as a source of tumor antigens, tumor derived-exosomes should either be pulsed onto *ex vivo* mature DC or secreted from heat activated cells to mediate immunostimulatory functions *in vivo*.

Exosomes and clinical immunotherapy

The sophisticated GMP manufacturing process [25] and the optimization of antigen loading [37, 38] together with their immunogenic properties convinced the regulatory authorities to agree to launch clinical trials using DEX in cancer patients. The first phase I clinical trial using autologous DEX was performed at the Gustave Roussy and

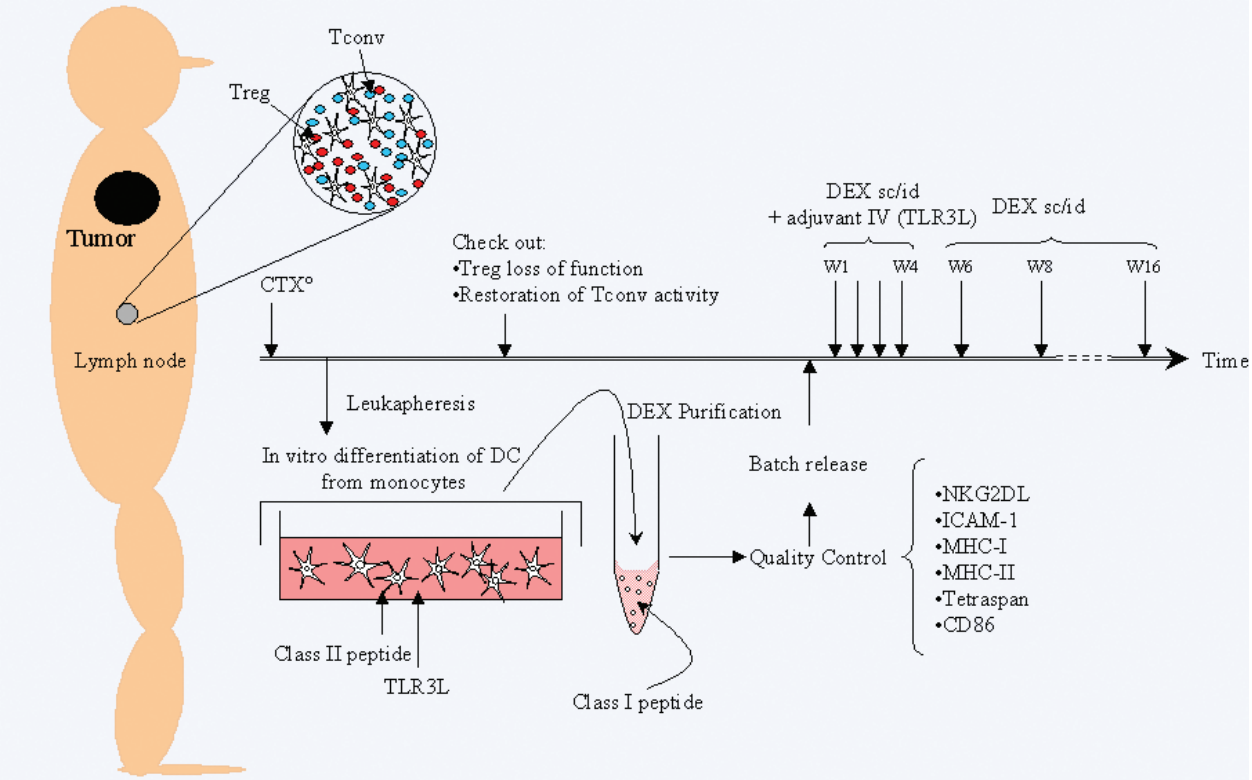

Fig. 1 Design of the Phase II clinical trial with DEX. One putative schema could be *i*) to inhibit the suppressive function of regulatory T cells by treating patients with oral cyclophosphamide at metronomic dosing, *ii*) to derive autologous MD-DC from a leukapheresis, *iii*) to promote DC maturation to enhance ICAM1 expression onto DEX, *iv*) to pulse MHC class II peptides onto DC and MHC class I peptides onto DEX, *v*) to vaccinate patients sc/id on a weekly basis using DEX along with a TLR3L adjuvant intravenously. The immunomonitoring studies should encompass T and NK cell effector functions.

Curie Institute in stage III/IV melanoma patients [59]. DEX were purified from the culture supernatant of day 7 MD-DC of fifteen patients bearing a tumor expressing MAGE3 antigen. MAGE3 peptides (HLAA1/B35) were loaded onto MD-DC (in the first six patients) or directly onto DEX (in nine patients). MAGE3 class II peptides (DP04) or tetanus toxin class II epitopes were also loaded onto MD-DC to confer a helper effect. Two dose levels of cryopreserved DEX (0.13 versus $0.40.10^{14}$ MHC class II molecules) or peptides (10 versus 100 µg/ml) were tested by weekly sc/id injection for 4 weeks, followed by vaccinations every 3 weeks in patients who achieved objective tumor response. GMP DEX were successfully prepared from all patients in the study. The therapy schedule was well tolerated with toxicities under grade II. Although the aim of a phase-I clinical trial is not designed to conclude that the treatment has a significant activity, some clinical benefits were observed (one partial response, one minor response, one mixed response and two stable diseases) in skin and lymph node target lesions. The T cell immunomonitoring did not reveal any significant anti MAGE3 A1/B35 CTL expansion nor clinically significant delayed type hypersensitivity (DTH) responses. Unexpectedly, the number of circulating CD3-/CD56+ NK cells/mm^3 significantly increased after three vaccinations with exosomes. More than 50% of patients exhibited enhanced NK cell cytotoxic capacity and IFNγ secretion following an *ex vivo* boost with IL-2 or DC. This first Phase I trial together with a second one [60] in non small cell lung cancers (NSCLC) established the feasibility and safety of DC-derived exosomes-based vaccination in metastatic patients

Based on these encouraging results, a Phase II/III trial using DEX in NSCLC has been designed as outlined in Fig. 1. In parallel, the group of Adams has launched a Phase I/II trial in

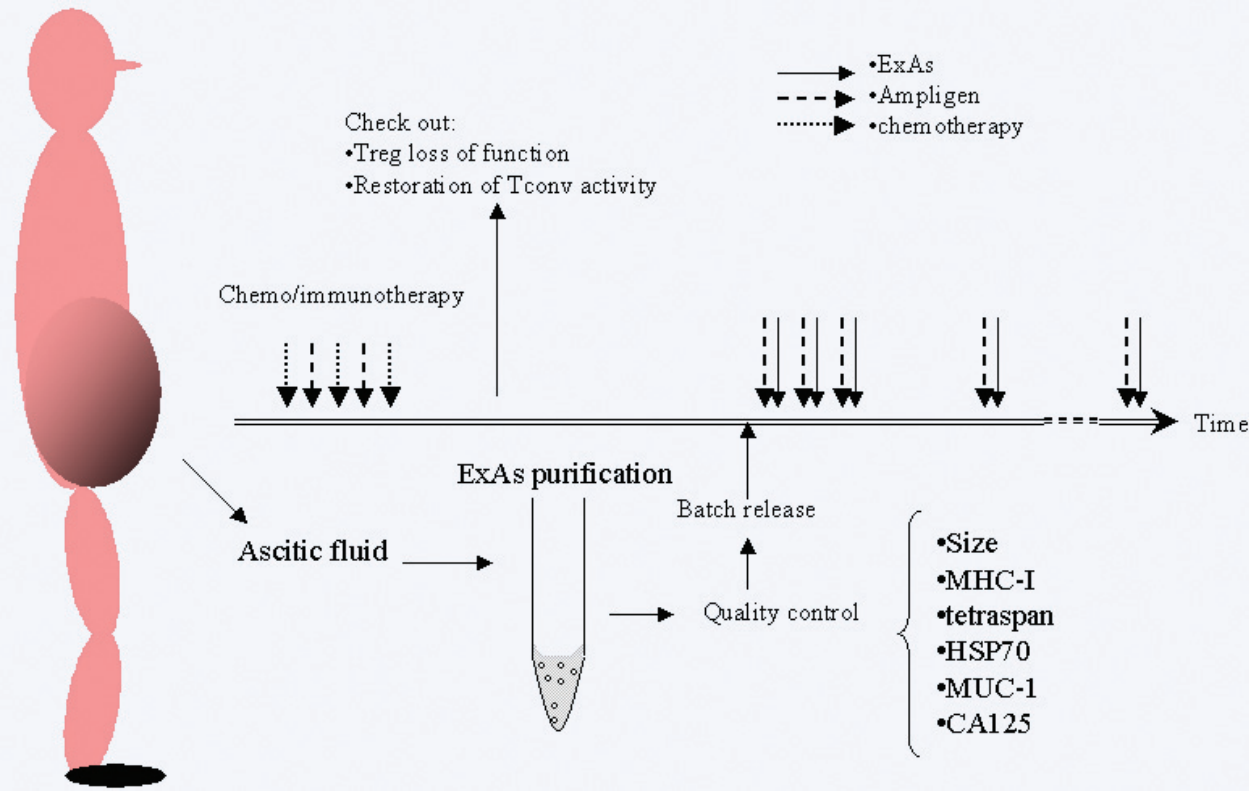

Fig. 2 Design of a phase I/II clinical trial using TEX. Some malignancies, like ovarian cancer, favour ascites formation, and exosomes can be harvested from ascitic fluids. Patients will receive conventionnal chemotherapy with TLR3L doses in between. ExAs (ascites derived exosomes) can be administrated sc/id after purification and quality control, which includes electron microscopic examination (size and morphology of vesicles) and protein expression by western blotting (MHC-I, CD81 and HSP70), and adsorption capture assay (for Muc-1). TLR3 ligand, such as Ampligen, will be co-administred iv to enhance DC maturation. There is no need to suppress Treg function, because conventional chemotherapy against such tumors inhibits Treg activity [65].

ovarian cancer patients aimed at assessing the association of chemotherapy/ immunotherapy with a TLR3 agonist followed by a vaccination with tumor cell-derived exosomes isolated from autologous ascitic fluids [personal communication and 61] as outlined in Fig. 2.

Other potential therapeutic applications of exosomes

Aline *et al.* established that *Toxoplasma gondii* antigens pulsed DC-derived exosomes could prime an antigen specific cellular and humoral immune response that provides protection against both acute and chronic toxoplasmosis in mice [62]. This was the first demonstration that DEX can be used for immunoprophylaxis against pathogens.

Recently, Robbins *et al.* demonstrated in two studies that exosomes produced by IL10 treated BM-DC or by genetically modified BM-DC expressing FAS-L could be immunosuppressive *in vivo* [63, 64]. These tolerogenic exosomes are able to suppress inflammation in a murine footpad model of DTH and to reduce the severity of an established collagen-induced arthritis. These effects were antigen- specific and required the presence of MHC class II on exosomes as well as the integrity of the vesicles. Taken together, these data suggest that immature or genetically modified DC confer immunosuppressive activity to the secreted exosomes. It is therefore conceivable that DEX might be useful in controlling autoimmune diseases.

Conclusion

Exosomes are produced by numerous cell types and can be defined by three major criteria: a size of 60–100 nm in diameter, a density of 1,13 to 1,21 g/dL in a sucrose gradient and an endocytic origin (enrichment in HSP 70, tetraspanins, Tsg101, Alix, MHC molecules). For immunologists, exosomes represent a cell free vaccine which may be a little bit easier to define, store and manipulate compared to a cell-based vaccine. To date, the physiological role of the different exosomes produced *in vivo* is not understood and remains difficult to evaluate. The *in vivo* stimulatory properties of DEX have been demonstrated in preclinical studies using DEX or TEX. It is established that ICAM-1 is necessary for their immunogenicity and that the use of adjuvants or the depletion of Tregs by cyclophosphamide strongly enhanced antitumor effects. The feasibility and the safety of DEX assessed in phase I clinical trials are encouraging for the future development of exosomes in cancer immunotherapy.

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References

1. **Henderson RA, Mossman S, Nairn N, Cheever M.** Cancer vaccines and immunotherapies: emerging perspectives. *Vaccine* 2005; 23: 2359–62.
2. **Davis ID, Jefford M, Parente P, Cebon J.** Rational approaches to human cancer immunotherapy. *J Leukoc Biol.* 2003; 73: 3–29.
3. **Schuler G, Schuler-Thumer B, Steinman RM.** The use of dendritic cells in cancer immunotherapy. *Curr Opin Immunol.* 2003; 15: 138–47.
4. **Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, Ricciardi-Castagnoli, Raposo G, Amigorena S.** Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nature Med*. 1998; 4: 594–600.
5. **Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S, Soares H, Brimnes MK, Moltedo B, Moran TM, Steinman RM.** *In vivo* targeting of antigens to maturing dendritic cells *via* the DEC-205 receptor improves T cell vaccination. *J Exp Med*. 2004; 199: 815–24.
6. **Tacken PJ, de Vries IJ, Gijzen K, Joosten B, Wu D, Rother RP, Faas SJ, Punt CJ, Torensma R, Adema GJ, Figdor CG.** Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells *via* a humanized anti-DC-SIGN antibody. *Blood* 2005; 106: 1278–85.
7. **Fevrier B, Raposo G.** Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr Opin Cell Biol*. 2004; 16: 415–21.
8. **Denzer K, Kleijmeer MJ, Heijnen HF, Stoorvogel W, Geuze HJ.** Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci*. 2000; 19: 3365–74.
9. **Johnstone RM, Bianchini A, Teng K.** Reticulocyte maturation and exosome release: transferrin receptor containing exosomes shows multiple plasma membrane functions. *Blood* 1989; 74: 1844–51.
10. **Fader CM, Savina A, Sanchez D, Colombo MI.** Exosome secretion and red cell maturation: Exploring molecular components involved in the docking and fusion of multivesicular bodies in K562 cells. *Blood Cells Mol Dis*. 2005; 35: 153–7.
11. **Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ.** B lymphocytes secrete antigen-presenting vesicles. *J Exp Med.* 1996; 183: 1161–72.
12. **Clayton A, Turkes A, Navabi H, Mason MD, Tabi Z.** Induction of heat shock proteins in B-cell exosomes. *J Cell Sci*. 2005; 118: 3631-8.
13. **Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ.** Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 1999; 94: 3791–9.
14. **Skokos D, Botros HG, Demeure C, Morin J, Peronet R, Birkenmeier G, Boudaly S, Mecheri S.** Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses *in vivo*. *J Immunol*. 2003; 170: 3037-45.
15. **Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, Flament C, Pouzieux S, Faure F, Tursz T, Angevin E, Amigorena S, Zitvogel L.** Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med*. 2001; 7: 297–303.
16. **van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bensussan N, Heyman M.** Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* 2001; 121: 337–49.
17. **Faure J, Lachenal G, Court M, Hirrlinger J, Chatellard-Causse C, Blot B, Grange J, Schoehn G, Goldberg Y, Boyer V, Kirchhoff F, Raposo G, Garin J, Sadoul R.** Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci.* 2006; in press.
18. **von Schwedler UK, Stuchell M, Muller B, Ward DM, Chung HY, Morita E, Wang HE, Davis T, He GP, Cimbora DM, Scott A, Krausslich HG, Kaplan J, Morham SG, Sundquist WI.** The protein network of HIV budding. *Cell* 2003; 114: 701–13.
19. **Babst M, Katzmann DJ, Estepa-Sabal EJ, Meerloo T, Emr SD.** Escrt-III: an endosome-associated het-

erooligomeric protein complex required for mvb sorting. *Dev Cell*. 2002; 3: 271–82.

20. **Babst M, Katzmann DJ, Snyder WB, Wendland B, Emr SD.** Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. *Dev Cell*. 2002; 3: 283–9.
21. **Wubbolts R, Leckie RS, Veenhuizen PT, Schwarzmann G, Mobius W, Hoernschemeyer J, Slot JW, Geuze HJ, Stoorvogel W.** Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J Biol Chem*. 2003; 278: 10963–72.
22. **de Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M.** Lipid raft-associated protein sorting in exosomes. *Blood* 2003; 102: 4336–44.
23. **Laulagnier K, Motta C, Hamdi S, Roy S, Fauvelle F, Pageaux JF, Kobayashi T, Salles JP, Perret B, Bonnerot C, Record M.** Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. *Biochem J.* 2004; 380: 161–71.
24. **Matsuo H, Chevallier J, Mayran N, Le Blanc I, Ferguson C, Faure J, Blanc NS, Matile S, Dubochet J, Sadoul R, Parton RG, Vilbois F, Gruenberg J.** Role of LBPA and Alix in multivesicular liposome formation and endosome organization. *Science* 2004; 303: 531–4.
25. **Lamparski HG, Metha-Damani A, Yao JY, Patel S, Hsu DH, Ruegg C, Le Pecq JB.** Production and characterization of clinical grade exosomes derived from dendritic cells. *J Immunol Methods.* 2002; 270: 211–26.
26. **Thery C, Boussac M, Veron P, Ricciardi-Castagnoli P, Raposo G, Garin J, Amigorena S.** Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol*. 2001; 166: 7309–18.
27. **Thery C, Regnault A, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P, Raposo G, Amigorena S.** Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J Cell Biol.* 1999; 147: 599–610.
28. **Hemler ME.** Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Annu Rev Cell Dev Biol.* 2003; 19: 397–422.
29. **Thery C, Zitvogel L, Amigorena S.** Exosomes: composition, biogenesis and function. *Nat Rev Immunol.* 2002; 2: 569–79.
30. **Laulagnier K, Grand D, Dujardin A, Hamdi S, Vincent-Schneider H, Lankar D, Salles JP, Bonnerot C, Perret B, Record M.** PLD2 is enriched on exosomes and its activity is correlated to the release of exosomes. *FEBS Lett*. 2004; 572: 11–4.
31. **Denzer K, van Eijk M, Kleijmeer MJ, Jakobson E, de Groot C, Geuze HJ.** Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. *J Immunol*. 2000; 165: 1259–65.
32. **Coutant F, Perrin-Cocon L, Agaugue S, Delair T, Andre P, Lotteau V.** Mature dendritic cell generation promoted by lysophosphatidylcholine. *J Immunol.* 2002; 169: 1688–95.
33. **Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C.** Exosomal-like vesicles are present in human blood plasma. *Int Immunol*. 2005; 17: 879–87.
34. **Navabi H, Croston D, Hobot J, Clayton A, Zitvogel L, Jasani B, Bailey-Wood R, Wilson K, Tabi Z, Mason MD, Adams M.** Preparation of human ovarian cancer ascites-derived exosomes for a clinical trial. *Blood Cells Mol Dis*. 2005; 35: 149–52.
35. **Andre F, Schartz NE, Movassagh M, Flament C, Pautier P, Morice P, Pomel C, Lhomme C, Escudier B, Le Chevalier T, Tursz T, Amigorena S, Raposo G, Angevin E, Zitvogel L.** Malignant effusions and immunogenic tumor-derived exosomes. *Lancet* 2002; 360: 295–305.
36. **Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ.** B lymphocytes secrete antigen-presenting vesicles. *J Exp Med*. 1996; 183: 1161–72.
37. **Hsu DH, Paz P, Villaflor G, Rivas A, Mehta-Damani A, Angevin E, Zitvogel L, Le Pecq JB.** Exosomes as a tumor vaccine: enhancing potency through direct loading of antigenic peptides. *J Immunother.* 2003; 26: 440–50.
38. **Thery C, Duban L, Segura E, Veron P, Lantz O, Amigorena S.** Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat Immunol*. 2002; 3: 1156–62.
39. **Andre F, Chaput N, Schartz NE, Flament C, Aubert N, Bernard J, Lemonnier F, Raposo G, Escudier B, Hsu DH, Tursz T, Amigorena S, Angevin E, Zitvogel L.** Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. *J Immunol*. 2004; 172: 2126–36.
40. **Chaput N, Schartz NE, Andre F, Taieb J, Novault S, Bonnaventure P, Aubert N, Bernard J, Lemonnier F, Merad M, Adema G, Adams M, Ferrantini M, Carpentier AF, Escudier B, Tursz T, Angevin E, Zitvogel L.** Exosomes as potent cell-free peptide-based vaccine. II. Exosomes in CpG adjuvants efficiently prime naive Tc1 lymphocytes leading to tumor rejection. *J Immunol*. 2004; 172: 2137–46.
41. **Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W.** Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004; 10: 942–9.
42. **Ghiringhelli F, Larmonier N, Schmitt E, Parcellier A, Cathelin D, Garrido C, Chauffert B, Solary E, Bonnotte B, Martin F.** CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol*. 2004; 34: 336–44.
43. **Shimizu J, Yamazaki S, Sakaguchi S.** Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol*. 1999; 163: 5211–8.
44. **Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E.** Tumor rejection by *in vivo* administration

of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. *Cancer Res*. 1999; 59: 3128–33.

45. **Polak L, Turk JL.** Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity. *Nature* 1974; 249: 654–6.
46. **Berd D, Mastrangelo MJ.** Effect of low dose cyclophosphamide on the immune system of cancer patients: depletion of CD4+, 2H4+ suppressor-inducer T-cells. *Cancer Res*. 1988; 48: 1671–5.
47. **North RJ.** Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. *J Exp Med*. 1982; 155: 1063-74.
48. **Taieb J, Chaput N, Schartz N, Roux S, Novault S, Ménard C, Ghiringhelli F, Terme M, Carpentier AF, Darrasse-Jèse G, Lemonnier F, Laurence Zitvogel.** Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. *J Immunol.* 2006; 176: 2722–9.
49. **Segura E, Amigorena S, Thery C.** Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses. *Blood Cells Mol Dis*. 2005; 35: 89–93.
50. **Segura E, Nicco C, Lombard B, Veron P, Raposo G, Batteux F, Amigorena S, Thery C.** ICAM-1 on exosomes from mature dendritic cells is critical for efficient naïve T-cell priming. *Blood* 2005; 106: 216–23.
51. **Veron P, Segura E, Sugano G, Amigorena S, Thery C.** Accumulation of MFG-E8/lactadherin on exosomes from immature dendritic cells. *Blood Cells Mol Dis*. 2005; 35: 81–8.
52. **Abusamra AJ, Zhong Z, Zheng X, Li M, Ichim TE, Chin JL, Min WP.** Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis. *Blood Cells Mol Dis*. 2005; 35: 169–73.
53. **Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL.** Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. *Clin Cancer Res.* 2005; 11: 1010–20.
54. **Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, Squarcina P, Accornero P, Lozupone F, Lugini L, Stringaro A, Molinari A, Arancia G, Gentile M, Parmiani G, Fais S.** Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J Exp Med*. 2002;195 :1303–16.
55. **Clayton A, Tabi Z.** Exosomes and the MICA-NKG2D system in cancer. *Blood Cells Mol Dis*. 2005; 34: 206–13.
56. **Riteau B, Faure F, Menier C, Viel S, Carosella ED, Amigorena S, Rouas-Freiss N.** Exosomes bearing HLA-G are released by melanoma cells. *Hum Immunol.* 2003; 64: 1064–72.
57. **Liu C, Yu S, Zinn K, Wang J, Zhang L, Jia Y, Kappes JC, Barnes S, Kimberly RP, Grizzle WE, Zhang HG.** Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function. *J Immunol.* 2006; 176: 1375–85.
58. **Dai S, Wan T, Wang B, Zhou X, Xiu F, Chen T, Wu Y, Cao X.** More efficient induction of HLA-A*0201-restricted and carcinoembryonic antigen (CEA)-specific CTL response by immunization with exosomes prepared from heat-stressed CEA-positive tumor cells. *Clin Cancer Res*. 2005; 11: 7554–63.
59. **Escudier B, Dorval T, Chaput N, Andre F, Caby MP, Novault S, Flament C,Leboulaire C, Borg C, Amigorena S, Boccaccio C, Bonnerot C, Dhellin O, Movassagh M, Piperno S, Robert C, Serra V, Valente N, Le Pecq JB, Spatz A, Lantz O, Tursz T, Angevin E, Zitvogel L.** Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J Transl Med*. 2005; 3: 10.
60. **Morse MA, Garst J, Osada T, Khan S, Hobeika A, Clay TM, Valente N, Shreeniwas R, Sutton MA, Delcayre A, Hsu DH, Le Pecq JB, Lyerly HK.** A phase I study of DEXosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med*. 2005; 3: 9.
61. **Adams M, Navabi H, Croston D, Coleman S, Tabi Z, Clayton A, Jasani B, Mason MD.** The rationale for combined chemo/immunotherapy using a Toll-like receptor 3 (TLR3) agonist and tumor-derived exosomes in advanced ovarian cancer. *Vaccine* 2005; 23: 2374–8.
62. **Aline F, Bout D, Amigorena S, Roingeard P, Dimier-Poisson I.** Toxoplasma gondii antigen-pulsed dendritic cell-derived exosomes induce a protective immune response against *T. gondii* infection. *Infect Immun*. 2004; 72: 4127–37.
63. **Kim SH, Lechman ER, Bianco N, Menon R, Keravala A, Nash J, Mi Z, Watkins SC, Gambotto A, Robbins PD.** Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J Immunol*. 2005; 174: 6440–8.
64. **Hee Kim S, Bianco N, Menon R, Lechman ER, Shufesky WJ, Morelli AE, Robbins PD.** Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive. *Mol Ther*. 2006; 13: 289–300.
65. **Coleman S, Clayton A, Mason MD, Jasani B, Adams M, Tabi Z.** Recovery of CD8+ T-cell function during systemic chemotherapy in advanced ovarian cancer. *Cancer Res.* 2005; 65: 7000–6.