Mechanisms of disease

Malignant effusions and immunogenic tumour-derived exosomes

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Summary

Background Exosomes derived from tumours are small vesicles released in vitro by tumour cell lines in culture supernatants. To assess the role of these exosomes in vivo, we examined malignant effusions for their presence. We also investigated whether these exosomes could induce production of tumour-specific T cells when pulsed with dendritic cells.

Methods We isolated exosomes by ultracentrifugation on sucrose and D_2O gradients of 11 malignant effusions. We characterised exosomes with Western blot analyses, immunoelectron microscopy, and in-vitro stimulations of autologous T lymphocytes.

Findings Malignant effusions accumulate high numbers of membrane vesicles that have a mean diameter of 80 nm (SD 30). These vesicles have antigen-presenting molecules (MHC class-I heat-shock proteins), tetraspanins (CD81), and tumour antigens (Her2/Neu, Mart1, TRP, gp100). These criteria, including their morphological characteristics, indicate the similarities between these vesicles and exosomes. Exosomes from patients with melanoma deliver Mart1 tumour antigens to dendritic cells derived from monocytes (MD-DCs) for cross presentation to clones of cytotoxic T lymphocytes specific to Mart1. In seven of nine patients with cancer, lymphocytes specific to the tumour could be efficiently expanded from peripheral blood cells by pulsing autologous MD-DCs with autologous ascitis exosomes. In one patient tested, we successfully expanded a restricted T-cell repertoire, which could not be recovered carcinomatosis nodules.

Interpretation Exosomes derived from tumours accumulate in ascites from patients with cancer. Ascitis exosomes are a natural and new source of tumour-rejection antigens, opening up new avenues for immunisation against cancers.

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Introduction

Results of previous work[1] have shown that a protective immune response against non-immunogenic tumours could be generated in mice and that immunogenicity might not occur in a patient because the tumour cannot activate the immune system rather than because tumour antigens are absent. Tumour antigens are also present in non-immunogenic tumours in human beings, and generate an immune response when injected with adjuvants. Activated dendritic cells derived from monocytes (MD-DC) that are pulsed with tumour peptides have antitumour effects in patients with melanoma,[2] prostate cancer,[3] lymphoma,[4] and renal cancer.[5] However, immunisation strategies for ovarian or breast cancers have not been effective for several reasons. First, most tumour antigens have been identified in tumours that are immunogenic (eg, melanoma). Second, although many investigators have tried to identify tumour antigens, they have not been able to specify which tumour antigens are tumour rejection antigens—ie, those resulting in an immune response from cytotoxic T lymphocytes. Third, most reports about a source of relevant tumour antigens have dealt with antigens that were extracted or derived from dying tumour cells and that needed an ex-vivo step of tumour manipulation (eg, irradiated tumour cells,[6] fused tumour cells,[7] APOPTOTIC BODIES or necrotic debris,[8] and extracted heat-shock protein[9]).

EXOSOMES are released in vitro by many types of cells, including tumour cell lines[10] and antigen presenting cells.[11,12] These membrane vesicles have a different pattern of proteins compared with plasma membranes and are enriched in molecules involved in antigen presentation (eg, MHC class I and II molecules, costimulatory and TETRASPANIN molecules, heat-shock protein CHAPERONS) and potentially in cell targeting (CD11b, ICAM, tetraspanins, lactadherin). Exosomes derived from tumour cell lines that are loaded on dendritic cells transfer shared tumour antigens, thus stimulating T cells in an MHC class I dependent way. These T cells allow cross-protection against syngeneic and allogeneic tumours in mice.[9] We aimed to isolate and characterise exosomes from malignant effusions, and assessed the immunogenicity of these exosomes.

Methods

Patients

We included patients if they presented with peritoneal or pleural carcinomatosis associated with ascitis or pleural effusion and had tumour cells in the biological fluid. All patients gave oral informed consent, and the internal review board of Institut Gustave Roussy, Villejuif, France, and the local ethical committee approved the protocol. Ascites were removed either at

GLOSSARY

APOPTOTIC BODIES
Apoptotic bodies are remnants of cells in the early stages of cell death induced by apoptosis. They express annexin V but do not stain with propidium iodide and are harvested by low-speed centrifugation in vitro.

CHAPERONS
Chaperons, also called heat-shock proteins, are intracellular proteins that bind and transport peptides between subcellular compartments. Gp96 is a specific heat-shock protein that occurs in the endoplasmic reticulum but not late endosomes (ie, exosomes).

EXOSOMES
Exosomes are membrane vesicles 60–90 nm in diameter that originate in the endosomes secreted by most living cells of haemopoietic origin, and by tumour cells. Exosomes are highly stable, and enriched in tetraspanin, heat-shock proteins, and lactadherin molecules.

T-CELL REPERTOIRE
The T-cell repertoire refers to the specificity of the pool of mature T cells—ie, the expression of unique rearranged gene products of the T-cell receptor. The CDR3 regions of the T-cell receptor, which are encoded by the hypervariable V-J (α chain of the T-cell receptor) or V-D-J (β chain of the T-cell receptor) junctions, are essential for binding the antigenic peptide.

TETRASPANINS
Tetraspanins are molecules of a superfamily (CD9, CD37, CD53, CD63, CD81, CD82, etc) characterised by four transmembrane domains. They are part of a multimolecular complex containing b1 integrins and MHC antigens. The function of these molecules is unclear.

the first debulking operation or examination under anaesthetic, or under local anaesthesia because of symptoms. We excluded patients if they had received chemotherapy within 4 weeks before removal of their ascitis, had a concentration of protein in the exudates that was below 30 g/L, or had haemorrhage associated with carcinomatosis.

Purification of exosomes
We centrifuged ascitis samples at 300 *g* to discard floating cells (figure 1). Supernatants were removed and centrifuged successively at 800 *g* for 30 min, 10 000 *g* for 30 min, and 100 000 *g* for 1 h. After the last centrifugation step, we recovered the pellet and resuspended it in phosphate-buffered saline. The solution was then centrifuged at 90 000 g for 1·25 h to allow irrelevant cell debris to pellet. Exosomes contained in the 30% sucrose/D_2O cushion were resuspended in phosphate-buffered saline, and were concentrated in a last step of 1 h ultracentrifugation at 100 000 *g*, as previously described.[13] We resuspended the pellet of exosomes (referred to ExAs) in phosphate-buffered saline and stored the solution at –80°C. We used a similar process to purify exosomes derived from tumour cell lines (referred to as ExLi) contained in in-vitro supernatants accumulated over 3–5 passages.

Electron microscopy
Exosomes obtained after differential ultracentrifugation were fixed in 4% paraformaldehyde and stored at 4°C. We then loaded exosomes onto electron-microscopy grids coated with formvar carbon, and contrasted and embedded them in a mixture of uranyl acetate and methylcellulose.[11] When indicated, we did single immunogold labelling before the contrasting step with mouse monoclonal antibodies to class I molecules (HC10), TRP1 (TA99), gp100/Pmel 17 (HMB45), and CD81, and a rabbit polyclonal antibody to HLA DR[11] (Novocastra, UK). The primary antibodies were visualised with protein A coupled to 10 nm gold particles (Department of Cell Biology, Utrecht Medical School, Utrecht, Netherlands).

Quantification of MHC class I molecules associated with exosomes
We incubated a mouse monoclonal antibody to human MHC class I molecules of a known concentration with excess exosomes that had been dried in a 96-well plate, and subsequently blocked the excess antibody with 6% non-fat milk. After 1 h incubation, the plate was washed and incubated with excess goat antibody to mouse conjugated to horseradish peroxidase for 1 h at room temperature. We developed the plate for 5 min using the electrochemiluminescence substrate system (Amersham Pharmacia, Orsay, France), and measured the chemiluminescence signal with the Wallac trilux chemiluminometer (Perkin Elmer, Gaithersburg, MA).

Western blot analysis
We extracted exosomal or cell-lysate proteins as described previously[10] and analysed them by Western blotting with a monoclonal antibody to human MHC class I molecules (clone HC10, Soldano Ferrone, Roswell Park Cancer Institute, NY, USA); to Hsc70 (clone N27F3, SPA 815, Stressgen, Canada); to gp96 (clone 9G4, SPA 850, Stressgen, Canada); to MHC class II molecules (clone 3B5); to Mart1/MelanA (clone A103, Novocastra, UK), or Her2/Neu (clone ncb11) at the suppliers' recommended dilutions, followed by secondary horseradish peroxidase coupled antibodies (Jackson Immunoresearch, West Grove, PA, USA) and chemiluminescence detection (Boehringer Mannheim, Penzberg, Germany).

Cell cultures
We pelleted tumour cells by centrifugation of the ascitis at 300 *g*, and cultured them in 5% carbon dioxide in RPMI 1640 supplemented with 50 U/mL penicillin, 50 mg/L streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate (Gibco-BRL, France), and 10% decomplemented (heat inactivated for 30 min at 56°C) fetal calf serum (FCS; Seromed, France). These primary tumour cultures were assessed on cytospins with May Grünwald Giemsa and anticytokeratin staining by pathologists.

We isloated peripheral blood mononuclear cells by Ficoll-Hypaque density gradient centrifugation and plated them at $3–5\times10^6$ cells/mL in AIMV (Gibco-BRL, France), supplemented with 50 U/mL penicillin, 50 mg/L streptomycin, 2 mM L-glutamine, 1mM sodium pyruvate, and 10% decomplemented FCS for 3 h at 37°C. Cells that did not adhere were mostly lymphocytes that were frozen at –80°C at day 0. We washed the adherent fraction three times in phosphate-buffered saline. Adherent cells were then propagated in AIMV complete medium containing 1000 IU/mL of recombinant human granulocyte macrophage colony stimulating factor and recombinant human interleukin 4 (Novartis-Schering Plough, NJ, Kenilsworth, USA). MD-DCs differentiate from CD14+ adherent cells and become floating cells with dendrites and cytoplasmic expansions by day 2–3, and lose CD14 and acquire CD1a expression. These cells were frozen at day 5, and used at day 6—ie, 1 day after thawing—for in-vitro stimulations. Fluorescence-activated cell-sorting scan analysis of these cells showed low expression of CD1a,

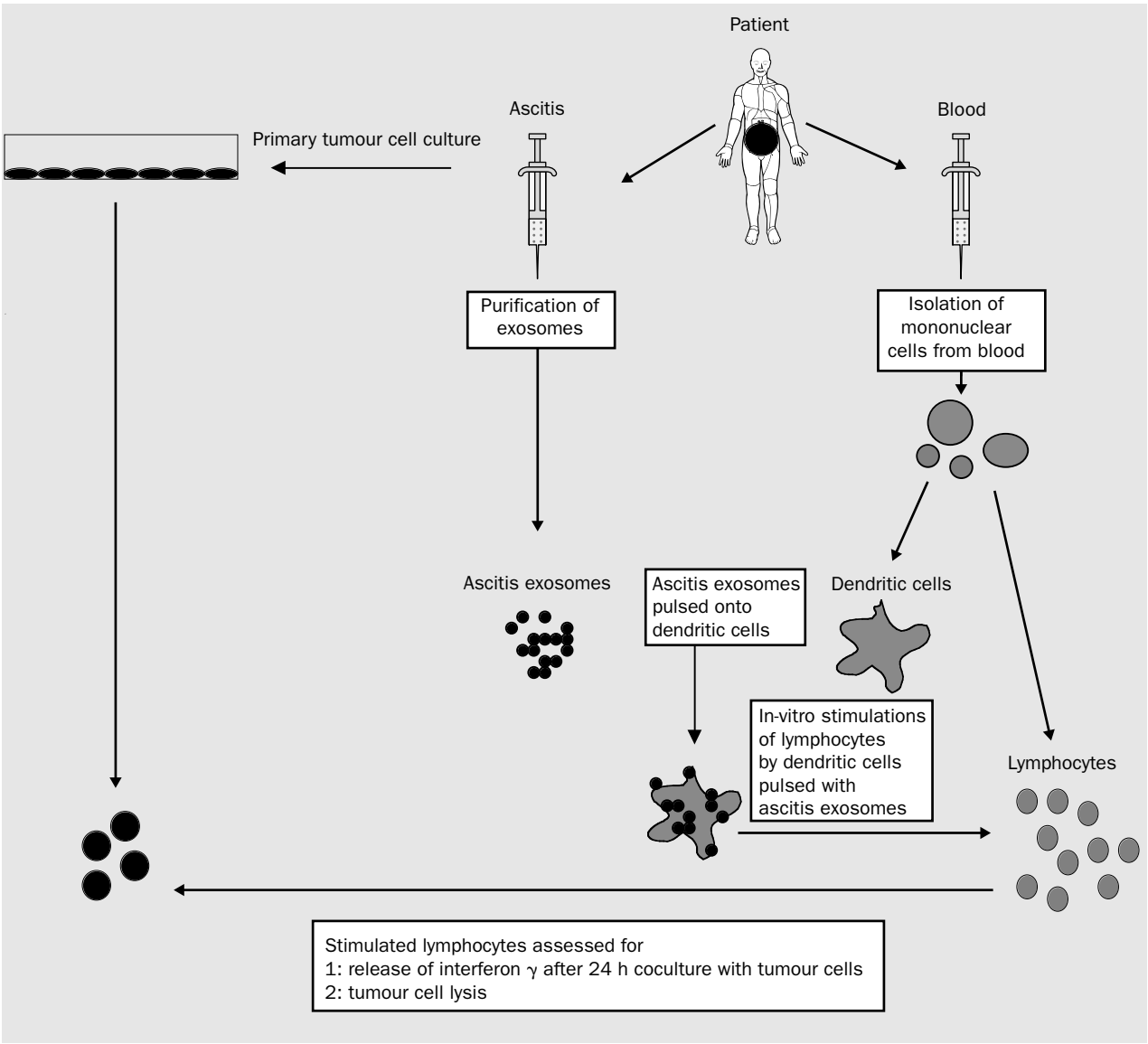

Figure 1: **Methods used to purify exosomes and assess T-lymphocyte activation**

HLA-DR, CD11c, CD40, CD80, and CD86 molecules, with no detection of CD83. MD-DCs could mature completely after stimulation by lipopolysaccharides (as assessed by upregulation of CD83 and enhanced allostimulatory activity in mixed lymphocyte reaction, data not shown).

In-vitro stimulation of peripheral blood lymphocytes

Mixed in-vitro microcultures were done in round-bottomed 96-well plates, in RPMI complete medium supplemented with 10% human pooled AB serum, 100 IU/mL interleukin 2 (Proleukine, Chiron), and T-cell growth factors.[14] We pulsed 3×10^4 autologous MD-DCs with autologous ascitis exosomes or autologous tumour exosomes for 2 h in 50 μL of culture medium. The number of exosomes pulsed onto the dendritic cell well was equivalent to 10 mL of pleural/peritoneal effusion or culture supernatant (patient 1–7) or was normalised to 10^{12} equivalent MHC class I molecules (patients 8 and 9). Autologous peripheral blood lymphocytes were then added at a ratio of three lymphocytes to one MD-DC in a final volume of 200 μL/well. We stimulated the peripheral blood lymphocytes in vitro once a week for a total of two or three stimulations. Alternative culture conditions and negative controls included stimulations with irradiated autologous tumour cells (10^4/well) for patients 1 and 9, and with unpulsed dendritic cells in seven patients. At days 15–21, we harvested stimulated lymphocytes, counted viable cells using trypan blue exclusion, phenotyped them with fluorescence-activated cell-sorting analysis with monoclonal antibodies to CD4, CD8, and CD56 (Coulter, Fullerton, USA), and tested them in Chromium-51 release assays and interferon γ release assays against autologous tumour cells with or without monoclonal antibodies to MHC class I molecules (produced from laboratory hybridoma of W632) or irrelevant tumour cells. Figure 1 summarises the methods.

Interferon γ release assay

After 2 or 3 rounds of in-vitro stimulation, we incubated 5×10^4 lymphocytes with or without 10^4 autologous tumour cells. Interferon γ was measured in 24 h super-

Patient	Sex	Age (years)	Primary tumour site	Histology	Stage and metastatic sites	Previous chemotherapy regimen	Follow-up (months)
1	F	67	Ovary	Papillary adenocarcinoma	FIGO IV Pleura/peritoneum	Carboplatin/doxorubicin×6, Melphalan×2, paclitaxel×2, Fluorouracil×2	Dead (2)
2	M	64	Kidney	Clear-cell adenocarcinoma	UICC IV Peritoneum/lung/bone/ lymph node	Interleukin 2/interferon alfa×2 Thalidomide	Dead (2)
3	F	51	Ovary	Papillary adenocarcinoma	FIGO IIIc Peritoneum	No	Alive (12)
4	F	68	Peritoneum	Adenocarcinoma	FIGO IIIc Peritoneum	No	Dead (9)
5	M	67	Lung	Adenocarcinoma	UICC IV Lung/peritoneum	Oxaliplatin/gemcitabine×6, Vinorelbine×7	Dead (3)
6	F	48	Breast	Ductal adenocarcinoma	UICC IV Peritoneum/bone/lymph node	Fluorouracil×2/doxorubicin/ cyclophosphamide×6	Dead (8)
7	F	80	Ovary	Papillary adenocarcinoma	FIGO IIIc Peritoneum	No	Alive (12)
8	M	38	Peritoneum	Mesothelioma	Unknown Peritoneum	No	Alive (10)
9	F	46	Breast	Ductal adenocarcinoma	UICC IV Peritoneum/bone	Doxorubicin/cyclophosphamide/ fluorouracil×6 Docetaxel/cisplatin×6	Alive (13)
10	M	62	Skin	Melanoma	UICC IV Peritoneum/lymph node	Dacarbazine×3	Dead (2)
11	F	42	Skin	Melanoma	UICC IV Peritoneum/liver/skin/lymph node	Dacarbazine/BCNU/cisplatin×3 Fotemustine×1	Dead (2)

FIGO=International Federation of Gynecology and Obstetrics. UICC= Union Internacional Contra la Cancrum.

Table 1: **Patients' characteristics**

natants by ELISA (Pharmingen, San Diego). To check for the MHC class I restriction of the tumour recognition, we incubated antibodies to MHC class 1 molecules for 1 h with tumour cells before coculture with lymphocytes.

In-vitro cross-presentation assays

LT11 (BV9) is a CD8 positive T-cell clone that carries Mart1 and is restricted to HLA-A2.[14] MD-DCs from healthy volunteers positive for HLA-A2 were plated at day 6 at 2×10^4 cells/well for 2 h with the ascitis exosomes of patients with melanoma or ovarian cancer that was HLA-A2 negative. We then added 2×10^4 LT11 to 2×10^4 of these cultures in a final volume of 200 μL in round-bottomed 96-well plates. Release of interferon γ in the 24-h supernatants was assessed with ELISA (Immunotech, Marseille, France).

T-cell cloning and analysis of T-cell receptor β-chain use

After three rounds of in-vitro stimulation with MD-DCs pulsed with ascitis exosomes, we did a limiting dilution assay with one patient's lymphocytes. We incubated plates containing about 0·6 cells/well with 5000 rad γ-irradiated autologous tumour cells (10^4/well) and Epstein-Barr virus transformed allogeneic B cell lines (10^5/well). Cultures were done in round-bottomed 96-well plates in RPMI 1640 supplemented with 10% human pooled AB serum, 50 IU/mL recombinant human interleukin 2 (Proleukine, Chiron, USA), and T-cell growth factors.[14] After 15 days of limiting dilution assays, we tested growing clones in a ^{51}Cr release assay against autologous tumour cells and K562 cells to assess non-specific lysis. We also did blocking experiments with antibodies to MHC class I molecules. Clones with lysis of autologous tumour cells restricted to MHC class I molecules were subsequently analysed for use of T-cell receptor β-chain V domains. First, we used the monoclonal antibodies specific to β-chain V to test the β-chain V profile of individual clones. Then we extracted RNA from T-cell clones ($1–5\times10^5$ cells) and

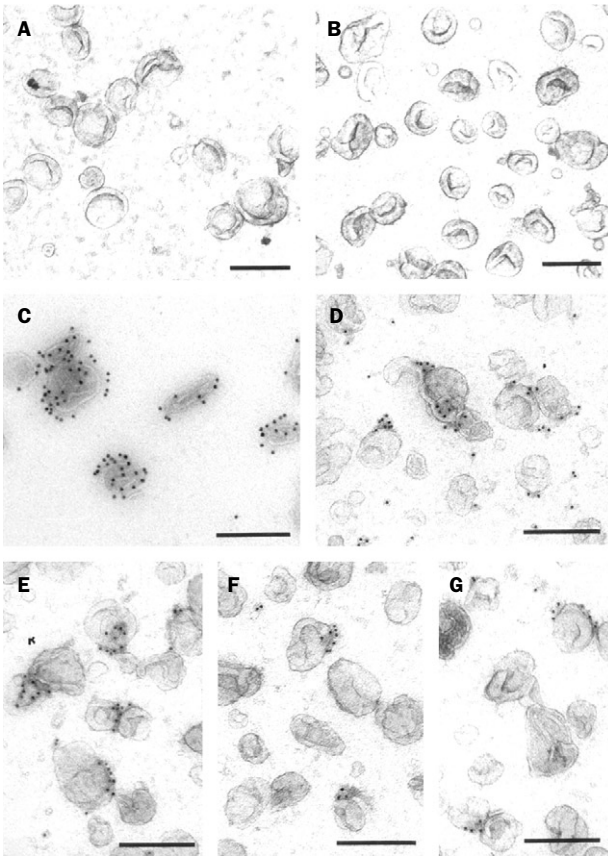

Figure 2: **Electron-microscopy images of ascitis samples**
Exosome-like vesicles from the ascitis samples of patients with ovarian cancer (A) and melanoma (B); exosomes from patient with melanoma were immunogold labelled with antibodies to tetraspanin CD81 (C), MHC class I molecules (D), MHC class II molecules (E), TRP1 (F), and gp100 (G), and protein A coupled to 10 nm gold particles. Bars=220 nm.

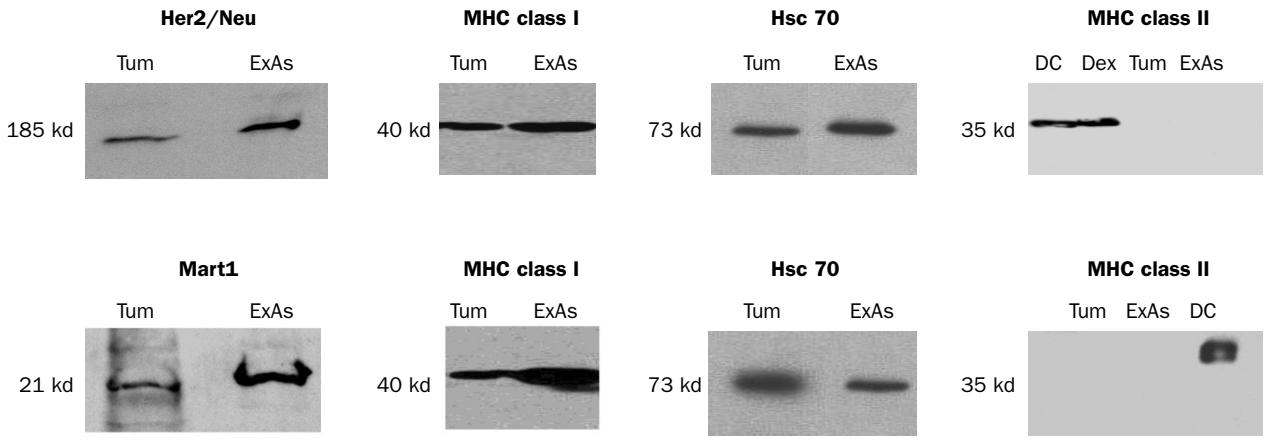

Figure 3: **Western blot analysis of ascitis vesicles**
Western blot of 10–20 μg of proteins extracted from exosomal ascitis pellets (ExAs) or ascitis tumour cell lysates (Tum) with antibodies directed against Mart1 or Her2/Neu native tumour antigens, against MHC class I and class II molecules, and against heat-shock protein 70 chaperons (Hsc 70). Lysates of exosomes derived from dendritic cells (Dex) and from monocytes (DC) were used as positive controls for MHC class II immunoblotting. Ascitis samples are from patient 3 (upper) and patient 10 (lower).

from carcinomatosis nodules containing tumour-infiltrating lymphocytes to analyse T-cell receptor β-chain V domains.[15] We extracted RNA with RNAzol, and converted it to cDNA by reverse transcriptase. A PCR based-runoff technology was done as previously described.[15]

Cytotoxicity assay
After two to three rounds of in-vitro stimulation, autologous lymphocytes were used as effector cells. Targets were either K562 cells or autologous primary tumour cells after 3–5 passages, both labelled with 100 μCi of ^{51}Cr for 60 min and washed three times before use. To inhibit non-specific lysis, unlabelled K562 cells were added at a ratio of 50 to 1 labelled tumour target. To check for MHC class I restriction of tumour recognition, we incubated antibodies to MHC class I molecules for 1 h with ^{51}Cr labelled tumour cells before coculture with the effector cells. Effector cells were added to corresponding targets at varying effector-to-target cell ratios. After 4 h, we harvested 50 μL of each supernatant, and measured release of ^{51}Cr (gamma counter; Packard Top-Count, Zurich, Switzerland). The mean of triplicate samples was calculated and the proportion of specific ^{51}Cr release was ascertained as follows:

proportion of specific lysis=(experimental ^{51}Cr release–control ^{51}Cr release)÷(maximum ^{51}Cr release–control ^{51}Cr release)×100.

Patient (type of exosome)	Exosomal MHC class I molecules*	Tumour cells†
2 (ascitis)	3×10^{10}	NA
4 (ascitis)	1×10^{11}	NA
5 (ascitis)	5×10^{10}	NA
6 (ascitis)	9×10^{10}	NA
7 (ascitis)	2×10^{10}	NA
8 (supernatant and ascitis)	8×10^{9} and 1×10^{11}	$2{\cdot}6\times10^{8}$ NA
9 (supernatant and ascitis)	7×10^{9} and 1×10^{11}	3×10^{8} NA
Mean (SD)	7×10^{10} (3×10^{10})	NA

NA=not assessable. *Immunocapture assay with antibodies to HLA-A, HLA-B, and HLA-C. Values are quantity/mL of ascitis or supernatant. †Number of primary tumour cells over five passages.

Table 2: **Quantification of exosomes**

The values for experimental ^{51}Cr release were counts from target cells mixed with effector cells, control ^{51}Cr release were counts from targets incubated with medium alone (spontaneous release), and maximum ^{51}Cr release were counts from targets exposed to 5% Triton X-100.

Role of the funding source
The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or in the writing of the report.

Results

We assessed malignant effusions for presence of exosomes in three patients with papillary adenocarcinoma, one with clear-cell adenocarcinoma,

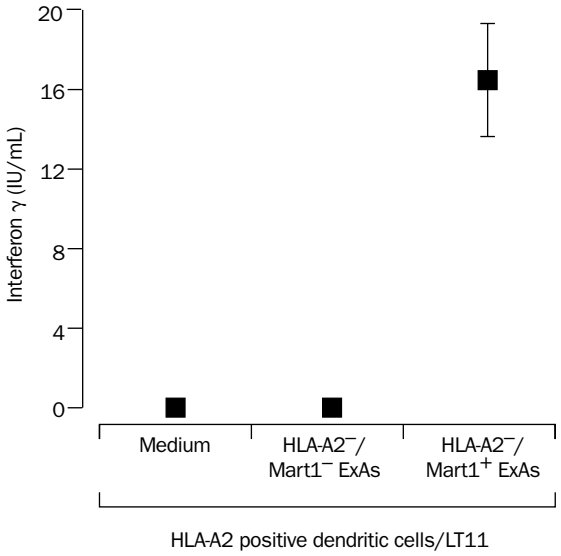

Figure 4: **Release of interferon γ when HLA-A2 positive dendritic cells combined with T-lymphocyte clones are cultured in medium, and with Mart1 negative (ovarian cancer) and Mart1 positive (melanoma) ascitis exosomes**
Results are from two independent experiments. Values are mean (SD) of triplicate wells. ExAs=ascitis exosomes.

Patient	In-vitro stimulation condition	Cycles	Proliferation index*	CD3+/CD8+	Specific lysis (SE)	Interferon γ release after coculture		Tumour specific interferon γ release (pg/mL/day)† (2–1)
						Medium (1)	Tumour cells (2)	
1	Auto tum	3	1	4%	0 (5)			
	DC/ExAs	3	10	14%	30 (7)‡	ND	ND	ND
2	DC	3	1	7%	0 (0)	38	56	18
	DC/ExAs	3	8	14%	12 (2)	155	681	526
3	DC	3	7	30%	30 (2)	80	160	80
	DC/ExAs	3	8	30%	45 (3)	40	1400	1360‡
4	DC	2	2	33%	0 (0)	82	60	0
	DC/ExAs	2	4	35%	5 (1)	53	57	4
5§	DC	2	2	28%	2 (2)	0	0	0
	DC/ExLi	2	3	23%	6 (2)	144	372	228
	DC/ExAs	2	4	30%	11 (1)	50	256	206
6	DC	3	5	34%	6 (2)	105	140	35
	DC/ExLi	3	10	32%	6 (2)	75	519	444
	DC/ExAs	3	12	35%	33 (0)¶	230	1378	1148
7	DC	3	3	17%	0 (1)	128	161	33
	DC/ExLi	3	5	23%	0 (1)	55	46	0
	DC/ExAs	3	5	24%	0 (2)	58	46	0
8¶	DC	2	2	10%	NA	0	0	0
	DC/ExLi	2	2	3%	NA	1	4	1
	DC/ExAs	2	10	12%	NA	0	252	252
9	Auto tum	2	3	10%	NA	0	36	36
	DC/ExLi	2	5	14%	NA	0	156	156
	DC/ExAs	2	5	13%	NA	61	220	159

ND=not done. NA=not assessable. Auto tum=autologous tumour cells. DC=dendritic cells. ExAs=ascitis exosomes. ExLi=tumour exosomes. *The fold increase in the absolute number of CD3+/CD8+ lymphocytes after stimulations. †Interferon γ release after coculture of lymphocytes with autologous tumour cells minus spontaneous release of interferon γ. ‡Lysis and interferon γ release are restricted to MHC class I molecules. §ExAs and ExLi were harvested from the same volume of ascitis or cell culture supernatant (patients 5, 6, 7). ¶Equivalent amounts of exosomes associated MHC class I molecules were pulsed onto DCs (patients 8, 9).

Table 3: **Functional activity of bulk lymphocytes stimulated with DC +/- exosomes or tumour cells**

two with adenocarcinoma, two with ductal adenocarcinoma, one with mesothelioma, and two with melanoma (table 1). As assessed by whole mount immunoelectronmicroscopy studies, the ultra-centrifugation pellets of the material floating at a density of 1·14–1·18 g/mL in a 30% sucrose/D_2O gradient contained many membrane vesicles (figure 2). 95% of these vesicles were labelled with a monoclonal antibody to CD81 (tetraspanin) and 60% with a monoclonal antibody to MHC class I molecules (figure 2). In the ascitis of two of five patients, exosomes had detectable concentrations of MHC class II molecules in Western blots or immunoelectronic microscopy (figure 2), suggesting that the ascitis of these two patients contained exosomes derived from specific antigen-presenting cells (eg, B cells, macrophages, dendritic cells). The membrane vesicles contained in the effusions were heterogeneous in size (50–100 nm diameter), as previously described for exosomes released in vitro. When extracted from patients with melanoma, membrane vesicles were labelled with antibodies directed against antigens from melanoma tumours such as TRP and gp100 (figure 2). Exosomes positive for TRP and gp100 were under-represented compared with those positive for MHC class I molecules and CD81 in patient 11 (figure 2). We noted little, if any, contamination with cell debris in these preparations.

Western blotting showed the typical features of exosomes derived from cells propagated ex vivo, in particular, expression of MHC class I molecules (figure 3), hsc70 (figure 3), and heat-shock protein 80 (not shown). As previously described from exosomes derived from melanoma cell lines, exosomes purified from the ascites of a patient with melanoma contained

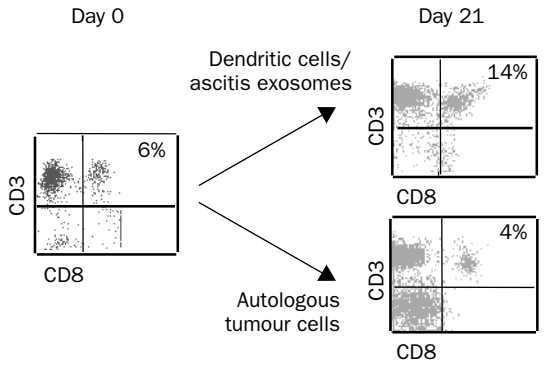

Figure 5: **Phenotype of bulk lymphocytes before (Day 0) and after in-vitro stimulations (Day 21) with either monocytic dendritic cells loaded with ascitis exosomes or irradiated tumour cells**
Measurements from patient 1. Lymphocytes were labelled with antibodies to CD3 and CD8.

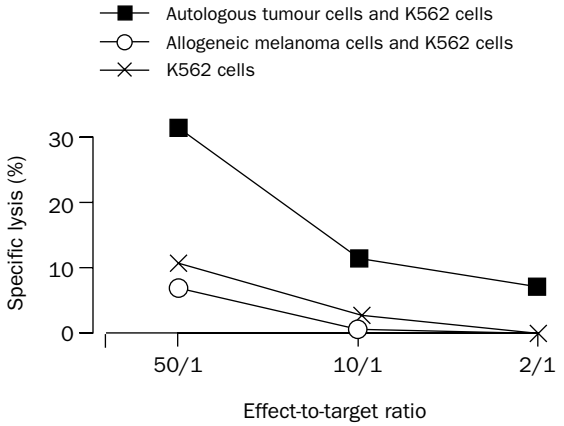

Figure 6: **Chromium release assay done on day 21 showing lysis of autologous tumours and irrelevant allogeneic tumour cells, both with K562 cells, and of K562 cells alone**
Measurements from patient 1. Cells labelled with ^{51}Cr and combined with unlabelled K562 cells.

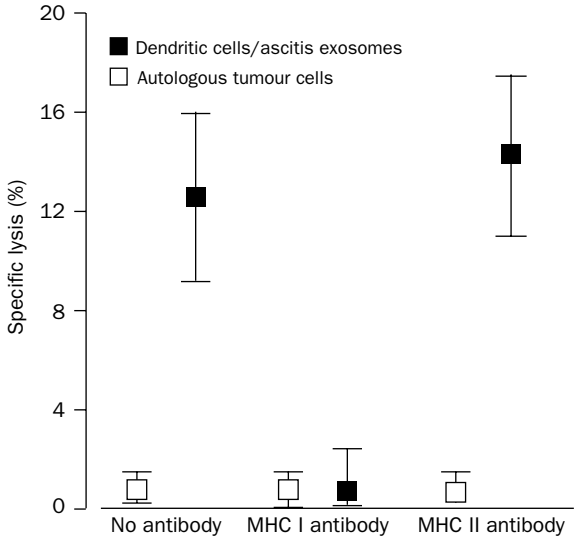

Figure 7: **Lysis of tumour cells by lymphocytes in patient 1**
Values show effect of no antibodies, and antibodies to MHC class I and II molecules on lysis. Values are mean (SD) of duplicate wells.

the whole native tumour antigen Mart1 (figure 3; patient 10). The presence of antigens to Mart1 tumours could be assigned to exosomes and not to cell debris since we did not detect any gp96 in exosome preparations from ascitis (not shown). Ascitis-exosomes purified from patients with ovarian and breast cancer also contain antigens to Her2/Neu tumours (figure 3). Quantitative analyses of ascitis exosomes showed that a mean of 7×10^{10} (SD 3×10^{10}) exosomal MHC class I molecules can be recovered from 1 mL ascitis fluid. For comparison, we recovered 8×10^{9} exosomal MHC class I molecules from 1 mL cell culture supernatant (table 2). We identified no MHC class II molecules in three of five exosomal preparations (figure 3).

We previously showed that exosomes derived from melanoma cell lines contain Mart1 native antigen that could be transferred to dendritic cells and stimulate cytotoxic T lymphocytes specific to Mart1 and restricted to HLA-A2.[9] Therefore, we tested whether ascitis exosomes from a patient with HLA-A2 negative melanoma, when loaded on MD-DCs positive for HLA-A2, allow activation of Mart1 specific, HLA-A2 restricted cytotoxic T lymphocyte clones. By contrast with exosomes derived from ovarian ascitis, those derived from melanoma ascitis contained high concentrations of Mart1 protein (patient 10, figure 3) in both patients tested. MD-DCs loaded with ascitis exosomes from patients with HLA-A2 negative, Mart1 positive melanoma (patient 10), but not from patients with ovarian cancer (HLA-A2 negative, Mart1 negative), induce production of interferon γ from the clone of cytotoxic T lymphocytes (figure 4). These data suggest that ascitis exosomes transfer antigens to MD-DCs for cross-presentation of antigens to specific cytotoxic T lymphocytes.

To test the ability of ascitis exosomes to prime peripheral CD8 positive T cells, we stimulated peripheral blood lymphocytes with MD-DCs with or without ascitis exosomes two to three times in vitro (table 3). The number of CD3/CD8 positive lymphocytes increased by two-fold to twelve-fold after stimulation of peripheral blood lymphocytes with MD-DCs pulsed with ascitis exosomes (table 3, figure 5). Irradiated autologous primary tumour cultures did not cause substantial proliferation of CD8 positive T cells (figure 5). The number of CD3/CD8 lymphocytes increased by one-fold to seven-fold after stimulation of peripheral blood lymphocytes with unloaded MD-DCs (table 3). In seven of nine patients, lymphocytes stimulated with MD-DCs loaded with ascitis exosomes (table 3) differentiated into tumour specific cytotoxic T lymphocytes. Lymphocytes stimulated with dendritic cells and ascitis exosomes, but not those stimulated with unpulsed dendritic cells or autologous tumour cells, were able to lyse autologous tumour cells, or release interferon γ, or both, after 24 h of coculture with lymphocytes and tumour cells (table 3). Recognition of autologous tumour cells was specific to the tumour and restricted to MHC class I molecules. With saturated amounts of unlabelled K562 cells, lysis of autologous tumour cells differed substantially from that of irrelevant allogeneic tumour cells. In addition, direct lysis of K562 cells was not substantial (figure 6). We recorded very little lysis of autologous tumour cells when antibodies to MHC class I molecules were added (figure 7). Secretion of interferon γ by lymphocytes incubated with autologous tumour cells could also be successfully blocked by these antibodies (figure 8). Day 0 lymphocytes before in-vitro stimulation did not show any pattern of specific tumour recognition (not shown). Furthermore, lymphocytes stimulated with purified exosomes in the absence of dendritic cells could not be expanded (data not shown).

We did a quantitative and qualitative comparison between primary tumour cultures and ascites to decide which was more suitable as a source of exosomes for vaccination. In patients 5, 6, and 7, we compared ExLis and ExAs on the bases of availability of the biological fluids. Exosomes harvested in 50 mL of ascitis or 50 mL of culture supernatants (where cells grew at 10^{6}/mL) were used to stimulate 5×10^{5} autologous lymphocytes. In two of three patients, cytotoxic T lymphocytes responses were only achieved with ExAs. However, immunocapture assays revealed a 1–2 log quantitative difference in exosome associated MHC class I molecules

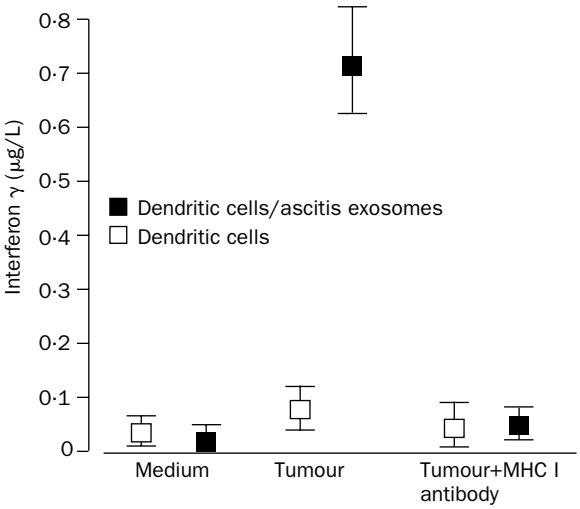

Figure 8: **Release of interferon γ by peripheral blood lymphocytes stimulated with or without ascitis exosomes at day 21**
Values are mean (SD) of duplicate wells.

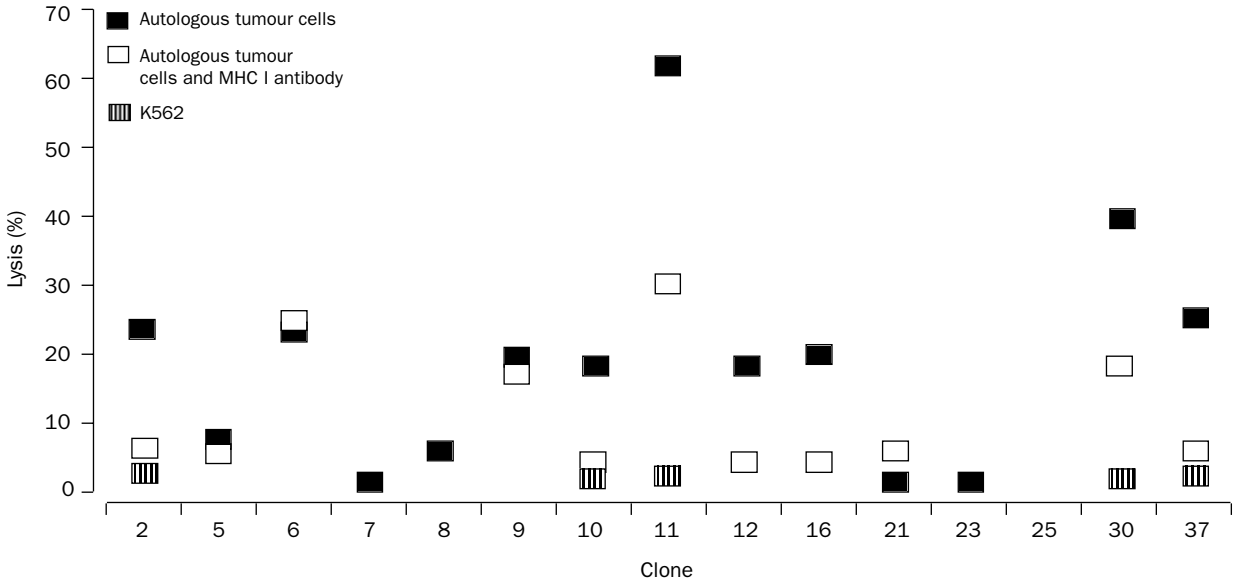

Figure 9: **Lysis of autologous tumour cells with and without antibodies to MHC class I molecules and of K562 cells in 15 clones**
Cytotoxic T lymphocytes were cloned by limiting dilution assays from bulk lymphocytes from patient 6 after three in-vitro stimulations. The effect-to-target ratio was 10 to 1.

in favour of ExAs, suggesting that many more exosomes were harvested from ascitic fluids than from primary cultures. In patients 8 and 9, the quantity of ExLi and ExAs was normalised on the basis of MHC class I molecules—ie, 5×10^{12} molecules were used to stimulate 500 000 peripheral blood lymphocytes. In both cases, ExAs promoted substantial expansion of specific cytotoxic T lymphocytes, whereas ExLi allowed activation of cytotoxic T lymphocytes in patient 9.

In patient 6, the analysis of T-cell receptor repertoire use was done on clones of lymphocytes expanded using MD-DCs pulsed with ascitis exosomes, and compared with that of tumour-infiltrating lymphocytes. Lymphocytes stimulated three times with MD-DCs and ascitis exosomes (patient 6, table 3) were cloned. In seven of 15 sucessfully expanded clones that showed substantial lysis of autologous tumour cells, the lysis was blocked by antibodies to MHC class I molecules (figure 9). These seven clones did not lyse K562 cells.

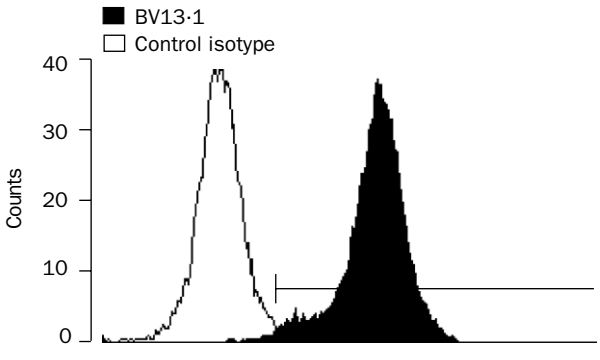

Figure 10: **BV13.1 staining in clone 2**
Analysis was done by fluorescence-activated cell-sorting scan with the series of β chain V domain antibodies (PE/FITC) of the T-cell receptor β chain V repertoire kit. Line defines zone of positive cells.

We assessed the T-cell receptor β-chain-V domain repertoire of all these clones with fluorescence-activated cell-sorting analysis. All clones had more than 90% of CD8 positive T lymphocytes stained with the monoclonal antibody to BV13.1 (eg, clone 2; figure 10). Analysis of T-cell receptor β-chain-V domain gene use was confirmed by reverse transcription PCR with a panel of primers specific for the 24 β-chain-V subfamilies. The T-cell receptor repertoire of clone 2 was restricted to BV13.1, BV16, and BV17 specificities (figure 11). Tumour-infiltrating lymphocytes had a restricted T-cell receptor β-chain-V domain repertoire (BV 5, BV16, BV17, BV19, BV22) that did not include T-cell receptor BV13.1 specificity (figure 11).

Ascitis exosomes caused expansion of a biased T-CELL REPERTOIRE from peripheral blood lymphocytes ex vivo, showing mostly a monoclonal BV13.1 pattern with antitumour effector functions that were not present originally in the breast tumour.

Discussion

We were able to isolate numerous exosomes from tumours in vivo. Exosomes were originally described in supernatants of propagated ex-vivo cultures and were shown to be antigenic in vitro and immunogenic in mice with tumours.[10–12] In our study, 11 patients had exosomes that could be purified from their ascitic fluid and that constituted a source of tumour antigens that could be transferred to MD-DCs. These MD-DCs then caused differentiation and expansion of tumour-specific cytotoxic T lymphocytes, even in people with poorly immunogenic tumours (eg, mesothelioma, lung, breast, or ovarian tumours).

Exosomes have been reported to be 60–90 nm vesicles in the membrane, originating from late endosomes or multivesicular bodies and released in the extracellular microenvironment spontaneously after fusion of these bodies with the plasma membrane.[11] We showed that at the ultrastructural level, vesicles purified from ascitis in tumours resemble exosomes derived from tumour lines ex vivo. They are cup shaped, heterogeneous in size,

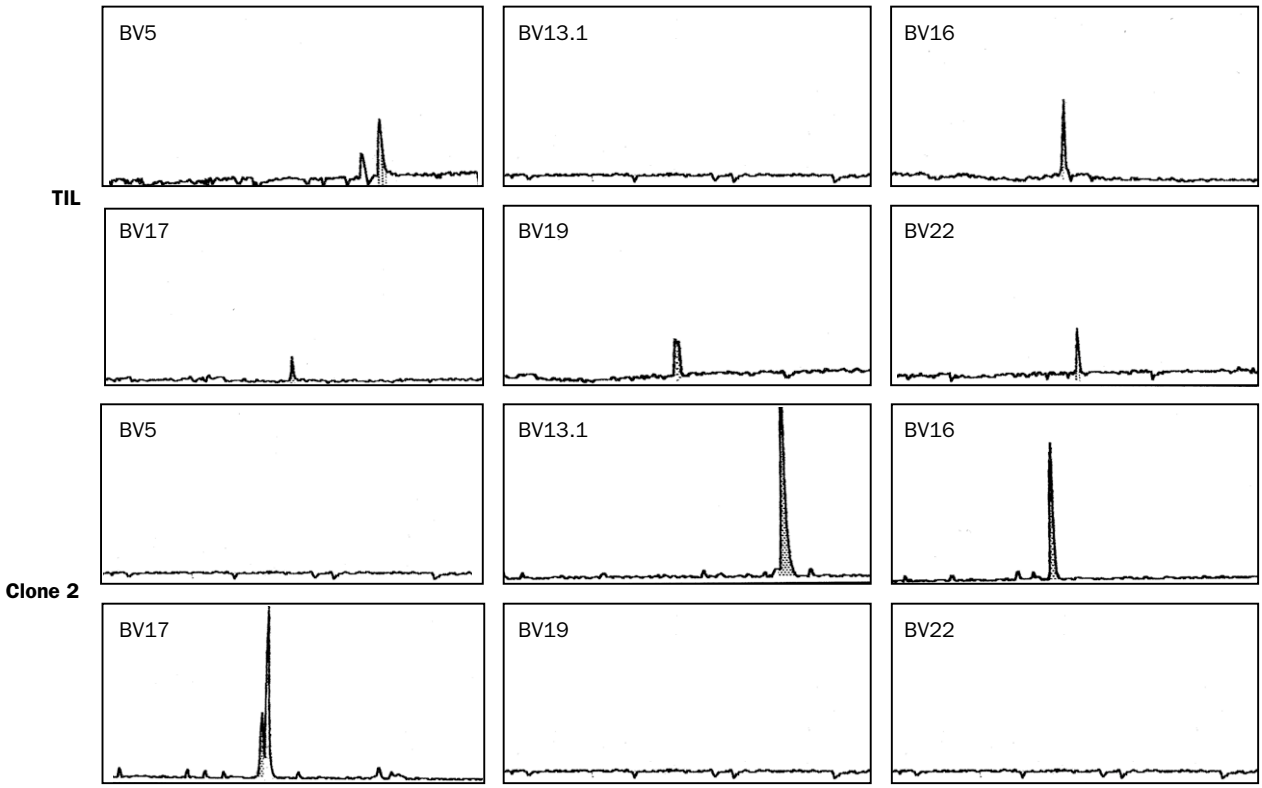

Figure 11: **Distribution patterns of T-cell receptor β chain V repertoire in tumour infiltrating lymphocytes (TIL) and in clone 2**

often aggregated, and contain tetraspanins such as CD81 and MHC class I molecules. Their biophysical properties (eg, flotation at a density of 1·14–1·18 g/mL in sucrose/D_2O gradient) are a specific attribute of these exosomes that was used for their purification. The molecular markers we identified on exosomes were those previously reported in exosomes purified from MD-DCs and tumour cell lines.[10,16] Hsc70 and heat-shock protein 80—recovered from ascitis exosomes—are constitutive chaperons overexpressed in exosomes derived from dendritic cells[16] and tumours in mice.[10] In our study, most ascitis exosomes were probably derived from peritoneal or pleural tumour cells since tumour antigens—ie, TRP, gp100 (immunoelectron microscopy), and Mart1/MelanA or Her2/Neu (Western blotting) were detected in the exosome preparations and because we identified no MHC class II molecules in three of five exosomal preparations in these patients. Two patients who might have had MHC class II positive exosomes in their ascitis also had exosomes derived from antigen presenting cells. The under-representation of exosomes positive for TRP and gp100 compared with those positive for MHC class I molecules and CD81 in patient 11 is probably due to the different affinities of the various antibodies, and the heterogeneity of tumour antigen expression in the tumour. It could also be explained by presence of exosomes derived from non-malignant cells in this patient.

The biological functions and relevancy of exosomes remain unclear. In reticulocytes, secretion of exosomes eliminates transferrin receptors and acetylcholinesterase. Exosomes derived from B lymphocytes have many MHC class II molecules and bind selectively to follicular dendritic cells that do not make their own MHC class II molecules in vivo, suggesting that B cell exosomes might have a function in humoral immune responses.[17] Exosomes derived from both tumours and dendritic cells transfer antigens to dendritic cells for efficient activation of T cells in vitro and in vivo.[10,12] Dendritic cells can respond to chemoattractants, can acquire and process antigens in the periphery, and can migrate to the lymphoid organs with many T cells to prime naive T cells.[18] However, the molecular or cellular pathways involved in transfer of tumour antigens to dendritic cells remain unknown. In-vitro results have shown that apoptotic bodies,[19] immune complexes,[20] mRNA,[21] heat-shock protein,[22] and tumour derived-exosomes[10] mediate transfer of antigens from peripheral cells to dendritic cells. Nevertheless, antigen transfer allowing MHC class I restricted-cross presentation has never been reported with materials directly purified from in-vivo samples. Here we show—in a Mart1 tumour antigen model system—that ascitis exosomes with irrelevant HLA haplotypes can induce activation of a clone of cytotoxic T lymphocytes once loaded onto dendritic cells with the appropriate HLA-A2 allele. Moreover, in an autologous model system for which tumour antigens are not characterised, dendritic cells loaded with ascitis exosomes induced priming of cytotoxic T lymphocytes in seven of nine patients. Therefore, the antigenic transfer could have been mediated by exosomes, supporting the hypothesis that exosomes are one potential pathway of antigenic transfer from peripheral tissues to the network of professional antigen presenting cells. Because ascitis exosomes contained few whole candidate tumour proteins, exosome immunogenicity probably relies on the broad antigenic peptide spectrum expressed in association with MHC class I molecules in the exosome. Such abundant sources of exosomal MHC class I molecules and peptide complexes could allow

high throughput mass spectrometry analysis of the repertoire and its relevancy for tumour recognition.

Irrespective of their relevance in vivo, ascitis exosomes could be a new method to load dendritic cells ex vivo allowing cross presentation of multiple epitopes, especially in tumour models for which few candidate rejection tumour antigens are available. Despite promising results in cancer clinical trials,[2–5] an optimum method of sensitising dendritic cells is needed for broad spectrum cancer vaccines. Up to now in ovarian cancer, few tumour antigens (Her2/Neu, MUC-1, CEA) have been identified,[23,24] accounting for the poor immunogenicity of irradiated tumour cells or tumour cell lysates used for in-vitro stimulations. Thus, immunotherapy strategies based on adoptive transfer of tumour infiltrating lymphocytes stimulated by interleukin 2, or LAK cells, or activated macrophages benefit only a few individuals.[25–28] New ideas such as fusion of dendritic cells with tumour cell suspension[6] or loading of dendritic cells with tumour lysates[29] or acid-eluated peptides[30] are awaiting clinical outcome. Nevertheless, all these strategies need tumour specimens to be processed ex vivo. Here, we report that ascitis exosomes are not only efficient at sensitisation of dendritic cells and priming of cytotoxic T lymphocytes but are also a feasible, high output process that is easy to scale up and is compatible with good laboratory practice. Ascitis exosomes prime cytotoxic T lymphocytes at least as efficiently as do those derived from tumour cell lines. The presence of exosomes derived from different cell types in the ascitis makes interpretation of this comparison in terms of exosome bioactivity difficult. Nevertheless, it suggests, in a preclinical setting, that immunotherapy based on ascitis exosomes is suitable for further studies. In addition, dendritic cells pulsed with ascitis exosomes can elicit efficient antitumour T-cell responses that do not naturally occur in patients.

Contributors

F Andre, N Schartz, E Angevin, S Amigorena, and L Zitvogel designed the study. F Andre, N Schartz, M Movassagh, C Flament, and G Rapaso gathered the data. P Pautier, P Morice, C Pomel, C Lhomme, B Escudier, T Le Chevalier, and T Tursz included patients in the study. L Zitvogel was the main investigator.

Conflict of interest statement

None declared.

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Uses of error

A fainting mechanic

Peter Sandercock

It was a typical Tuesday morning neurology clinic; over-booked and running late. A 19-year-old mechanic came in with his father. The father had read the appointment letter in which I requested that patients bring a witness to any blackouts they may have suffered. The young man told his story. On the Friday night he had been out on the town with his friends and had had quite a lot to drink, got home late, and gone straight to bed. Next morning he woke late, got dressed in a hurry, skipped breakfast, and had gone on his motorbike to the garage where he worked as a mechanic and forklift driver. As he clocked in for work, he said he felt a bit dizzy and lightheaded. He said he thought it was probably because he had missed breakfast. He passed out briefly, but thought he had just fainted, recovered quickly, got up and got on with his job. The second attack had occurred the following Saturday morning under identical circumstances. I asked the father whether he had witnessed either of these events. The father said that he hadn't, but that the garage manager who had, was supposed to be coming this morning but hadn't arrived yet.

I now use this story in the teaching session I give to medical students on "blackouts, fits, and faints". The seminars are fairly informal and I often ask one of the students what they think the diagnosis is. Most of them will say a simple vasovagal faint. My reply is, "yes, that's exactly what I diagnosed". The next question I ask is "can you spot my error?" On a good day, a few will note that I had not elicited a history from a witness. I then usually tell them that the patient had no other significant medical history and physical examination of the cardiovascular system and nervous system was normal. I ask the students whether any tests are indicated (none, if it is indeed a straightforward vasovagal syncope, precipitated by the combination of a bad hangover and lack of food). I then ask whether the patient should be allowed to ride his motorbike (yes, if the syncope was provoked, that's fine) and, of course, there is no specific treatment.

If the clinic had not been running late, I would normally have telephoned the garage to get the witness' account, but on this occasion I didn't have time. I made a quick decision, which later proved to have been dramatically wrong. I said confidently to the father and the young lad that this was just straightforward vasovagal syncope, no further investigations or treatment would be needed and he could continue riding his motorbike and driving the forklift at work.

Half an hour later, the nurse said that the garage manager had arrived and was anxious to talk to me about his young employee. I fortunately made the time to see him. He said that, on the first occasion, the young man had come up the metal staircase outside his office and was standing outside when he seemed to stare into space, make lip smacking movements, and then turn his head to the left. He then went stiff and fell backwards down the steel staircase coming to rest at the bottom of the first flight. He then jerked all over. The manager gave a clear account of a generalised tonic-clonic seizure. The young man was then quite confused for about an hour afterwards and the manager was particularly concerned that he might come to some harm operating the machinery in the workshop.

As I hadn't taken a witness' account of this episode, I had made the wrong diagnosis, given the wrong advice about investigation and treatment, and potentially put the boy's life at risk. Fortunately, I was able to reach him at home by telephone, and to tell him that the witness account clearly indicated that he had had two seizures. It sounded as though the seizures were complex partial seizures, probably arising in the temporal lobe with secondary generalisation. It was therefore important to exclude a focal brain lesion and he would therefore need computed tomography, an electroencephalogram, and treatment to prevent further attacks. He would need to stop driving, and have a period off work until his seizures were sufficiently controlled. As you might imagine, none of this news went down well either with him or with his father.

I use this case to illustrate the sound reasoning behind the standard advice to defer a diagnosis on the nature of blackouts until one has a witness' account. I hope that it emphasises for the medical students the importance of avoiding it themselves. If we do not identify and learn from our mistakes then medicine will not do well as a profession. The next time a patient with blackouts has no available witness, please don't make a definite diagnosis until at least you have lifted up the telephone to get a description of the attack. Failing to do so may be a fatal error.

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