Targeting Tumor Antigens to Secreted Membrane Vesicles *In vivo* Induces Efficient Antitumor Immune Responses

Ingrid S. Zeelenberg,[1,2] Matias Ostrowski,[1,2] Sophie Krumeich,[1,2] Angélique Bobrie,[1,2] Carolina Jancic,[1,2] Alexandre Boissonnas,[1,2] Alain Delcayre,[4] Jean-Bernard Le Pecq,[4] Béhazine Combadière,[3] Sebastian Amigorena,[1,2] and Clotilde Théry[1,2]

[1]Institut National de la Santé et de la Recherche Médicale U653 and [2]Section Recherche, Institut Curie; [3]Institut National de la Santé et de la Recherche Médicale U543, Hôpital Pitié-Salpêtrière, Paris, France; and [4]ExoThera LLC, Menlo Park, California

Abstract

Expression of non-self antigens by tumors can induce activation of T cells *in vivo*, although this activation can lead to either immunity or tolerance. CD8$^+$ T-cell activation can be direct (if the tumor expresses MHC class I molecules) or indirect (after the capture and cross-presentation of tumor antigens by dendritic cells). The modes of tumor antigen capture by dendritic cells *in vivo* remain unclear. Here we examine the immunogenicity of the same model antigen secreted by live tumors either in association with membrane vesicles (exosomes) or as a soluble protein. We have artificially addressed the antigen to secreted vesicles by coupling it to the factor VIII–like C1C2 domain of milk fat globule epidermal growth factor-factor VIII (MFG-E8)/lactadherin. We show that murine fibrosarcoma tumor cells that secrete vesicle-bound antigen grow slower than tumors that secrete soluble antigen in immunocompetent, but not in immunodeficient, host mice. This growth difference is due to the induction of a more potent antigen-specific antitumor immune response *in vivo* by the vesicle-bound than by the soluble antigen. Finally, *in vivo* secretion of the vesicle-bound antigen either by tumors or by vaccination with naked DNA protects against soluble antigen-secreting tumors. We conclude that the mode of secretion can determine the immunogenicity of tumor antigens and that manipulation of the mode of antigen secretion may be used to optimize antitumor vaccination protocols. [Cancer Res 2008;68(4):1228–35]

Introduction

Activation of tumor-specific T cells is observed in mice bearing growing tumors, although this T-cell response does not necessarily lead to tumor eradication and can even result in tolerance (1, 2). Because only professional antigen-presenting cells, especially dendritic cells, are able to prime immune responses, activation of T cells specific for tumor antigens must involve an indirect pathway of antigen presentation: antigen-presenting cells acquire antigens from tumors and present them as MHC class I-peptide and MHC class II-peptide complexes to CD8$^+$ and CD4$^+$ T cells. Studies done *in vitro* have shown that dendritic cells can capture antigens from tumors and cross-present them to CD8$^+$ T cells using various sources of antigenic material: dead (apoptotic or necrotic) tumor cells (3), heat shock protein-peptide complexes purified from tumor lysates (4), plasma membrane fragments that dendritic cells capture by "nibbling" from live tumors (5), soluble proteins (6), or vesicles secreted by live tumor cells, called exosomes (7).

When injected *in vivo*, apoptotic and necrotic tumor cells, heat shock proteins, soluble proteins, and exosomes also induce antigen-specific CD8$^+$ T-cell activation (7–10). The actual source of tumor antigens used by dendritic cells *in vivo* to capture antigens from growing tumors remains, however, controversial: in the absence of extensive tumor cell death (i.e., in the beginning of tumor development, and in the absence of therapy), proteins secreted by live tumor cells and/or subcellular secreted compartments, such as exosomes, could represent a source of tumor antigens for the activation of tumor-specific T cells.

To test this hypothesis, we have analyzed the immune response induced *in vivo* by tumors secreting an antigen either specifically coupled to membrane vesicles or freely as a soluble protein. To generate such tumors, we used the exquisite membrane-binding properties of MFG-E8/lactadherin (11). Lactadherin is a secreted protein with two functional domains: NH_2-terminal epidermal growth factor (EGF)–like domains, which contain an Arg-Gly-Asp sequence that binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, and COOH-terminal domains called C1 and C2, which are similar to the blood clotting factor VIII domains and which bind with strong affinity to lipid membranes especially when they contain phosphatidylserine (12, 13). Because of its C1C2 domain, lactadherin is secreted in association with membrane vesicles: it was originally identified in milk on mammary epithelial cell-derived fat globules (11) and, more recently, on small membrane vesicles or exosomes secreted *in vitro* by live cells such as mammary epithelial cells (14) and mouse dendritic cells (15, 16). The C1C2 domain of lactadherin can target other amino acid sequences to exosomes: when fused to interleukin 2 (IL-2) or granulocyte macrophage colony-stimulating factor (GM-CSF), the fusion protein is secreted by live cells in association with exosomes (17).

Here, we have targeted a model antigen, the chicken egg ovalbumin (OVA), to secreted vesicles (i.e., exosomes) by fusing it to the C1C2 domain of lactadherin. We show that tumor cell lines secreting OVA *in vivo* as a vesicle-associated form induce more efficient antitumor immune responses, and as a consequence grow slower than tumor cells secreting the same antigen as a soluble protein. Thus, in this murine fibrosarcoma model, vesicle-associated antigens are more immunogenic than soluble antigens. Indeed, when used in DNA vaccination protocols to transfect muscle cells *in vivo*, the OVAC1C2 fusion cDNA induced stronger delay in OVA-expressing tumor growth than the cDNA encoding

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Current address for I.S. Zeelenberg: Department of Tumor Immunology, NCMLS 278, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands.

Requests for reprints: Clotilde Théry, Institut National de la Santé et de la Recherche Médicale U653, Institut Curie, 26 rue d'Ulm, 75005 Paris, France. Phone: 33-1-42-34-67-16; E-mail: clotilde.thery@curie.fr.

doi:10.1158/0008-5472.CAN-07-3163

the soluble OVA form. Our results thus suggest new means to improve endogenous antitumor immune responses and to delay tumor growth *in vivo*.

Materials and Methods

Mice. C57Bl/6 mice were obtained from Charles River. C57Bl/6 CD45.1, C57Bl/6 Rag$^{-/-}$ $\gamma c^{-/-}$, OT-I Rag$^{-/-}$, and OT-I CD45.1 mice were bred in our animal facility (Curie Institute, Paris, France). Mice were housed in specific pathogen-free conditions. Experiments were done in accordance with the guidelines of the French Veterinary Department.

Plasmids. The pcDNA3-hygro and pcDNA6-Myc/His-blasticidine expression plasmids were from Invitrogen. C1C2 was amplified by PCR from the mouse MFG-E8/lactadherin cDNA; OVA was amplified by PCR from the chicken OVA cDNA; and the leader sequence of MFG-E8/lactadherin was synthesized as two complementary oligonucleotides. All three sequences (for OVAC1C2) or only the leader sequence and OVA (for sOVA) were first cloned in-frame into pcDNA6 and then inserted (including Myc/His tags) into pcDNA3-hygro (Fig. 1*A*).

Antibodies and reagents. Fluorescence-activated cell scanning (FACS): fluorophore-coupled antibodies to mouse H2-K^b, CD9, CD8, CD69, CD45.2 (BD PharMingen); rabbit anti-OVA (Sigma-Aldrich); and FITC-coupled anti–rabbit IgG (Jackson ImmunoResearch). Western blot: antibodies to CD9 (BD PharMingen), hsc70 and gp96 (Stressgen Biotech), tsg101 (Santa Cruz Biotech), MFG-E8/lactadherin (18), and horseradish peroxidase (HRP)–conjugated secondary antibodies (Jackson Immunoresearch). ELISA: anti-myc 9E10 monoclonal antibody and purified rabbit polyclonal anti-OVA (Sigma-Aldrich). Immunoprecipitation: goat-anti-OVA (MP Biomedicals) and protein G-sepharose (Amersham).

Cells. The MCA101 C57Bl/6 fibrosarcoma was cultured in DMEM supplemented with 10% FCS (Abcys) and penicillin/streptomycin (Invitrogen). Stable cell lines expressing sOVA or OVAC1C2 were obtained by electroporation with the corresponding plasmids, selection in the presence of 1 mg/mL hygromycin (Roche), and cloning by limiting dilution. CD8$^+$ OT-I T cells (specific for the OVA$_{257–264}$ peptide in the H2-K^b context) were obtained by mechanical dissociation of lymph nodes from OT-I Rag$^{-/-}$ mice, or from OT-I Rag$^{+/+}$ mice, followed by negative selection with a CD8$^+$ depletion kit (Miltenyi Biotec). The B3Z hybridoma, expressing the same T-cell receptor as OT-I T cells, as well as IL-2–regulated *β-galactosidase* gene (19), was cultured in RPMI supplemented with 10% FCS, β-mercaptoethanol (Invitrogen), and penicillin-streptomycin.

Exosome purification and characterization. Exosome production, purification, and characterization were done as previously described (20). Briefly, MCA101 cells were cultured for 48 h in medium depleted from serum-derived exosomes by overnight centrifugation at 100,000 × *g*. Exosomes were purified by successive centrifugations, and the concentration of exosomal proteins was quantified by Bradford assay (Bio-Rad). Exosomes and total cell lysates [cells lysed in 50 mmol/L Tris (pH 7.5), 0.3 mol/L NaCl, 0.5% Triton X-100, 0.1% sodium azide] were loaded on SDS-PAGE for Western blot analysis or coated on 4-μm aldehyde-sulfate latex beads (Interfacial Dynamics) for FACS analysis.

ELISA. For detection of secreted OVA by ELISA, anti-myc 9E10 (12 μg/mL) was coated on MaxiSorp 96-well plates. Washes were done in PBS-0.05% Tween 20, blocking in PBS-5% milk, and after incubation with the samples, bound OVA was revealed by polyclonal rabbit anti-OVA serum (3.5 μg/mL), followed by HRP-conjugated anti–rabbit antibody (0.16 μg/mL) and TMB substrate reagent (BD OptEIA). Reaction was stopped with 6N HCl, and absorbance was read at 450 nm.

Immunoprecipitation. Samples were obtained from 3 × 150 mm dishes of cells cultured for 4 days in depleted medium. Cells were harvested and lysed and exosomes were purified from the supernatants. Complete EDTA-free protease inhibitors (Roche) were added to cells, exosomes, and exosome-depleted supernatants. Half of the supernatant (after exosome purification), half of the cells, and all the exosomes (resuspended in lysis buffer) were subjected to immunoprecipitation. Sepharose-4G beads bearing covalently linked goat anti-OVA antiserum (4 μg/sample) were incubated with samples overnight at 4°C. After extensive washes in lysis buffer, beads were resuspended in Laemmli sample buffer and analyzed by Western blotting with the rabbit anti-OVA antiserum.

Immuno-electron microscopy. Whole mounts of exosomes were immuno-gold labeled as previously described (20) with goat anti-OVA IgG (10 μg/mL), followed by rabbit anti-goat antiserum (1/200) and 10-nm protein A-gold particles. Samples were observed and photographed under a Philips CM120 Electron Microscope (FEI Company). Images were acquired using the ITEM program.

***In vitro* T-cell stimulation assay.** MCA cell lines were treated overnight with 10 ng/mL IFNγ (BD Biosciences), washed extensively, harvested, and seeded at increasing doses (10^2–3×10^5 per well) in flat-bottomed 96-well plates with 10^5 B3Z hybridoma cells. After 18-h incubation at 37°C, T-cell activation was quantified, as previously described (19), as β-galactosidase activity (i.e., $A_{595\ nm}$ after 2-h incubation with 20 μg/mL chlorophenol red β-galactopyranoside and 0.5% NP40).

***In vivo* T-cell stimulation assay.** OT-I T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen Molecular Probes; 5 μmol/L in PBS-0.5% bovine serum albumin for 10 min at 37°C) before i.v. injection in CD45.1 C57Bl/6 mice bearing 6-day-old tumors. Cells from the ipsilateral inguinal lymph nodes were harvested 1 or 6 days later and analyzed by FACS on a FACSCalibur (BD PharMingen) after staining with antibodies to CD45.2, CD8, and CD69.

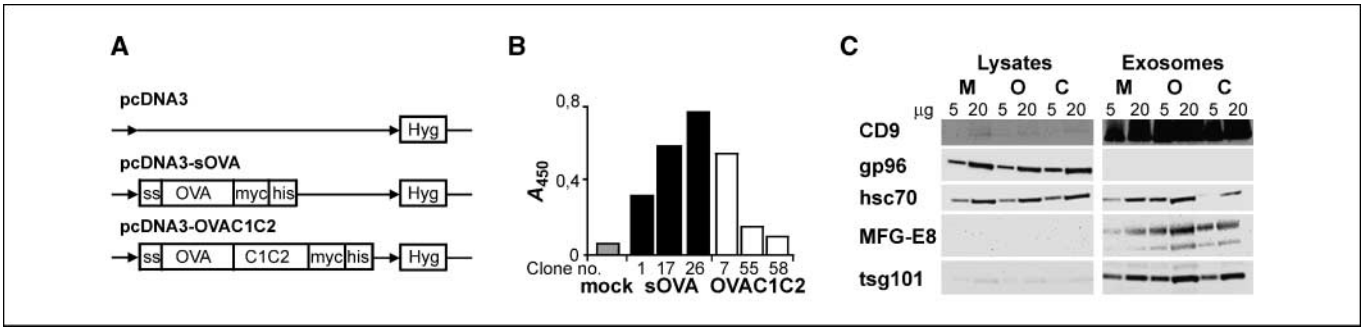

Figure 1. Generation of tumor cell lines secreting either free or vesicle-bound OVA. *A,* schematic diagrams of the constructs made in pcDNA3, encoding secreted soluble OVA (sOVA), secreted vesicle-bound OVA (OVAC1C2), or no OVA (pcDNA3). *SS,* signal sequence from the MFG-E8/lactadherin cDNA; *OVA,* chicken egg ovalbumin cDNA; *C1C2,* factor VIII–like domains of the MFG-E8 cDNA; *myc,* myc tag; *his,* 6-histidine tag; *Hyg,* hygromycin resistance gene. *B,* MCA101 cells transfected with pcDNA3 (*mock*) or three different subclones of MCA101 cells transfected with pcDNA3-sOVA (sOVA: *1, 17, 26*) or pcDNA3-OVAC1C2 (OVAC1C2: *7, 55, 58*) were grown to 90% confluency in 96-well plates, and the amount of OVA present in each well (cells together with supernatant) after addition of detergent was quantified by ELISA. Subclones sOVA 17 and OVAC1C2 7 were used for all subsequent experiments. *C,* characterization by Western blot of total cell lysates and exosomes secreted by the mock (*M*), sOVA (*O*), and OVAC1C2 (*C*) cells, using antibodies to characteristic exosomal proteins (CD9, hsc70, MFG-E8, and tsg101) and to a non-exosomal protein (gp96).

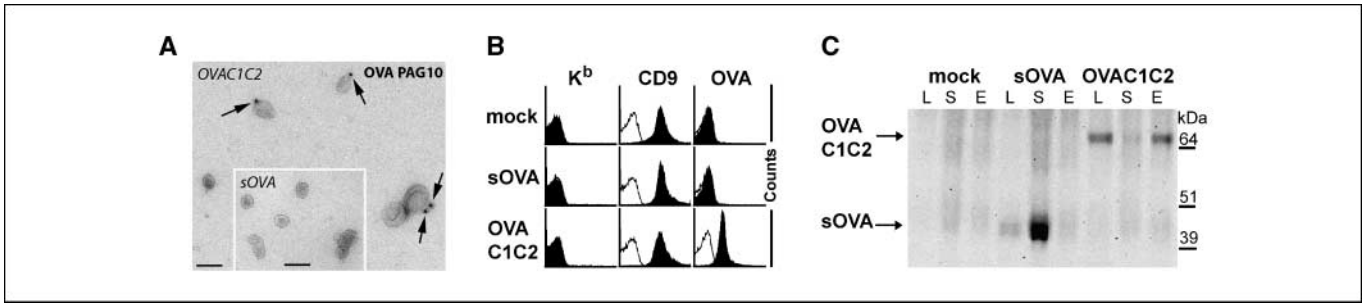

Figure 2. Characterization of OVA secretion by the tumor cell lines. *A,* immuno-electron microscopy analysis of purified exosomes secreted by OVAC1C2 and sOVA (*inset*) cells, after staining with goat anti-OVA and 10-nm protein A coupled to gold particles. *Arrows,* specific anti-OVA staining. *Bar,* 100 nm. *B,* FACS analysis of beads coated with exosomes secreted by mock, sOVA, and OVAC1C2 MCA cells using antibodies to MHC class I (K^b), CD9, or OVA. Only exosomes secreted by MCA/OVAC1C2 cells bear OVA. *C,* immunoprecipitation of OVA from cell lysates (*L*), cell supernatant (after exosome depletion; *S*), or purified exosomes (*E*) from mock, sOVA, and OVAC1C2 MCA cells, followed by Western blotting with anti-OVA antiserum. sOVA (50 kDa) is present in the supernatant and not in exosomes, whereas OVAC1C2 (66 kDa) is present in exosomes and not in the supernatant.

***In vivo* tumor growth assays.** Tumor cells (2×10^5) obtained from subconfluent cultures were injected s.c. in the shaved flank and tumor size was measured every 3rd day with a caliper. Tumor volume was calculated as length × width × [(length + width) / 2]. Mice were killed when tumor volume reached 1,500 mm^3.

DNA vaccination. pcDNA3, pcDNA3-sOVA, and pcDNA3-OVAC1C2 plasmids were purified from 500-mL bacteria cultures using Nucleobond endotoxin-free plasmid DNA purification kit (Macherey-Nagel). Fifty micrograms of DNA diluted in sterile H_2O were injected in both calves of each mouse, twice at 1-month interval.

Results

Generation of tumor cell lines secreting either free or vesicle-bound OVA. To generate tumor cell lines secreting the OVA antigen either as a soluble or as a membrane-associated protein, we cloned the OVA cDNA downstream a signal sequence either directly (soluble secreted OVA, sOVA) or as a fusion with the C1C2 domains of lactadherin (vesicle-bound form, OVAC1C2) in a modified pcDNA3 plasmid (Fig. 1*A*). Either plasmid or empty pcDNA3 was transfected into MCA101 cells, a poorly immunogenic methylcholanthrene-induced fibrosarcoma cell line (21). Several subclones secreting various amounts of OVA were selected (Fig. 1*B*). The MCA/sOVA 17 and MCA/OVAC1C2 7 subclones, which secrete similar amounts of total OVA as shown by ELISA (Fig. 1*B*), were used for most experiments and compared with the MCA/mock bulk population expressing the empty pcDNA3 plasmid. Figure 1*C* shows that the exosomes from the three cell

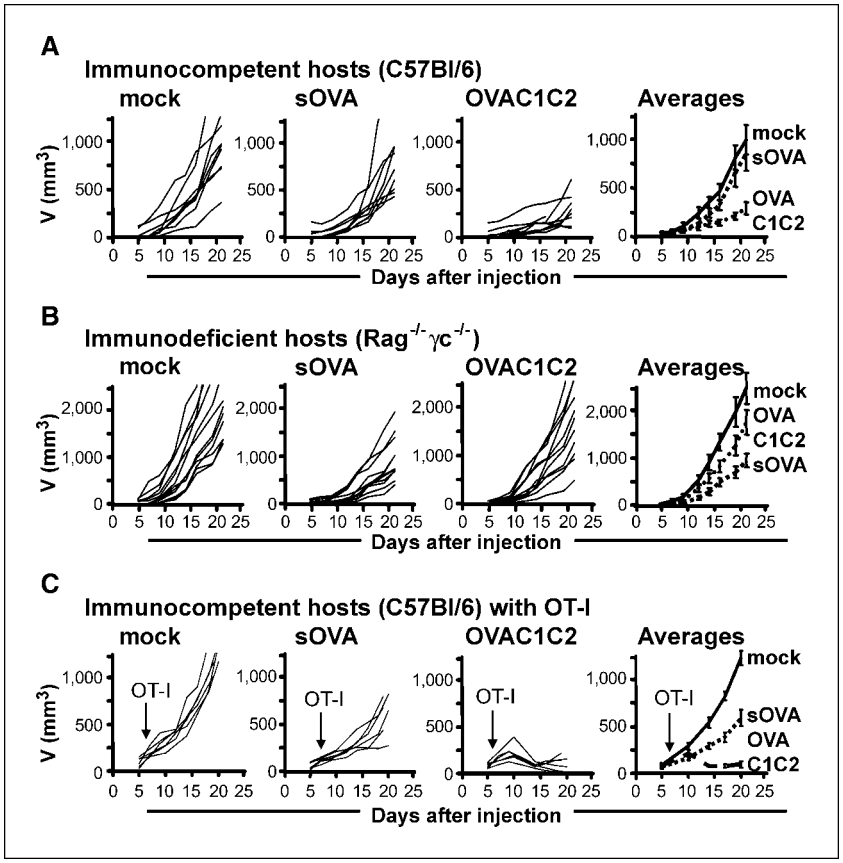

Figure 3. Tumors secreting vesicle-bound OVA grow slowly in immunocompetent hosts. *A* to *C,* growth of mock, sOVA, or OVAC1C2 tumors after s.c. injection in C57Bl/6 wild-type (*A*) or $Rag^{-/-}$ $\gamma c^{-/-}$ (*B*) hosts, or in wild-type hosts after adoptive transfer of OT-I T cells (*arrows*; *C*). *First three columns,* tumor growth in individual mice. *Last column,* mean growth of 9 (*A*), 11 (*B*), or 6 (*C*) mice pooled from two independent experiments; *bars,* SEM. *A,* at day 21, OVAC1C2 tumors were significantly (Student's *t* test) smaller than both mock ($P = 0.0008$) and sOVA ($P = 0.009$) tumors. *B,* at day 21, OVAC1C2 tumors were not significantly smaller than mock tumors ($P = 0.09$, Student's *t* test). *C,* only the MCA/OVAC1C2 tumors are rejected by OT-I T cells. At day 20, OVAC1C2 tumors are significantly smaller than mock ($P < 0.0001$) and sOVA ($P = 0.001$) tumors.

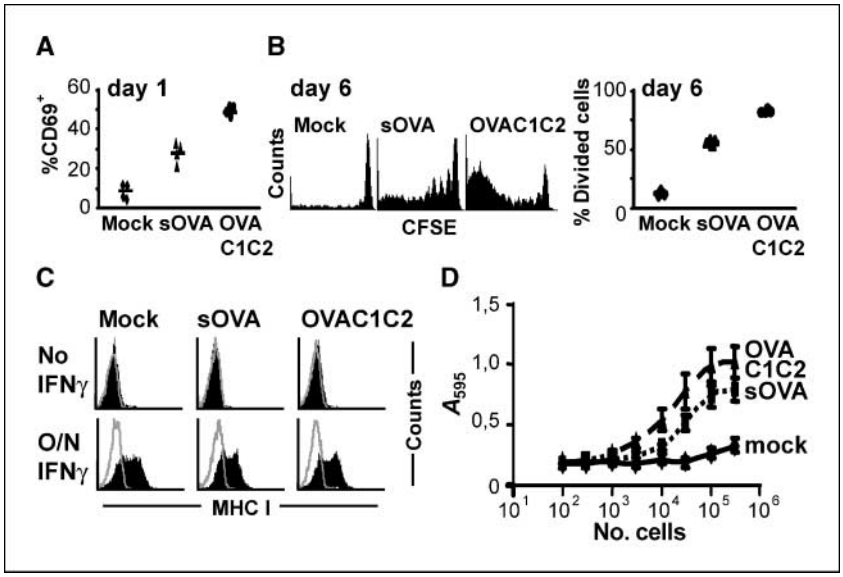

Figure 4. Tumors secreting vesicle-bound OVA induce efficient activation of OVA-specific $CD8^+$ T cells *in vivo*. *A* and *B,* anti-OVA OT-I T cells were injected i.v. in hosts bearing 6-d-old mock, sOVA, or OVAC1C2 tumors. *A,* activation of OT-I T cells 1 d after transfer. Percent of $CD69^+$ cells among the $CD8^+$ $CD45.2^+$ cells (OT-I T cells) from tumor-draining lymph nodes, obtained in four mice pooled from two independent experiments. The percent of activated OT-I T cells is significantly higher in OVAC1C2-bearing than in sOVA-bearing mice ($P = 0.002$, Student's *t* test). *B,* proliferation of CFSE-labeled OT-I T cells 6 d after transfer. *Left,* representative histograms of CFSE staining on OT-I ($CD8^+$ $CD45.2^+$) T cells from tumor-draining lymph nodes. *Right,* percent divided cells obtained in four mice pooled from two independent experiments. The percent of divided OT-I T cells is significantly higher in OVAC1C2-bearing than in sOVA-bearing mice ($P < 0.0001$, Student's *t* test). *C* and *D, in vitro* presentation of MHC class I and K^b-OVA-peptide complexes by mock, sOVA, and OVAC1C2 cells. *C,* FACS analysis of surface expression of MHC class I (*black histogram*) on cultured mock, sOVA, and OVAC1C2 MCA101 cells in control conditions (*No IFN*γ) or after overnight incubation with IFNγ (*O/N IFN*γ). *White histograms,* isotype control. *D,* activation of B3Z hybridoma, evidenced by β-galactosidase activity ($A_{595\ nm}$), in the presence of increasing numbers of IFNγ-treated mock, sOVA, and OVAC1C2 tumors. *Points,* mean of five experiments; *bars,* SEM. No significant difference between sOVA and OVAC1C2 at any cell concentration ($P > 0,05$, Student's *t* test).

lines are similar, as defined by enrichment of the tetraspanin CD9, the heat shock protein hsc70, and the ESCRT protein tsg101, and absence of the endoplasmic reticulum protein gp96. These exosomes also bear the endogenous MFG-E8/lactadherin, detected by an antibody to the EGF-like domain. Observation by electron microscopy of purified exosomes showed the typical cup-shaped morphology and size (60–110 nm diameter) for exosomes from both MCA/sOVA and MCA/OVAC1C2 (Fig. 2*A*). Staining with anti-OVA antiserum was observed on MCA/OVAC1C2 exosomes (Fig. 2*A, arrows*), but not on MCA/sOVA (Fig. 2*A*) or MCA/mock (data not shown) exosomes. Purified exosomes were analyzed by FACS after coating on beads (Fig. 2*B*): equivalent amounts of exosomes were adsorbed to the beads, as shown by the strong CD9 staining, but only exosomes purified from MCA/OVAC1C2 bear detectable levels of OVA (Fig. 2*B*). Finally, OVA immunoprecipitation followed by Western blotting was done on cell lysates, exosome-depleted supernatants, and exosomes (Fig. 2*C*). sOVA was strongly detected in the supernatant but was absent from exosomes secreted by MCA/sOVA cells, whereas OVAC1C2 was present on exosomes and almost undetectable in the supernatant of MCA/OVAC1C2 cells. As expected, no OVA was detected in any of the MCA/mock samples. These results show that the OVAC1C2 fusion protein behaves like previously described IL2-C1C2 and GMCSF-C1C2 fusion proteins in terms of specific enrichment on exosomes secreted by live cells (17). We have thus generated tumor cell lines expressing OVA either as a protein freely secreted in the extracellular medium (MCA/sOVA) or as a protein secreted in association with vesicles, such as exosomes (MCA/OVAC1C2).

Tumors secreting vesicle-bound OVA grow slowly in immunocompetent hosts. Growth of the three tumors *in vivo*, after s.c. injection in C57Bl/6 hosts, was then analyzed. As shown in Fig. 3*A*, the MCA/OVAC1C2 tumor grew slower than both the MCA/mock and the MCA/sOVA tumor in immunocompetent hosts. This result was also obtained for the two other subclones of MCA/sOVA and of MCA/OVAC1C2 (Supplementary Fig. S1*A*). The less efficient growth of MCA/OVAC1C2 was not due to an intrinsic slower rate of proliferation of this particular clone because all cell lines grew at the same rate *in vitro* (data not shown). It was also not due to an inherent inability to grow after s.c. injection *in vivo* because MCA/OVAC1C2 tumors grow even faster than MCA/sOVA tumors when injected in $Rag^{-/-}$ $\gamma c^{-/-}$ C57Bl/6 mice, devoid of B and T lymphocytes and of natural killer (NK) cells, and only slightly less than MCA/mock tumors (Fig. 3*B*). This observation suggests that tumors secreting the OVA antigen on exosomes induce an efficient adaptive antitumor immune response *in vivo*, which is not the case for tumors secreting the soluble OVA antigen.

Tumors secreting vesicle-bound OVA elicit a stronger anti-OVA immune response. To test this hypothesis, we adoptively transferred naïve $CD8^+$ anti-OVA T cells obtained from OT-I mice into tumor-bearing hosts, and analyzed their activation and proliferation 1 or 6 days later in the tumor-draining lymph nodes. At the time of OT-I T-cell transfer (i.e., day 6 after tumor injection), the three types of tumors were of similar sizes (Fig. 3*C, arrows*; no statistically significant differences, $P > 0.3$). As shown in Fig. 4*A*, a larger proportion of OT-I T cells were activated in MCA/OVAC1C2-bearing than in MCA/sOVA-bearing mice, as soon as 24 h after transfer (up-regulation of the early activation marker CD69). Five days later (Fig. 4*B*), proliferation of OT-I T cells was also more extensive in MCA/OVAC1C2-bearing than in MCA/sOVA-bearing mice. Eventually, in the mice in which extensive activation and

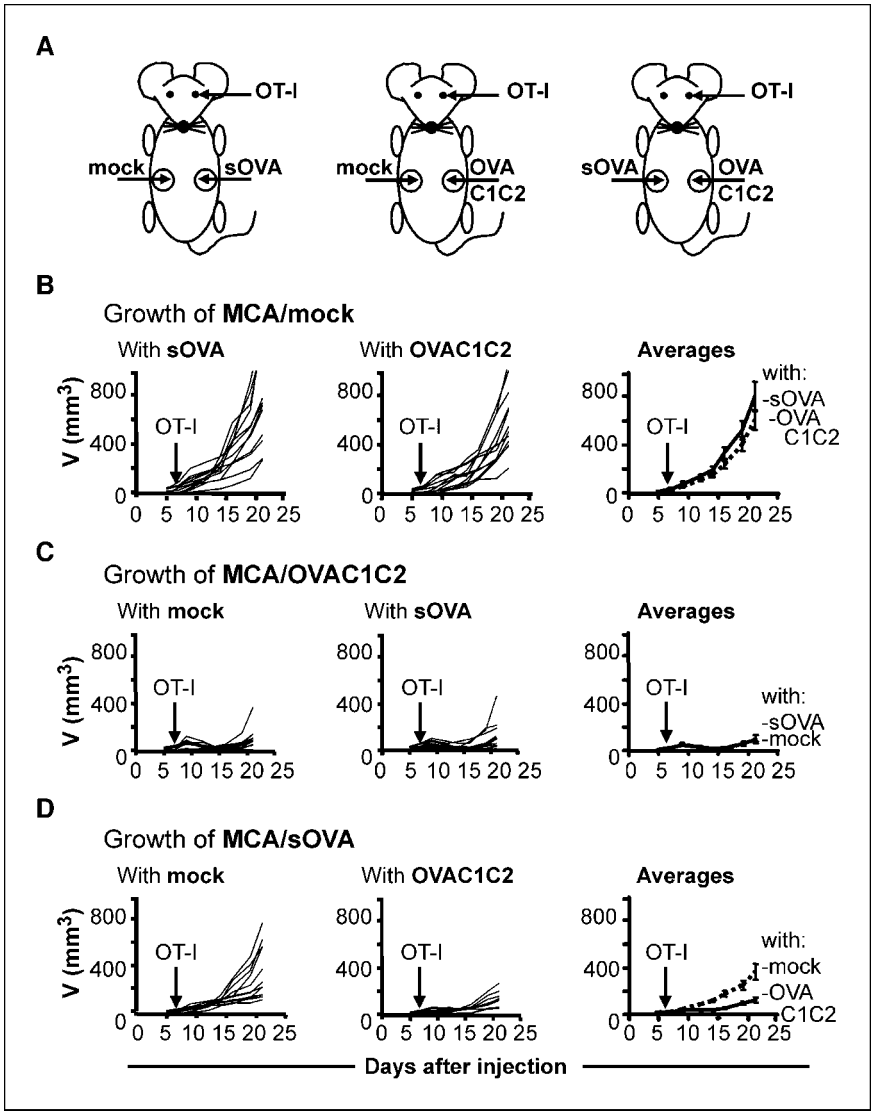

Figure 5. Tumors secreting vesicle-bound OVA induce rejection of OVA-secreting tumors *in vivo*. *A,* schematic diagrams of the experimental setting. OT-I T cells were injected in mice bearing two 6-d-old tumors, as indicated. *B* to *D, left* and *middle,* growth of MCA tumors in individual mice. *Right, points,* averages; *bars,* SEM. Twelve individual mice in two independent experiments are represented. *B,* growth of MCA/mock tumors in mice bearing either MCA/sOVA (*left*) or MCA/OVAC1C2 (*middle*) tumors. *C,* growth of MCA/OVAC1C2 tumors in mice bearing either MCA/mock (*left*) or MCA/sOVA (*middle*) tumors. *D,* growth of MCA/sOVA tumors in mice bearing either MCA/mock (*left*) or MCA/OVAC1C2 tumors (*middle*). At day 20, MCA/sOVA tumors growing next to mock tumors were significantly bigger than sOVA tumors growing next to OVAC1C2 tumors ($P = 0.004$, Student's *t* test).

proliferation of OT-I T cells had taken place (MCA/OVAC1C2-bearing hosts), tumors started regressing 3 days after OT-I T-cell injection and became barely detectable another 6 days later (Fig. 3*C*). In contrast, the MCA/sOVA tumors were not rejected by the OT-I T cells, which barely affected their growth. We confirmed that the other MCA/sOVA subclones were also not rejected after OT-I T-cell transfer, even if they secreted more OVA than the OVAC1C2 subclones, whereas all the MCA/OVAC1C2 subclones, even if they expressed low levels of OVA, were rejected (Supplementary Fig. S1*B*).

We verified that the OVAC1C2 fusion protein was not more immunogenic per se independently of its association to vesicles: sOVA and OVAC1C2 were purified from the culture supernatants and covalently coupled to beads, making them identically suitable for phagocytosis and processing by antigen-presenting cells (Supplementary Fig. S2*A*). Beads bearing sOVA or OVAC1C2 were identically efficient to induce OT-I T-cell activation *in vitro* in the presence of dendritic cells (Supplementary Fig. S2*B*) and *in vivo* after s.c. injection (Supplementary Fig. S2*C*). Thus, the sOVA and the OVAC1C2 proteins per se are identically immunogenic.

Increased activation of OVA-specific $CD8^+$ T cells in mice bearing MCA/OVAC1C2 tumors could be due to higher number of MHC-OVA-peptide complexes at their surface as compared with the MCA/sOVA tumors. We tested this hypothesis by measuring activation *in vitro* by our MCA-derived cell lines of B3Z, a costimulation-insensitive K^b-OVA-specific T-cell hybridoma. As previously described for the parental MCA101 cell line (21), our MCA101-derived cells express undetectable levels of MHC class I *in vitro*, but treatment with IFNγ induces surface expression of MHC class I (Fig. 4*C*). In these conditions, B3Z activation by MCA/sOVA was as efficient as by MCA/OVAC1C2 (Fig. 4*D*). Thus, the two cell lines present similar levels of K^b-OVA-peptide complexes at their surface.

MCA/sOVA tumors are killed by the MCA/OVAC1C2-induced immune response. Our hypothesis is that enhanced T-cell activation by vesicle-bound secreted OVA allows generation of CTLs able to kill the OVA-expressing tumors, whereas soluble OVA secreted freely *in vivo* induces only limited T-cell activation, insufficient to generate a fully functional antitumor immune response. Another possibility, however, is that both tumors induce CTLs, but MCA/sOVA are more resistant than MCA/OVAC1C2 tumor cells to killing. Because both tumors grew too fast to evaluate their killing by CTLs *in vitro*, we designed an *in vivo* double-tumor growth experiment to address this question.

Mice were injected respectively in the left and right flanks with MCA/mock and MCA/sOVA, or with MCA/mock and MCA/OVAC1C2, or with MCA/sOVA and MCA/OVAC1C2 tumor cells (Fig. 5*A*). Six days later, naïve OT-I T cells were transferred, and growth of both tumors was monitored for the next 10 days. As shown in Fig. 5*B*, the MCA/mock tumors grew at the same rate, whether MCA/sOVA or MCA/OVAC1C2 tumors grew on the other side. Similarly, MCA/OVAC1C2 tumors were rejected in the same manner, irrespective of the tumors growing on the other flank of the mice (Fig. 5*C*). In contrast (Fig. 5*D*), whereas MCA/sOVA tumors were not rejected in MCA/mock-bearing hosts, as seen before when injected alone (see Fig. 3*C*), they underwent almost complete rejection when growing in MCA/OVAC1C2-bearing hosts. This observation shows that OT-I $CD8^+$ T cells activated by MCA/OVAC1C2 were able to kill both the original tumor and the distant MCA/sOVA tumor. MCA/sOVA is thus not resistant to killing by CTLs but fails to prime an efficient immune response, as opposed to the MCA/OVAC1C2 tumor.

Our results thus indicate that vesicle-associated antigens are more immunogenic than soluble antigens when secreted by tumor cells *in vivo*.

Vaccination with OVAC1C2 cDNA delays growth of OVA-expressing tumors. We next asked whether *in vivo* expression of the vesicle-bound antigen by endogenous cells, in the absence of any *in vitro* culture step, would also induce antigen-specific immune responses. To answer this question, we used vaccination with naked DNA to induce expression of the DNA-encoded antigen by cells at the site of vaccination. We injected sOVA or OVAC1C2 DNA (purified in endotoxin-free conditions) i.m. in mice, twice at 1-month interval (Fig. 6*A*). MCA/sOVA cells were injected s.c. 2 weeks after the last vaccination, when endogenous OVA-specific CTLs became detectable in sOVA- and OVAC1C2-vaccinated mice (Supplementary Table S1). As shown in Fig. 6*B*, DNA vaccination with both sOVA and OVAC1C2 increased the survival time (mice were killed when the tumors reached 1,500 mm^3) as compared with vaccination with empty pcDNA3, but OVAC1C2 induced a significantly better protection. Although in both sOVA and OVAC1C2 groups 50% of the mice did not develop any tumor, the growth of the remaining tumors was strikingly delayed in the OVAC1C2-vaccinated as compared with the sOVA-vaccinated group. At the termination of the experiments (day 88), tumors that developed in the sOVA-vaccinated mice were all larger than 1,500 mm^3, whereas the tumors in the OVAC1C2-vaccinated mice were still smaller. Thus, although tumor growth is not completely prevented, vaccination with OVAC1C2 expression plasmid significantly protects mice from tumor progression.

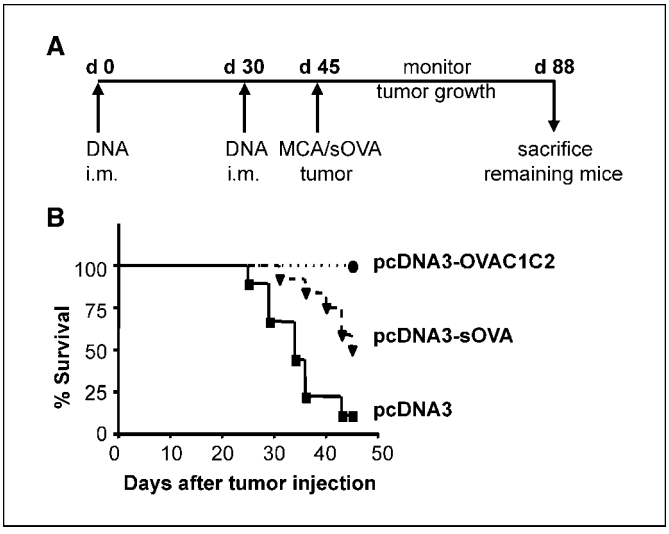

Figure 6. *In vivo* expression of vesicle-bound OVA after DNA vaccination protects against growth of OVA-secreting tumors. *A,* schematic representation of the experimental setting. DNA was injected i.m. at days 0 and 30, followed by s.c. injection of the MCA/sOVA tumor. Tumor growth was monitored twice per week after tumor injection. Mice were killed when the size of their tumor reached 1,500 mm^3, and the remaining mice were sacrificed at the end of the experiment. *B,* survival curve after vaccination with empty pcDNA3, pcDNA3-sOVA, or pcDNA3-OVAC1C2 plasmids (9–12 mice per group, from two independent experiments). Increased survival in vaccinated mice was due to prevention of tumor growth in some mice (tumor-free mice: 0 of 9 in pcDNA3-vaccinated, 6 of 12 in sOVA-vaccinated, and 6 of 12 in OVAC1C2-vaccinated mice), and delay in tumor growth in the remaining ones. Log-rank test shows statistically significant protection by sOVA vaccination, as compared with pcDNA3 ($P = 0.006$), but even enhanced protection by OVAC1C2 as compared with pcDNA3 ($P < 0.0001$) and sOVA ($P = 0.006$).

Discussion

We show here that *in vivo* secretion by live cells of an antigen fused to the C1C2 domain of MFG-E8/lactadherin promotes antigen-specific immune responses, resulting in delay of the growth of antigen-bearing tumors. The C1C2-fused antigen could be secreted *in vivo* either by genetically engineered tumor cells (Figs. 3–5) or by *in vivo* transfected cells (Fig. 6). In both cases, antigen-specific immune responses induced by the modified antigen delayed the growth of a tumor expressing a soluble form of the antigen. In contrast, *in vivo* expression of the soluble antigen induced T-cell activation, but this immune response did not result in tumor growth delay.

The C1C2 domain of lactadherin, which is similar to blood coagulation factor VIII, mediates lactadherin binding to phosphatidylserine-exposing membrane vesicles (12). Phosphatidylserine is also exposed at the surface of cells undergoing apoptosis, and purified recombinant lactadherin binds to apoptotic cells (22, 23). However, lactadherin secreted by live cells cultured *in vitro* is concentrated on the exosomes or membrane microvesicles they secrete (14, 15). In contrast, apoptotic vesicles purified from the supernatant of lactadherin-secreting cells induced to apoptose do not bear detectable lactadherin (24),[5] most probably because lactadherin production is shut off when cells start dying. Like whole lactadherin, and as previously shown (17), we confirm here that the C1C2 domain of lactadherin, when fused to another amino acid sequence than the EGF-like domains of lactadherin, is also recovered on secreted exosomes, and not as a soluble protein, in the supernatant of cells growing *in vitro* (Fig. 2). Therefore, *in vivo*, the OVAC1C2-expressing cells secrete OVA as a vesicle-bound form, which, in this tumor model, allows more efficient induction of antitumor immune responses than the native OVA secreted as a soluble form. We do not want to conclude from our results, however, that secreted vesicles generally play a role in T-cell priming *in vivo*. Indeed, it is likely that secreted vesicles, depending on the tumor (which may or may not secrete exosomes containing immunosuppressive molecules) and on the immunologic state of the host, could induce priming or tolerance. Depending on the type of tumor antigen secreted with vesicles, the outcome of the immune response may also be different, and the model antigen

[5] Our unpublished observation.

OVA used here is relevant to non-self tumor antigens arising from mutations of endogenous proteins but not to other tumor antigens, such as differentiation or testis tumor antigens.

Increased efficiency of T-cell activation after *in vivo* injection of cell-associated as compared with soluble OVA (10), or by transfected fibroblasts expressing plasma membrane-bound as compared with secreted OVA (25), has been reported before. In our work, however, not just any membrane-bound form of OVA induced efficient antitumor immune responses: the growth of tumor cells expressing OVA coupled to the Fc receptor (FcROVA), a transmembrane protein present at the cell surface but absent from exosomes (24), was only marginally slower in immunocompetent, as compared with immunodeficient mice (Supplementary Fig. S3). FcROVA-expressing tumors hence behaved like sOVA-expressing tumors, and differently from tumors expressing OVAC1C2, the growth of which was strongly impaired in immunocompetent mice (Fig. 3; Supplementary Fig. S3). Thus, for an efficient antigen-specific immune reaction, the antigen expressed by a tumor must be bound to membrane vesicles secreted by the cells, rather than simply bound to the surface of the cells.

Recent approaches have also shown that virus-like particles (i.e., membrane vesicles released by cells manipulated to express virus gag and/or env proteins; refs. 26, 27), if they bear antigens, also favor induction of T-cell activation as compared with the non–particle-bound antigen. Our work thus extends these observations to tumor immunology and gives them a physiologic meaning by showing that membrane vesicles spontaneously secreted by cells *in vivo* induce efficient immune responses.

Our hypothesis is that dendritic cells capture the antigen more efficiently *in vivo* when it is bound to exosomes or membrane vesicles, leading to activation of antigen-specific $CD8^+$ T cells. To favor such a cross-presentation mechanism, we choose to use the MCA101 cell line, which expresses low levels of MHC class I *in vivo* (ref. 21, and our observations). Surface-displayed MHC I-peptide complexes are sufficient for the MCA101 cell line to be killed by previously activated $CD8^+$ CTLs, but not to induce CTL generation from naïve $CD8^+$ T cells per se (28). Because MHC I-OVA-peptide complexes are equally displayed at the surface of MCA/sOVA and MCA/OVAC1C2 (Fig. 4*D*), both cells can be equally efficiently destroyed by OVA-specific CTLs (Fig. 5), and the difference in immune responses depends on the difference in cross-presentation efficiency for priming of $CD8^+$ T cells. Accordingly, we show here that antigen-specific $CD8^+$ T cells proliferate more in mice bearing OVAC1C2 than sOVA tumors (Fig. 4*B*). We also observed more OVA-specific IFNγ-secreting $CD8^+$ T cells in the blood of mice bearing the OVAC1C2 tumors (Supplementary Table S1).

Although these results fit very closely with our original model, we cannot rule out that binding of the OVAC1C2 antigen to other structures than exosomes or secreted vesicles plays a role in the efficiency of immune responses. Indeed, because exosomes and vesicles are too small to be visualized in whole tissues, and because the tumor cells secrete only low amounts of the vesicle-bound OVA, we could not follow the behavior of the fusion protein after its secretion *in vivo*. In particular, it is possible that, if apoptotic cells are present near the live cells secreting OVAC1C2, the protein could eventually equilibrate between phosphatidylserine exposed at the surfaces of apoptotic cells and secreted vesicles. For instance, when tumor destruction begins, after initial activation of antigen-specific CTLs, capture of apoptotic bodies coated with OVAC1C2 by dendritic cells could also help to amplify the extent of anti-OVA immune responses. However, even if apoptotic cells may play a role in the late steps of antitumor reactions, we observed increased activation of anti-OVA $CD8^+$ T cells in MCA/OVAC1C2-bearing mice very early after transfer, before any sign of tumor shrinkage (thus tumor cell death; Fig. 4*A*). In conclusion, the C1C2-fused protein secreted by live tumor cells allows better initial activation of antigen-specific T cells than the soluble form. Interestingly, we observed that subclones of MCA/OVAC1C2 cells expressing lower levels of the OVA antigen than the MCA/sOVA cells were also destroyed by OT-I T cells, and their growth was impaired by the endogenous immune system (Supplementary Fig. S1). Thus, it is not the absolute amount of antigen displayed by the tumor that determines the efficiency of activation of the immune system, but rather the form of the antigen (i.e., bound to secreted vesicles).

We also show in the double-tumor experiments (Fig. 5) that the way OVA-specific T cells are activated when the antigen is presented as a fusion with C1C2 allows them to destroy not only the tumor secreting the modified antigen but also the tumor secreting the soluble antigen. These experiments show that the sOVA-expressing tumors are responsive to the destruction mechanisms led by the immune system, provided that these mechanisms are properly set up by the OVAC1C2 antigen. As for the exact nature of these mechanisms, we show that antigen-specific CTLs are essential players (because adoptive transfer of OVA-specific $CD8^+$ T cells alone is sufficient to induce tumor destruction) and that expression of the antigen by tumor cells themselves is required (because MCA/mock tumor is not destroyed). However, whether CTLs only kill tumors directly or also indirectly by acting on tumor stroma is an open question. Furthermore, CTLs are most probably not acting alone, and other components of the immune system could be activated or inhibited by tumors secreting OVA and exosomes. Full-range analysis of the cellular mechanisms of the immune responses taking place in mice with growing OVAC1C2 tumors was beyond the scope of the present work but is an important question that we are currently investigating. It will be particularly important to decipher the involvement of dendritic cells, of the other players of the adaptive immunity, and of the innate arm of the immune responses.

These questions will be especially relevant for future use of the C1C2 approach on other tumor models. Indeed, exosomes purified from tumor cells can induce antitumor immune responses *in vivo* (7), but immunosuppressive effects of exosomes secreted by human (29–31) and mouse tumors (32) have also been described *in vitro*. Such immunosuppressive effects include T-cell killing due to enrichment of Fas ligand on tumor exosomes (29), NK cell inhibition (31, 32), or inhibition of monocyte differentiation into dendritic cells, probably mediated by transforming growth factor β (30). Exosomes secreted by the MCA101 tumor cell line used here may not have immunosuppression abilities, or if they have, our results show that antigen transfer *in vivo* via exosomes is not impaired by them. It is possible, however, that depending on the tumors, the balance between suppressive effects and increased antigen-specific immune response induced by exosomes will control tightly the final outcome on tumor growth.

Finally, our DNA vaccination experiments represent a very promising approach for future developments of antitumor treatments. Indeed, in this preventive setting, the OVAC1C2-fusion cDNA protected 50% of the mice from a challenge with the MCA/sOVA tumor and strongly delayed tumor growth in the other half. Protection against tumor growth by DNA vaccination was observed in the absence of any additional help to the endogenous immune system, especially without transfer of exogenous anti-OVA CTLs.

The purpose of these DNA vaccination experiments was mainly to confirm, in a fully *in vivo* experimental system, a role of expression of the vesicle-bound antigen in immunologic responses. Their striking efficiency to inhibit tumor growth, however, strongly encourages future use of the OVAC1C2 expression plasmid in curative protocols of DNA vaccination, which are more clinically relevant. Indeed, our preliminary observations suggest tumor growth–preventing effects of OVAC1C2-cDNA vaccination in mice bearing already growing tumors.

In conclusion, our results suggest a new way to increase the efficiency of induction of antitumor antigen immune responses: by coupling a tumor antigen to the C1C2 domain of mouse lactadherin and thus targeting the antigen to secreted vesicles.

Acknowledgments

Received 8/16/2007; revised 12/11/2007; accepted 12/18/2007.

Grant support: Institut National de la Santé et de la Recherche Médicale, Institut Curie, Association pour la Recherche sur le Cancer, Cancéropôle Ile de France, Fondation Schueller Bettencourt, European Grant DC-THERA 512074, Dutch Cancer Society postdoctoral fellowship (I.S. Zeelenberg), Institut National du Cancer postdoctoral fellowship (M. Ostrowski), and Fondation pour la Recherche Médicale postdoctoral fellowship (C. Jancic).

Conflict of interest: As partners of the ExoThera company, A. Delcayre and J.B. Le Pecq declare financial conflict of interest.

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We thank Lucien Cabanié and Alexander Nussbaum for help with protein purification and DNA vaccination, respectively, and Olivier Lantz, Claire Hivroz, and Gaël Sugano for critical reading of the manuscript.

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