Antigen Localization Controls T Cell-Mediated Tumor Immunity


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Antigen Localization Controls T Cell-Mediated Tumor Immunity


Effective antitumor immunotherapy requires the identification of suitable target Ags. Interestingly, many of the tumor Ags used in clinical trials are present in preparations of secreted tumor vesicles (exosomes). In this study, we compared T cell responses elicited by murine MCA101 fibrosarcoma tumors expressing a model Ag at different localizations within the tumor cell in association with secreted vesicles (exosomes), as a nonsecreted cell-associated protein, or as secreted soluble protein. Remarkably, we demonstrated that only the tumor-secreting vesicle-bound Ag elicited a strong Ag-specific CD8+ T cell response, CD4+ T cell help, Ag-specific Abs, and a decrease in the percentage of immunosuppressive regulatory T cells in the tumor. Moreover, in a therapeutic tumor model of cryoablation, only in tumors secreting vesicle-bound Ag could Ag-specific CD8+ T cells still be detected up to 16 d after therapy. We concluded that the localization of an Ag within the tumor determines whether a robust immunostimulatory response is elicited. In vivo, vesicle-bound Ag clearly skews toward a more immunogenic phenotype, whereas soluble or cell-associated Ag expression cannot prevent or even delay outgrowth and results in tumor tolerance. This may explain why particular immunotherapies based on these vesicle-bound tumor Ags are potentially successful. Therefore, we conclude that this study may have significant implications in the discovery of new tumor Ags suitable for immunotherapy and that their location should be taken into account to ensure a strong antitumor immune response. The Journal of Immunology, 2011, 187: 1281–1288.

The immune system has to protect the host against foreign pathogens and avoid damage to normal tissues. Because tumors are derived from these normal cells, the antitumor immune response is not always effective and sometimes results in tolerance (1). Immunotherapy against cancer aims to activate the immune system to destroy the tumor cells (2–4). For example, specific tumor-associated Ags can be loaded onto professional APCs, dendritic cells, which are then activated and reinjected back into the patient. Many well-known Ags used for these clinical studies, such as Her2/Neu, Mart1, TRP, and gp100, have been found in vesicles secreted by tumor cells, called exosomes (5). Therefore, we wondered whether the localization of the Ag was important for the antitumor immune response.

APCs can capture Ag from tumors using various sources of material: apoptotic or necrotic tumor cells, heat shock protein–peptide complexes, plasma membrane fragments, soluble proteins, or vesicles secreted by live tumor cells, called exosomes (6–10). However, the actual source of Ag in vivo is probably a combination of all of the above. It is known that APCs can direct soluble Ags into lysosomes, resulting in presentation on MHC-II molecules or target them to early endosomes, leading to MHC-I–restricted presentation (11). Particulate Ags are phagocytosed and result in presentation on both MHC-I and MHC-II. Particulate Ag in a cell-associated form was shown to result in increased T cell activation compared with soluble Ag (12, 13). Also, virus-like particles, which contain Ags, were shown to result in a stronger T cell response compared with soluble proteins (14). Because tumors continuously secrete membrane vesicles, such as exosomes, providing another source of particulate Ag, we wondered whether this source of Ag in vivo leads to a more profound T cell-mediated immune response compared with secreted soluble or nonsecreted cell-associated Ags.

Previously, we reported that a tumor expressing the model Ag OVA coupled to the factor VIII-like C1C2 domain of milk fat globule EGF factor VIII (MFG-E8)/lactadherin (OVAC1C2), secreted OVA in association with vesicles, whereas the SOVA tumor secreted OVA only in a soluble form and not associated with vesicles (15) (Fig. 2A). In addition, we constructed a fusion protein of FcγRII and the Ag OVA (FcROVA tumor, Fig. 2A), because FcγRII is present on the plasma membrane and was shown to be excluded from exosomes (16). The FcROVA tumor expresses OVA only in a cell-associated membrane-bound form; it does not secrete OVA in a soluble form nor in association with vesicles (Fig. 2A).

To obtain a strong T cell response, naive CD8+ T cells first need to encounter the Ag on MHC-I molecules. Because most tumors, including the murine fibrosarcoma MCA101 used in this study, do
not express sufficient levels of MHC-I to induce CTLs from naive T cells, the T cells need to be activated by professional APCs (17). The naturally occurring T cell response in tumor-bearing mice does not always lead to tumor eradication and can even result in tolerance (1, 18). Because of their specific killing capacities, CD8⁺ T cells have the ability to eliminate malignant cells. For the rejection of a tumor it is important that the CTLs reach and deeply infiltrate the tumor, which is dependent on the presence of Ag in the tumor (19). However, help from CD4⁺ T cells is needed for maximal proliferation and effector function of CD8⁺ T cells (20). CD4⁺ T cells are also necessary to provide help to B cells to start proliferation and induce Ab class switching and Ab production, leading to Ab-dependent cell-mediated cytolysis (21). By contrast, CD4⁺ regulatory T cells (Tregs) can inhibit or weaken the immune response and limit the efficacy (22, 23). Naturally occurring Tregs originate from the thymus, and adaptive Tregs are induced in the periphery from conventional CD4⁺ T cells (24). Tregs exert a strong, suppressive activity on multiple components of the immune system. Depending on the model, they can suppress priming or proliferation of the T cells, inhibit proliferation, or even kill dendritic cells in an Ag-specific manner (23, 25). In general, the influx of CD8⁺ T cells is assumed to be beneficial for the patient, whereas a higher Treg number in the tumor implies a worse prognosis (26). The attribution of these diverse T cell responses in respect to differently localized Ag in tumors has not been investigated.

In this study, we compared the potential of differently localized Ags to induce T cell-mediated tumor immunity. We investigated the endogenous CD8⁺, as well as the CD4⁺ T cell response in mice bearing tumors with these differently localized Ags. To determine the impact of the localization of Ags in a more therapeutical setting, we analyzed the Ag-specific CD8⁺ T cell response after cryoablation, a tumor-destruction technique using a thermal energy source used to treat solid tumors (27). We showed that only tumors secreting vesicle-bound Ag induced a strong antitumor immune response, which is prolonged after therapy, whereas tumors secreting soluble Ag or containing nonsecreted cell-associated Ag showed a weak immune response, unable to prevent or even delay tumor growth.

Materials and Methods

Mice

C57BL/6J mice were obtained from Charles River and maintained under specific pathogen-free barrier conditions at the Central Animal Laboratory (Nijmegen, The Netherlands). OT-II mice were bred at the Central Animal Laboratory. Drinking water and standard laboratory food pellets were provided ad libitum. The experiments were performed in accordance with the guidelines for animal care of the Nijmegen Animal Experiments Committee.

Plasmids

The pcDNA3-hygroycin expression plasmids were from Invitrogen. The OVA/C12 and sOVA constructs were made as described previously (15). The FcγRIIB receptor was amplified by PCR from mouse cDNA; the cytosolic part containing the ITIM sequence was deleted, replaced with OVA, and inserted into the pcDNA3 vector (for FcROVA).

Cells

The MCA101 fibrosarcoma was cultured in DMEM supplemented with 10% bovine calf serum (Greiner Bio-One) and antibiotic-antimycotic (Life Technologies). Stable cell lines were obtained by electroporation, followed by selection with 1 mg/ml hygromycin (Roche) and cloning by limiting dilution, as described previously (15).

Exosome purification

Exosome production, purification, and characterization were done as previously described (28). Briefly, transfected MCA 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. Concentrations were quantified by micro protein BCA assay kit (Pierce), and routine characterization was done by Western blotting and FACS analysis after coating on 4-μm aldehyde-sulfate latex beads (Invitrogen Molecular Probes).

ELISA

For detection of OVA in supernatant, cells, and purified exosomes by ELISA, goat anti-OVA (MP Biomedicals) was coated on MaxiSorp 96-well plates (NUNC). Washes were done in PBS supplemented with 0.05% Tween 20, blocking in PBS/5% milk. After incubation with the samples, bound OVA was revealed by polyclonal rabbit anti-OVA serum (Sigma Aldrich), followed by HRP-conjugated anti-rabbit Ab and 3,3’-5,5’-tetramethylbenzidine substrate reagent (Sigma). Reaction was stopped with 0.8 M H2SO4, and absorbance was read at 450 nm. For detection of OVA-specific Abs, OVA was coated on MaxiSorp 96-well plates. ELISA was performed as described above, except that wells were blocked with PBS with 0.05% Tween and 1% BSA before diluted serum (as stated in the figure legends) was added to the plates. Biotinylated anti-mouse IgG1 Ab (The Binding Site) was followed by HRP-conjugated streptavidin (Invitrogen). A monoclonal mouse anti-OVA Ab was used as a positive control to obtain a standard curve from which the concentrations were calculated.

[^15S] metabolic labeling and pulse-chase immunoprecipitation

Transfected MCA cells were starved in methionine and cysteine-free DMEM medium, supplemented with dialyzed FBS, for 2 h. Cells were labeled with 100 μCi/ml TRAN[^15S]-label (MP Biomedicals) for 15 min, washed, and then incubated in chase medium (complete DMEM) for 0, 30, or 120 min. Subsequently, cells were lysed in 50 mmol/l Tris (pH 7.5), 0.3 mol/l NaCl, and 0.5% Triton X-100, containing a mixture of protease inhibitors. Lysates and supernatants were incubated with Protein G-agarose beads prebound with rabbit anti-OVA Ab overnight at 4°C. After extensive washings, the samples were boiled with nonreducing sample buffer and separated on SDS/10% PAGE gels. The gels were fixed, washed with DMSO, and treated with PPO before drying and exposure to film at ~80°C.

B2Z T cell-activation assay

Transfected MCA cells were incubated overnight at 37°C with 10 ng/ml IFN-γ to enhance MHC-I expression. After washing, 1 × 10⁶ MCA cells were coincubated for 16 h with 5 × 10⁴ B2Z T cells, which carry a lacZ (β-galactosidase) construct driven by NFAT elements from the IL-2 promoter and recognize the OVA peptide (257–264) SIINFEKL in the context of Kb MHC-I molecules (29). Cell pellets were lysed with PBS containing 0.125% Nonidet P-40, 9 mM MgCl2, 100 mM 2-ME, and 15 mM chloroform red/β-D-galactopyranoside (Calbiochem). After 3 h at 37°C, the plate was read at 595 nm using a 96-well plate reader.

In vivo tumor growth

Transfected MCA cells (4 × 10⁶) obtained from subconfluent cultures were injected s.c. in the shaved flank, and tumor size was measured every 3–4 d with a caliper. Tumor volume was calculated as length × width × (length + width)/2. Mice were killed at day 20 for Treg analysis.

Intracellular cytokine staining

Blood was drawn from the tail vein of animals at multiple time points. Erythrocytes were lysed using ACK lysis buffer (containing 0.15 M NH₄Cl, 1 μM KHCO₃, 0.1 μM EDTA) before cells were divided in 96-well plates and incubated for 5 h with 5 μg/ml brefeldin A with no peptide, irrelevant peptide, CD8⁺ OVA SIINFEKL peptide, or a combination of PMA with ionomycin as a positive control. After stimulation, cells were stained with fluorophore-coupled Abs to mouse CD4 and CD8 (BD Pharmingen), followed by fixation, permeabilization, and subsequent intracellular cytokine staining with anti–IFN-γ Ab, according to the manufacturer’s protocol (BD Pharmingen). Cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson) or a Cyan (Beckman Coulter).

In vivo T cell-stimulation assay

OT-II mice were sacrificed, lymph node suspensions were made, and their CD4⁺ CD25⁻ T cells were sorted using the CD4⁺ T cell isolation kit combined with CD25-PE and PE beads (Miltenyi Biotec). Those sorted OT-II T cells were labeled with CFSE (Invitrogen Molecular Probes) before i.v. injection in C57BL/6J mice bearing 8-d-old tumors. Three days later, cells from the draining and nondraining lymph nodes were analyzed...
by flow cytometry after staining with fluorophore-coupled Abs to CD4 and TCRVα2 (BD Pharmingen).

T cell analysis

Draining lymph nodes and tumors were excised and mashed using needles or scissors, respectively, and digested in collagenase type III (Worthington Biochemical) and DNase (Roche) for 20 min. After addition of EDTA and resuspension, cells were applied to a filter to remove debris. Cells were stained with Abs against CD4, CD8, and CD25. Intracellular staining for Foxp3 was performed according to the manufacturer’s protocol (Foxp3-staining kit; eBiosciences).

Suppression assay

Draining lymph nodes of five tumor-bearing animals were pooled, and Tregs were sorted from the regulatory T cell-depleted splenocytes, for 3–4 d. Proliferation of the conventional T cells was assessed by CFSE dilution, as analyzed by flow cytometry.

Cryoablation

Tumor cells (4 × 10⁵) were resuspended in a mixture of PBS and Matrigel (2:1) and injected s.c. at the right femur. At day 10, when all tumors were of similar size (∼500 mm³), cryoablation was performed under isoflurane anesthesia using a liquid nitrogen cryoablation system (CS76; Frigifield). Intracellular cytokine staining was performed as described above. Blood was drawn 1 d before and 7 and 16 d after cryoablation. Intraglottic cytokine staining was performed as described above.

Statistical analysis

The data were analyzed with one-way (bars) or two-way (curves) ANOVA, followed by a Bonferroni post test. Statistical significance is shown in the graphs only if it is significant compared with all other groups.

Results

Characterization of MCA tumor cells expressing differentially localized Ags

To study the effect of the localization of Ags in the antitumor immune response, we compared murine MCA101 fibrosarcoma tumors transfected with three OVA constructs and a mock construct. Previously, we coupled the model Ag OVA to the factor VIII-like C1C2 domain of MFG-E8/lactadherin, which resulted in secretion associated with vesicles (exosomes) (15). The fibrosarcoma tumor MCA101 expressing this OVA1C2 construct grew much more slowly in immunocompetent (but not in immunodeficient) mice than did the tumor secreting soluble OVA (sOVA tumor) (Fig. 1A) (15). We could not discriminate whether this was due to a vesicle-bound or membrane-bound form of the Ag. Therefore, in this study we also included a membrane-bound, but only cell-associated, form of the Ag OVA, FcROVA (Fig. 2A). The supernatant, purified exosomes, and cellular fraction of the FcROVA tumor were thoroughly characterized like the sOVA and the OVA1C2 tumor, and OVA was only expressed in a cellular form and not on exosomes (Fig. 2B) (15).

For the recognition and subsequent killing of tumors by cytotoxic T cells, it is very important that the MHC–peptide complexes on the cell surface are similar. Therefore, we checked the capability of the four MCA cell lines to activate the T cell line B3Z, which recognizes the OVA peptide (257–264) SIINFEKL in the context of Kb MHC-I (29). As shown in Fig. 2C, all cell lines were equally efficient in activating B3Z.

Shen and Rock (13) suggested that the stability of the protein is an important factor for cross-presentation. Thus, we performed a [35S] pulse-chase assay with the different transfected OVA tumor cell lines. As shown in Fig. 2D, all proteins degraded at a similar rate when comparing the 30-min pulse with the 120-min pulse. The sOVA and FcROVA seemed to be synthesized faster than was the OVA1C2 protein, but the degradation pattern was similar. In the supernatant only, the sOVA tumor showed a strong secretion of the protein; the OVA1C2 tumor did as well, but to a lesser extent. As previously verified by ELISA, the FcROVA tumor did not secrete any protein. Overall, these data showed that all proteins were equally stable.

Vesicle-bound Ag induces profound Ag-specific CD8⁺ T cell responses

As shown in Fig. 1A, only the OVA1C2 tumors secreting vesicle-bound OVA showed reduced growth. Remarkably, even the FcROVA tumor grew similarly to the mock-transfected tumor, showing that the presence of only a cell-associated membrane-bound, but not secreted form, of OVA did not inhibit the growth of tumors. Previously, we showed that adoptively transferred OVA-specific CD8⁺ OT-I T cells injected at day 6 proliferated more in the OVA1C2 tumor expressing vesicle-bound OVA compared with the sOVA tumor secreting soluble OVA. We followed the endogenous CD8⁺ T cell response in the blood of mice bearing the different OVA-expressing tumors over time. As shown in Fig. 1B, the tumors bearing the various forms of OVA showed different kinetics of the endogenous OVA-specific CD8⁺ T cell response. The OVA1C2 tumor showed the strongest immune response, which peaked at ~days 12–15 and decreased at ~day 20, in accordance with the contraction phase of the CTL response (31). The sOVA tumor showed weaker, but similar, kinetics compared with the OVA1C2 tumor. Interestingly, the FcROVA tumor, which has a membrane-bound form
of OVA similar to the OVAC1C2 tumor, although only nonsecreted cell-associated and not bound to secreted vesicles, exhibited a much weaker and earlier OVA-specific CD8+ T cell response. We concluded from these data that membrane-bound Ag must be associated with secreted vesicles to induce a strong Ag-specific CD8+ T cell immune response in vivo.

Only vesicle-bound Ag induces proliferation of OVA-specific CD4+ OT-II cells

CD4+ T cell help was shown to be essential for a strong CD8+ CTL response (20). Thus, we investigated the CD4+ Th cell response. Less than 0.5% of the total CD4+ T cells in the blood of mice bearing the different tumors were able to produce Th cell-specific cytokines, such as IFN-γ, IL-4, and IL-17, after incubation with PMA and ionomycin (data not shown). Because the detection level of OVA-specific CD4+ T cells in blood seemed to be too low, we took a different approach. We adoptively transferred OVA-specific CD4+ OT-II cells at day 8. Three days later, the mice were sacrificed, and the draining and nondraining lymph nodes were analyzed for proliferation of OT-II cells. Significant proliferation of the OT-II cells was only observed in the draining lymph nodes of mice bearing the OVAC1C2 tumor secreting vesicle-bound OVA (Fig. 3A). As expected, no proliferation was found in the nondraining lymph nodes (Fig. 3B).

Vesicle-bound Ag results in an OVA-specific IgG1 Ab response

Subsequently, we studied the capacity of CD4+ T cells to provide help to B cells to induce Ab class switching and produce Ag-specific Abs. Therefore, we measured the amount of Abs against the Ag OVA in the serum of mice bearing the tumors with the differently localized Ags over time. Early in the immune response (day 15), the mice with the OVAC1C2 tumors secreting vesicle-bound OVA already showed a significant OVA-specific IgG1 response (Fig. 4A). At day 25, 1 µg/ml OVA-specific IgG1 Abs was detected in the OVAC1C2 tumor-bearing mice (Fig. 4B). As a control, we measured the amount of total IgG1 Abs; no significant differences were observed. We detected only small amounts of OVA-specific IgG2b and IgG2c Abs at day 25 in serum of the mice bearing the sOVA tumors, secreting soluble OVA, and FcROVA tumors, containing nonsecreted cell-associated OVA (<50 ng/ml; data not shown). We concluded from these data that the Ag needs to be secreted in a vesicle-bound form to induce production of OVA-specific Abs of the IgG1 isotype.

Cell-associated or soluble Ag does not decrease the percentage of Tregs

To study whether Ag localization has an effect on the induction of tumor tolerance, we investigated the influx of Tregs in the tumors,

**FIGURE 2.** Schematic overview and characterization of the different tumor cell lines. A, The different constructs made in pcDNA3 to generate the MCA101 tumors expressing soluble OVA (sOVA), vesicle-bound OVA (OVAC1C2), and nonsecreted cell-associated OVA (FcROVA), as well as the non-OVA–expressing (mock) tumor cells, and where OVA is found, as assessed by ELISA and immunoprecipitation assays. B, An OVA ELISA was used to compare the amount of OVA present in the cellular fraction, supernatant, and purified exosomes of the respective tumor cell lines, as depicted in A. A standard curve of OVA was used to calculate the amount of OVA. Black bars, expression in the exosome-free supernatant; white bars, cellular expression; gray bars, expression on the exosomes. Data are means ± SEM of four purifications/cell line from two independent ELISAs in triplicate. All differences were highly significant (p < 0.001), as determined by two-way ANOVA, followed by a Bonferroni post test. C, All cell lines are equally capable of activating the OVA-specific T cell line B3Z, suggesting that similar amounts of OVA peptide in the context of MHC-I are expressed. A representative experiment is shown out of four; data are averages of triplicates ± SEM. D, All OVA proteins are equally stable, as shown by a [35S] pulse-chase assay. The indicated stable transfectants were metabolically labeled with TRAN[35S]-label for 15 min (pulse) and chased for 30 and 120 min. Cell lysates were immunoprecipitated with rabbit anti-OVA Ab bound to protein A agarose beads and separated by SDS-PAGE. C1C2, factor VIII-like domains of the MFG-E8 cDNA; Cells, cellular expression; Exo, expression on the exosomes; FcR, extracellular part of FcγRIIb; his, 6-histidines tag; Hyg, hygromycin resistance gene; myc, myc tag; ss, signal sequence from the MFG-E8 cDNA; Sup, expression in the exosome-free supernatant.
because this was demonstrated to be a negative prognostic factor (26). We analyzed the percentage of Tregs in the draining lymph nodes and in the tumors expressing the different forms of Ag. No difference was seen in the draining lymph nodes of all tumors when comparing the percentage of Tregs in the total CD4+ T cell pool (Fig. 5A). Also, the functionality of the Tregs in the draining lymph nodes was not affected, as demonstrated by their ability to suppress the proliferation of conventional CD4+ T cells (Fig. 5B).

When focusing on the tumors, we found that the sOVA tumor secreting soluble OVA, as well as the FcROVA tumor with cell-associated OVA, contained equal percentages of Tregs compared with the mock tumor (Fig. 5C). The presence of the immunodominant Ag OVA in itself proved insufficient to skew the balance from tumor tolerance toward immunity. However, when the Ag was secreted in a vesicle-bound form, as in the OVAC1C2 tumor, the percentage of Tregs among CD4+ T cells in the tumor was significantly less. Because the ratio of Tregs/conventional T cells in the draining lymph nodes and the tumor is important in the balance between immunity and tolerance, we also analyzed the percentages of total CD4+ and CD8+ T cells in the draining lymph nodes and tumors (Supplemental Fig. 1). The percentage of CD4+ and CD8+ T cells was equal in all tumors, confirming that the decrease in the percentage of Tregs observed in the OVAC1C2 tumor reflected a decrease in the ratio of Tregs/conventional CD4+ T cells. Thus, in contrast to the vesicle-bound Ag (OVAC1C2), secretion of a soluble OVA or expression of a nonsecreted cell-associated OVA by tumor cells was not sufficient to induce changes in the ratio of tolerogenic T cells/effector CD4 and CD8 T cells.
The vesicle-bound form of the Ag induces a prolonged OVA-specific CD8+ T cell response after cryoablation

The secreted vesicle-bound form of the Ag clearly skews the immune system toward antitumor immunity, whereas a secreted soluble form or a nonsecreted cell-associated form of the Ag does not. We wanted to investigate whether this effect was still apparent after therapeutic interference, such as cryoablation (27), which is a tumor-destruction technique that uses a thermal-energy source to freeze the tumor. It is known that following tumor ablation in situ, large amounts of tumor debris are released that could be taken up by the immune system and induce a strong tumor-specific immune response (30, 32). We wondered whether the Ag localization within the tumor cells in this particular therapeutic setting, where large amounts of Ags are released upon cryoablation, is important for the skewing of the immune response. The tumors with differentially localized Ags were grown in immunocompetent mice, as in Fig. 1A; at day 10, when all tumors were still of similar sizes, we performed cryoablation, as described previously (30). The tumors were completely destroyed, and no recurrence was observed. Without cryoablation, the contraction phase of the CTL response had started at day 19 (Fig. 1B), and few OVA-specific T cells were present in blood. However, after cryoablation, when the tumor had completely disappeared, a strong OVA-specific CD8+ T cell response was still present at day 19, and even at day 26, only in the mice bearing OVAC1C2 tumors secreting vesicle-bound OVA (Fig. 6). By contrast, cryoablation of the other tumors did not result in strong antitumor immunity, despite the release of all Ags, including the highly immunogenic protein OVA, in tumor debris. Taken together, these results demonstrated that only vesicle-bound Ags can lead to a boost in the immune response after therapeutic intervention and result in tumor immunity.

Discussion

The magnitude and type of the immune response are of crucial importance for many diseases, including cancer. We show that in vivo secretion of vesicle-bound Ag is of eminent importance in obtaining strong immunity against malignant tumors. It resulted in a stronger CD8+ T cell response, as well as in the proliferation of OVA-specific CD4+ T cells and more OVA-specific Abs. By contrast, tumors expressing the immunogenic OVA protein in a soluble or a cell-associated form behaved like the mock-transfected tumor and did not mount an effective immune response, resulting in outgrowth of the tumor.

At first glance, our findings may seem to contrast with observations from Shen and Rock (13), who showed that in vivo membrane-bound forms of OVA induced higher levels of CTL activity than did OVA expressed in the cytosol. However, in that study, fibroblasts were transfected with a fusion protein of the transferrin receptor and OVA in a cell-associated membrane-bound form. Most likely, this fusion protein will not be excluded from exosomes, because the transferrin receptor is present in exosomes secreted by different cell types (reticulocytes, oligodendroglial cells, and dendritic cells) (33–35). Therefore, all preclinical mouse studies in which fusion proteins of OVA and the transferrin receptor are used cannot be regarded as exclusively nonsecreted cell-associated forms of OVA, because the chance is high that they will be secreted through exosomes. By contrast, our constructs are either secreted in association with vesicles (OVAC1C2) or remain nonsecreted cell-associated forms (FcROVA). This may explain the apparent differences between the studies. Furthermore, considering the stability of the protein, Shen and Rock (13) showed clear differences, whereas this was not the case in our different OVA-containing constructs (Fig. 2D).

CD8+ cytotoxic T cells are essential for the rejection of tumors (36, 37). In this study, a significant endogenous OVA-specific CD8+ T cell response was only observed in the blood of the mice bearing the OVAC1C2 tumor, secreting vesicle-bound OVA. The sOVA tumor expressing soluble OVA or cell-associated OVA showed only a minor CD8+ T cell response. In addition to localization of the Ag, immune responses can be influenced by the form of the Ag, the presentation of the peptide on MHC-I necessary to activate T cells, and the stability of the protein. Previously, we showed that when purified sOVA and OVAC1C2 proteins were bound to beads, equal immune responses were elicited in vitro and in vivo, suggesting that the forms of the Ag did not alter the immune response (15). Also, no differences were found with regard to the ability of the different transfectants to activate OVA-specific T cells. Last, the stability of the protein was also equal in all MCA transfectants, as shown by the pulse-chase experiment (Fig. 2D). Because of these findings, we concluded that the difference in the magnitude of the immune response is due to the localization of the Ag (i.e., soluble, vesicle bound versus cell-associated).

CD4+ T cells provide essential help to CD8+ T cells via the production of cytokines to induce and maintain a strong effector response (20). Indeed, we showed, by adoptive transfer of OVA-specific CD4+ OT-II T cells, that only in the mice bearing the OVAC1C2 tumor did these CD4+ T cells proliferate and, therefore, could provide the necessary help to acquire a strong OVA-specific CD8+ T cell response. It was shown that CD4+ T cells are programmed to undergo only a limited number of divisions, whereas CD8+ T cells undergo extensive proliferation (38). Considering the low percentage of endogenous OVA-specific CD8+ T cells, it was not surprising that we did not detect endogenous OVA-specific CD4+ T cells in blood.

In our study, we observed a significant OVA-specific IgG1 response in the OVAC1C2 tumor secreting vesicle-bound OVA and minimal responses in the sOVA and FcROVA tumors. Because CD4+ T cells are also necessary for a functional B cell response (21), this result is consistent with the observation that OVA-specific CD4+ T cells only proliferated in the OVAC1C2 tumor. A few earlier in vivo studies compared the ability of membrane-bound and soluble Ags to provoke Ab responses (39, 40). These DNA-vaccination studies reported a strong IgG1 response after cryoablation at day 10 when all tumors were of similar sizes (Fig. 1A). Blood was collected from the mice just before cryoablation, as well as 9 and 16 d after cryoablation. After restimulation for 5 h with OVA-specific peptides and brefeldin A, the percentage of IFN-γ-producing CD8+ T cells was measured by FACS analysis. Data are means ± SEM of eight mice. *p < 0.05, ***p < 0.001, two-way ANOVA followed by a Bonferroni post test against all groups.
vaccination with the soluble form of OVA but not with the membrane-bound form. Again, as discussed above, the transferrin receptor-OVA fusion protein was used as a membrane-bound form of OVA. This construct is also present on exosomes, as explained above, but on the inside of the vesicle, thereby limiting its availability for internalization by the BCR. By contrast, the OVAC1C2 protein used in our study is detected on the outside of exosomes, rendering it more accessible for the B cells, which can explain the IgG1 Ab production. The lack of a strong Ab response in the mice with the sOVA andFcROVA tumor can be explained by the absence of proliferation of the CD4+ T cells, which are necessary to provide help for class switching, resulting in the observed impaired IgG responses.

A higher Treg number in the tumor is often associated with a worse prognosis in patients (26, 41–44). In the OVAC1C2 tumor, we saw a reduction in Tregs relative to the sOVA and the FcROVA tumor, but, in contrast, in these latter mice, fewer OVA-specific CD8+ T cells were present (Fig. 1B). However, Tregs are important for the outcome of the immune response in this tumor model, because we showed previously that the addition of more OVA-specific naive CD8+ OT-I T cells alone was not sufficient to prevent growth of the sOVA tumor (15). Only when the Tregs were depleted in the sOVA tumor could OT-I T cells induce regression of the tumor, showing that Tregs are important in this tumor model (25). It is of note that Tregs suppress in an Ag-independent manner (Fig. 5B) and that the “tolerance” we observed was against the whole tumor and was not Ag-specific tolerance. For a nonimmunogenic tumor, such as MCA, Tregs clearly play an important role, and introduction of an immunogenic protein per se could not prevent or even delay outgrowth of the tumor, unless it was secreted as a vesicle-bound form. Moreover, Tregs in this same MCA model secreting sOVA were shown to kill dendritic cells in an OVA-specific manner; thus, in addition to direct suppression, they indirectly blocked the formation of a strong OVA-specific T cell-mediated immune response (25). We did not find differences in the percentage of total dendritic cells (I.S. Zeelenberg, unpublished observations) or Tregs (Fig. 4A) in the tumor-draining lymph nodes, but Tregs were significantly decreased in the OVAC1C2 tumor itself, which could explain the decreased immunosuppression observed in this tumor. Further investigations are required to determine whether Ag-specific killing of DCs by Tregs occurs differently in the draining lymph nodes of the different tumors.

Accumulation of Tregs in the tumor is a result of increased migration, conversion from Foxp3− conventional T cells, increased survival, or increased proliferation. In ovarian cancer, macrophages within the tumor were shown to secrete CCL22, which can attract Tregs via CCR4 (42). Therefore, we analyzed the amount of macrophages in the tumor and observed that the OVAC1C2 tumor did not have a reduced number (I.S. Zeelenberg, unpublished observations), suggesting that the decrease in Tregs is not due to reduced migration. It is well known that TGF-β secreted by the tumor can contribute to the induction of Tregs (45).

In vitro, all four tumor cell lines secreted equal amounts of TGF-β (unpublished observations), indicating that this alone cannot explain our findings. However, the amount of TGF-β in the tumor in vivo can be quite different because of the presence of other TGF-β-secreting cells, such as the Tregs themselves, or an increase in secretion by the tumors. We were not able to analyze the survival and proliferation of the Tregs in the tumor. It is likely that the decrease in Tregs seen in the OVAC1C2 tumor resulted from a combination of all of the factors described above.

Cryoablation is a technique that destroys the tumor without exciting it and, thereby, leaves the tumor debris in situ for uptake by immune cells, resulting in weak, but tumor-specific, immunity (30, 32). Using this type of treatment, we expected that differences between the three differently localized Ags would be negligible, because all tumors create a similar mass of tumor debris containing a similar amount of Ag, which could increase the immunogenicity and, thus, break tumor tolerance. Remarkably, we only observed an increase in OVA-specific CD8+ T cells in the OVAC1C2 tumor, and not in the sOVA or FcROVA tumors, after cryoablation (Fig. 6). Whether this prolonged T cell response is due to a delay in T cell contraction, changes in the peak of the T cell response, or a booster response cannot be concluded from these time points. However, when comparing the time points in Figs. 1B and 6, it is unlikely that the prolonged T cell response is due to changes in the peak of the response or a delay in T cell contraction, because at day 19 we never observed any OVA-specific immune response without cryoablation, whereas a week later a strong OVA-specific response was still visible after cryoablation. These data suggested that this therapeutic intervention is most effective in increasing immune responses when an immune response is present before cryoablation. Thus, the initial immune response elicited by vesicle-bound Ag is essential for the outcome of the immune response after therapeutic intervention, such as cryoablation.

We concluded from our study that the location of Ag is of eminent importance for the immune system to induce tumor immunity. Vesicle-bound Ag clearly skew toward a more immunogenic phenotype, whereas soluble or a nonsecreted membrane-bound form of the Ag results in tumor tolerance. This has important implications for tumor therapies, such as cryoablation, because the additional advantage of a prolonged Ag-specific CD8+ T cell immune response is only visible in the OVAC1C2 tumor secreting vesicle-bound Ag. In the current study, we chose to use the model Ag OVA, which is a good model for nonself tumor Ags arising from mutations of endogenous proteins that have been described in human cancer. It is interesting to note that many of the tumor Ags already exploited in immunotherapies are found in preparations of tumor exosomes, such as Her2/Neu, Mart1, TRP, and gp100 (5). This may explain why immunotherapies based on these tumor Ags are potentially successful. Furthermore, certain forms of cancer therapy, such as irradiation, were shown to increase exosome secretion (46), which might also lead to an increase in the immunogenicity of tumors that secrete vesicle-bound Ag. Therefore, this study may have significant implications in the discovery of new tumor Ags suitable for immunotherapy and suggest that their location should be taken into account to ensure a strong antitumor immune response.

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Disclosures
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