An Effective Vaccine Strategy Protective against Antigenically Distinct Tumor Variants

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Abstract

Antigenically distinct tumor variants can emerge in response to selective pressures inherent to host-tumor interactions. The development of successful immunotherapeutic strategies can be limited by these disparate antigenic profiles. Using the immunomodulator B7-DC XAb to activate cytolytic T cells specific for tumor-associated antigens, we found that the specificity of immune responses elicited by live tumors are distinct from the specificity of the responses elicited by soluble proteins derived from the same tumors. Remarkably, whereas the induced antitumor immunity generated against live variants of the B16 melanoma and EL4 thymic lymphoma tumors were highly specific for the original tumor variant used in the challenge, immunity generated using soluble proteins derived from tumor lysates was broadly reactive, recognizing the challenge tumor, as well as antigenically distinct variants. The antigens detected using live tumor and tumor lysate vaccines could be distinguished biochemically, demonstrating that they are structurally distinct. We show that vaccines using antigens present in tumor cell lysates induce protective immunity with strong memory against distantly related tumor variants. The existence of a class of antigens shared among tumor variants provides an attractive target for vaccine development. [Cancer Res 2008;68(7):2471–8]

Introduction

One of the challenges to successful immunotherapy is finding antigens that will elicit a potent and specific response against targeted tumors (1). Antigens that drive a cytotoxic lymphocyte response must be available for processing and presentation on MHC I molecules by dendritic cells and the tumors themselves. This provides several challenges; tumor cells may not shed sufficient amounts of antigen for cross-presentation by dendritic cells, and self-antigens derived from tumors are processed in a tolerant environment, which may not lead to strong T cell activation.

Several methods have been employed to identify potential tumor antigens (2) including the identification of MHC class I–bound peptides (3). One problem with this approach is that MHC class I–restricted peptides will only benefit a limited subset of patients (4). Evidence suggests that the breadth and diversity of the T cell response is important for cancer immunity (5) and monospecific T cell responses may not be effective (6). Vaccination strategies have been employed that elicit immune responses against multiple epitopes (7). Because these vaccines have shown increased potential, it will be important to consider alternative sources of antigen that can elicit tumor-specific responses against multiple antigens. An allogeneic melanoma lysate vaccine (8) contains several melanoma-specific antigens (9). Clinical trials using melanoma lysates and its derivatives have been done and have shown significant response rates (10–12). Future studies may benefit from the addition of immunomodulating agents that can more aggressively activate immunity. Antibodies that bind CTLA4 and CD40 have been used to some effect in this regard (13, 14). Both have shown promise for the treatment of cancer.

Our laboratory has been studying the immunomodulating IgM antibody, B7-DC XAb, that activates the immune functions of human and mouse dendritic cells (15). This antibody has been shown to interact with the B7 family member B7-DC and its activity includes restored antigen uptake in dendritic cells and the promotion of strong T cell responses (16, 17). Furthermore, this antibody showed treatment potential against melanoma in both prophylactic and therapeutic regimens (18). We have shown that tumor protection is due to the development of a very rapid and robust MHC class I–dependent CTL response that is directed against the challenge tumor (19). Remarkably, this antibody treatment promoted highly specific immunity, in which protection was observed against the challenge tumor, but not against closely related variants. The current report uses tumor cell lysates (TCL) in conjunction with B7-DC XAb to generate robust vaccines that induce antitumor immunity, not only against the challenge tumor, but also against its antigenically distinct variants.

Materials and Methods

Mice. C57BL/6 mice were purchased from The Jackson Laboratory. Housing and care of animals was in compliance with the Mayo Clinic Institutional Animal Care and Use Committee.

Cells lines and antibodies. The B16, B16-OVA, B78H1, and B78H1K^bD^b lines were kindly provided by Dr. Richard Vile (Department of Immunology, Mayo Clinic, Rochester, MN). B16, B78H1, B78H1 K^bD^b, C57SV, and EL-4 lines were maintained in DMEM (Life Technologies Invitrogen) containing 10% cosmic calf serum (Hyclone). EG.7 and B16-OVA cells lines were maintained in the same media supplemented with 1 mg/mL of Geneticin (Life Technologies Invitrogen). Recombinant B7-DC XAb was generated as described previously (20). Purified serum IgM (sHIgM39) from a patient with a chronic lymphoproliferative disorder was used as an isotype control.

MHC class I expression. B16, B16-OVA, B78H1, and B78H1K^bD^b cells were plated at a density of 30,000 cells per well onto six-well plates. The following day, cells were treated with 1,000 units/mL of IFN-γ (Life Technologies Invitrogen). After overnight incubation, cells were removed by trypsinization, and analyzed by flow cytometry or pelleted for RNA isolation.

Nontreated and IFN-γ–treated cell lines were stained with mouse monoclonal antibodies to H-$2K^b$ (B8-24-3) and H-$2D^b$ (B22-249.R1). Phycoerythrin-labeled polyclonal goat anti-mouse IgG (BD Biosciences) was used as a secondary antibody for detection and analysis by flow

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

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cytometry. Cells stained with secondary antibody alone were used as negative controls.

RNA was isolated according to the manufacturer's protocol using the RNEasy kit (Qiagen, Inc.). cDNA was generated using the Roche Transcriptor First-Strand cDNA Synthesis Kit. We used 500 ng of total RNA to synthesize cDNA according to the manufacturer's protocol and 4 μL of the cDNA to analyze expression of H-2D^b and H-2K^b. The Universal Probe Library Assay Design Center[1] was used to design an assay that differentiates H-2D^b and H-2K^b expression using the following primers to amplify H-2D^b (5′GAAGTGGGCATCTGTGGTG, 5′TTGGAGTCGGTGGATAGAGG) and H-2K^b (5′GGTGGTGTTAAGATGTGGATGA, 5′GCTCCAGAGACAAGTCAGAGGT) transcripts. Universal probe numbers 12 and 98 were used to detect H-2D^b and H-2K^b amplicons, respectively. The protocol for amplification using the Lightcycler 2.0 instrument (Roche Diagnostics Corporation) was as follows: preincubation at 95°C for 10 min, amplification for 45 cycles (denature at 95°C for 10 s, annealing at 55°C for 30 s, extension at 72°C for 5 s with a single signal acquisition after every cycle) cooling to 20°C. Data was analyzed using the Lightcycler Software 4.0 (Roche Diagnostics Corporation) and was normalized to expression of glyseraldehyde-3-phosphate dehydrogenase.

Gene expression analysis. We used Illumina Beadchip Technology (Illumina, Inc.) to analyze the expression of 46,120 individual transcripts from individual cell lines. RNA was isolated from B16, B16-OVA, B16-N, B16-OVA-N, EL4, and EG.7 using the RNEasy kit (Qiagen). Samples were processed and labeled by the Mayo Clinic Advanced Genomics Technology Center. Raw data was analyzed by the Mayo Clinic Division of Biostatistics. Briefly, raw data was normalized using Illumina BeadStudio software; data were filtered based on a detection threshold of 20. Values <1 were given value of 1 to avoid arbitrarily large fold change values. Fold change data was analyzed for genes passing the detection threshold of 20 in at least one of the samples.

Histology. B16 and B16-OVA cells were plated onto two-well chamber slides at a density of 5,000 cells per well. Slides were incubated overnight followed by fixation with 4% paraformaldehyde for 15 min and cold methanol for 15 min. After three rinses with PBS, cells were stained with hemotoxylin, dehydrated through a graded alcohol series, and coverslipped. Stained cells were photographed using an Olympus DP70 camera attached to an Olympus AX70 research microscope (Olympus America, Inc.).

Preparation of TCLs and fractionation. B16 and B16-OVA lysate were prepared as described previously (21). Cells were grown until confluent, then trypsinized, pelleted, and resuspended in PBS at a concentration of 1×10^7 cells per milliliter before being frozen at −80°C. When sufficient numbers had been collected, cells were subjected to three cycles of freeze/thawing. After the final thaw, cell lysate suspensions were separated into 25 mL fractions and were sonicated with three 30-s bursts to further disrupt the cell suspension. The suspension was then centrifuged at 12,000 rpm for 30 min and filtered through a 2-μm filter to clarify the tumor lysate preparation.

Separation of TCL was accomplished by first precipitating the tumor lysate with 50% ammonium sulfate (Sigma). Lysates were allowed to precipitate overnight before the supernatant was removed and the pellet was resuspended in PBS. Lysates were then run over a high-capacity Superdex 200 size-exclusion column (GE Healthcare) to separate the lysate by size and to eliminate large protein aggregates. Protein fractions eluting after an initial large peak were then pooled and fractionated by charge using a Mono Q 10/10 anion exchange column (GE Healthcare). Samples were exchanged into 20 mmol/L of Tris (pH 8.0) before addition to the column and a stepwise increase of NaCl to 1 mol/L was used to elute fractions. Fractions were exchanged into PBS and concentrated before use in downstream assays.

Generation of mouse dendritic cells. Dendritic cells derived from mouse bone marrow were generated as previously described (22). Erythrocyte depleted of bone marrow isolated from the long bones of the

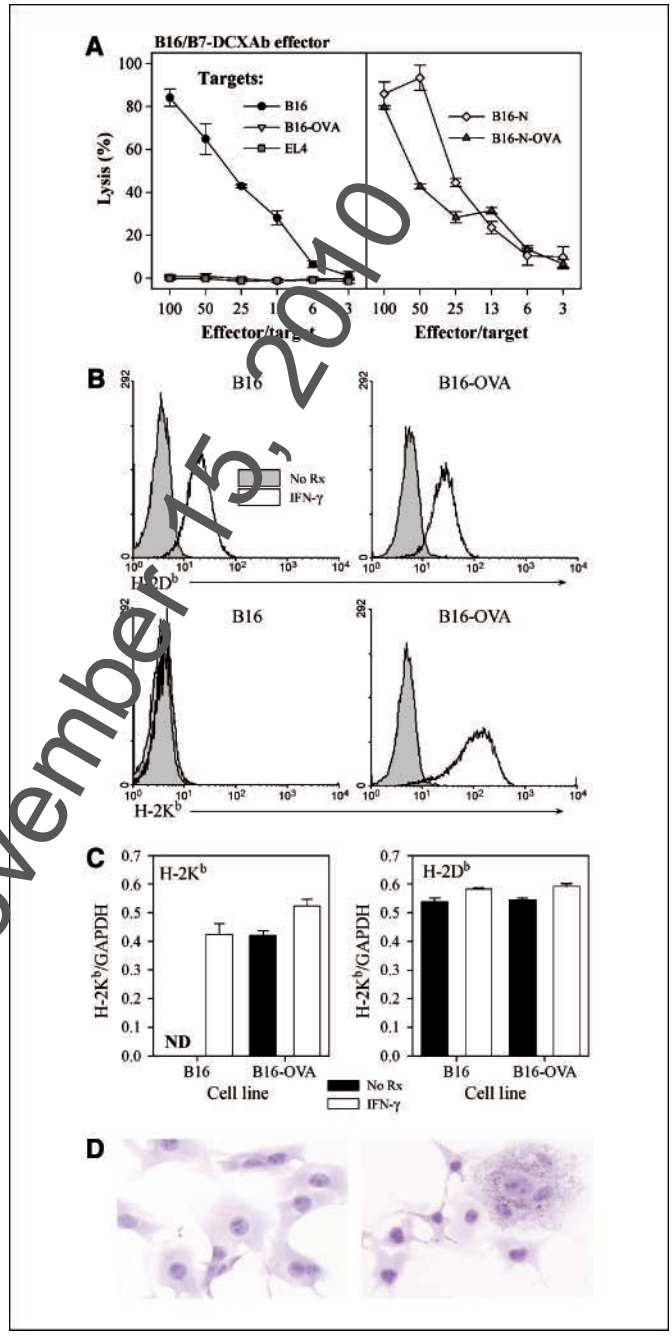

Figure 1. B16 and B16-OVA are unique cell lines as determined by B7-DC XAb–induced CTL, MHC class I expression, and melanin synthesis. *A,* mice were treated i.v. with 30 μg of recombinant B7-DC XAb 1 day prior to B16 tumor challenge and CTL harvested 6 days later were assessed by chromium release assay. CTL recognized B16 tumor cells (●) but not B16-OVA (▽) or the control cell line EL4 (□). These same CTL recognized the newly subcloned line B16-N (◇) as well as the OVA-transfected line B16-N-OVA (▲). *B,* B16 and B16-OVA cell lines were treated with IFN-γ and assessed by flow cytometry for H-2D^b (*top*) or H-2K^b (*bottom*) expression. B16 and B16-OVA up-regulate H-2D^b in response to IFN-γ treatment. B16 cells show minimal up-regulation of H-2K^b in response to IFN-γ treatment. In contrast, B16-OVA cells up-regulate H-2K^b in the presence of IFN-γ. *C,* RNA from IFN-γ–treated and –untreated B16 and B16-OVA was assessed by real-time reverse transcription-PCR to determine H-2K^b and H-2D^b expression. In the absence of IFN-γ, H-2K^b transcripts were not detected in B16, whereas transcripts were present in B16-OVA. H-2K^b transcripts were up-regulated after treatment with IFN-γ in both B16 and B16-OVA. B16 and B16-OVA showed similar levels of H-2D^b transcripts before and after IFN-γ treatment. *D,* B16 melanoma cells were noted for their lack of melanin content, in which B16-OVA cells consistently showed the presence of melanin pigment granules.

[1] http://www.roche-applied-science.com

hind legs were plated at a density of 1×10^6/mL in six-well plates in RPMI 1640 containing 10 ng/mL of murine granulocyte macrophage colony-stimulating factor and 1 ng/mL of murine interleukin-4 (PeproTech). The cells were incubated at 37°C with 5% CO_2. After 48 h, the cells were washed and media was replaced. Dendritic cells were harvested for *in vitro* assays after an additional 4 days of incubation.

Cytotoxicity assay and lymphocyte depletion. Briefly, C57BL/6 mice received an i.v. injection with 20 or 30 μg of either control antibody or B7-DC cross-linking antibody. On the following day, mice were challenged in the right flank with 5×10^5 melanoma cells or 200 μg of TCL. Six days after antigen challenge, cells from the draining lymph nodes from groups of five mice were harvested and pooled. Cells were isolated and titrated in triplicate and tested against ^{51}Cr (GE Healthcare) labeled cells in a standard 4-h cytotoxicity assay. Magnetic beads coated with anti-CD8, anti-CD4 or anti-CD49b (DX5) were used according to the manufacturer's protocol (Miltenyi Biotec) to remove specific cells from the effector population. Depletion was verified by flow cytometry.

Prophylactic and therapeutic treatment protocols. Mice were given an i.v. injection with 30 μg of B7-DC XAb or control antibody in 100 μL of PBS 1 day prior to or 6 days after B16 challenge. Mice received a s.c. injection with 5×10^5 B16 cells or 200 μg of B16-TCL on the right flank in a 100-μL volume. Mice were monitored for the development of tumor and euthanized when tumor size reached 225 mm^2. The size of the tumors was determined in two dimensions. Mice were monitored for 90 days. Animals that cleared the initial B16 tumor were re-challenged in the right flank with 5×10^5 B16-OVA cells and were monitored for tumor development an additional 35 days.

Statistical analysis. Statistical analysis was performed using SigmaStat3.1 software (Systat Software). We performed Kaplan-Meier survival analysis using the log-rank method to determine statistical differences between treatment groups. Pairwise multiple comparisons were performed using the Holm-Sidak method to isolate differences among the groups. Significance was determined by $P < 0.05$.

Results

Autologous tumor vaccination with B7-DC XAb treatment generates a cytotoxic lymphocyte response that differentiates related tumor lines. The human IgM antibody B7-DC XAb is a potent immunomodulator that promotes strong antitumor responses in animal models (17, 18). It binds to the B7 costimulatory family member B7-DC on dendritic cells and initiates a unique activation program that leads to increased homing, antigen uptake, and secretion of interleukin-12 (15). One surprising observation was the exquisite specificity of the CTL response (Fig. 1*A*; ref. 19) raised against autologous tumors. We challenged mice with the B16 melanoma line 1 day after injection of B7-DC XAb and assessed CTL function by chromium release assay 6 days later, the CTL killed the challenge B16 tumor in a CD8-dependent manner (Supplementary

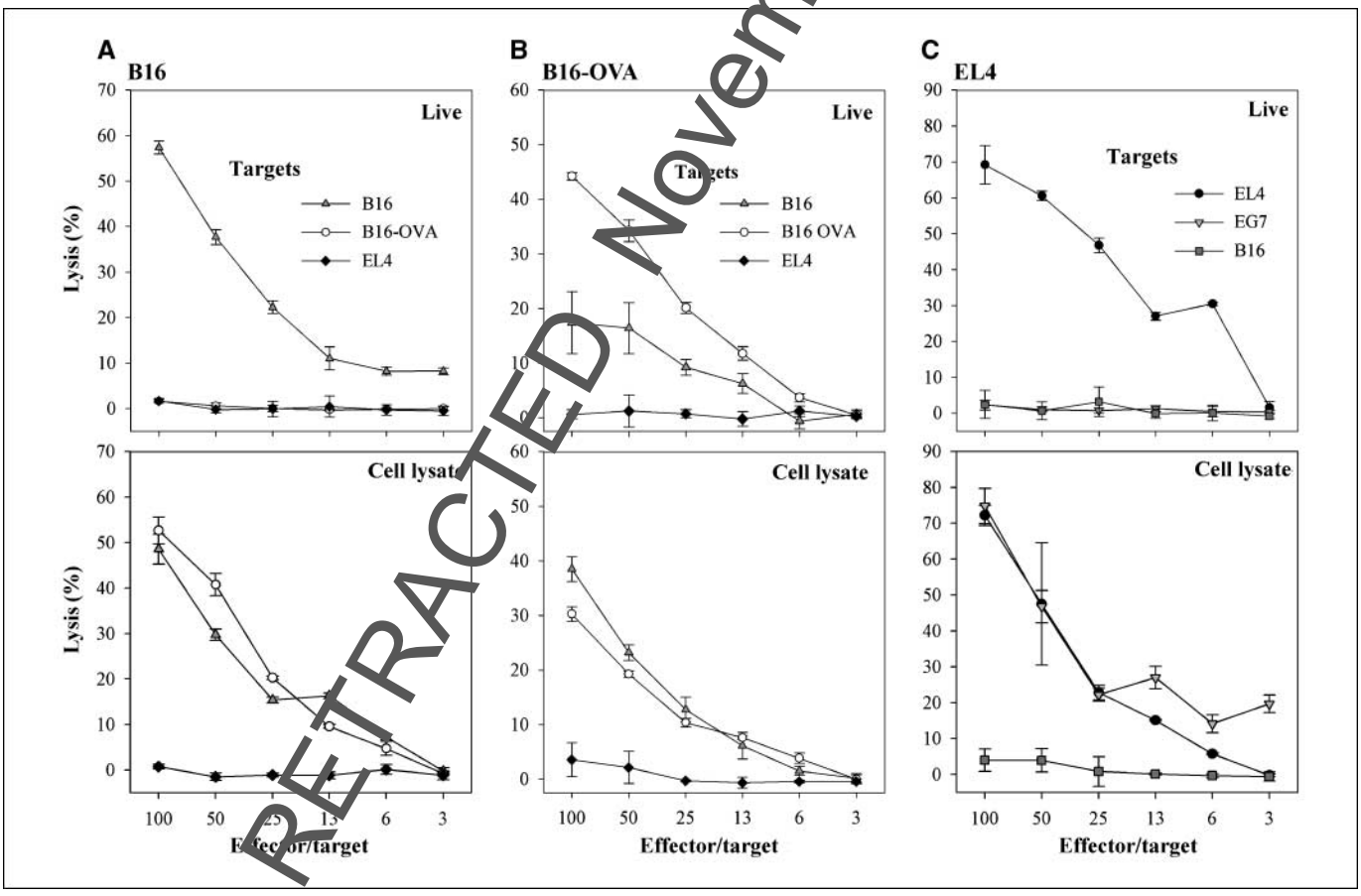

Figure 2. CTL elicited with TCLs were more broadly reactive than CTL induced with live tumor. Mice were treated i.v. with B7-DC XAb 1 day prior to injection of tumor cells or TCL. Six days after tumor or lysate challenge, draining lymph node cells were isolated and assessed by chromium release assay against the appropriate targets. *A,* live B16 immunization resulted in CTL that recognized the challenged cell line B16, but not B16-OVA or the control tumor line EL4. Immunization with B16-TCL generated CTL that recognized both B16 and B16-OVA, but not EL4. *B,* CTL induced by live B16-OVA killed B16-OVA, and to a lesser extent, B16, but not EL4. B16-OVA TCL immunization resulted in CTL that killed both B16-OVA and B16, but not EL4. *C,* live EL4 immunization resulted in CTL that killed EL4, but not EG.7 or B16. Immunization with EL4 lysate resulted in CTL that recognized both EL4 and EG.7, but not B16.

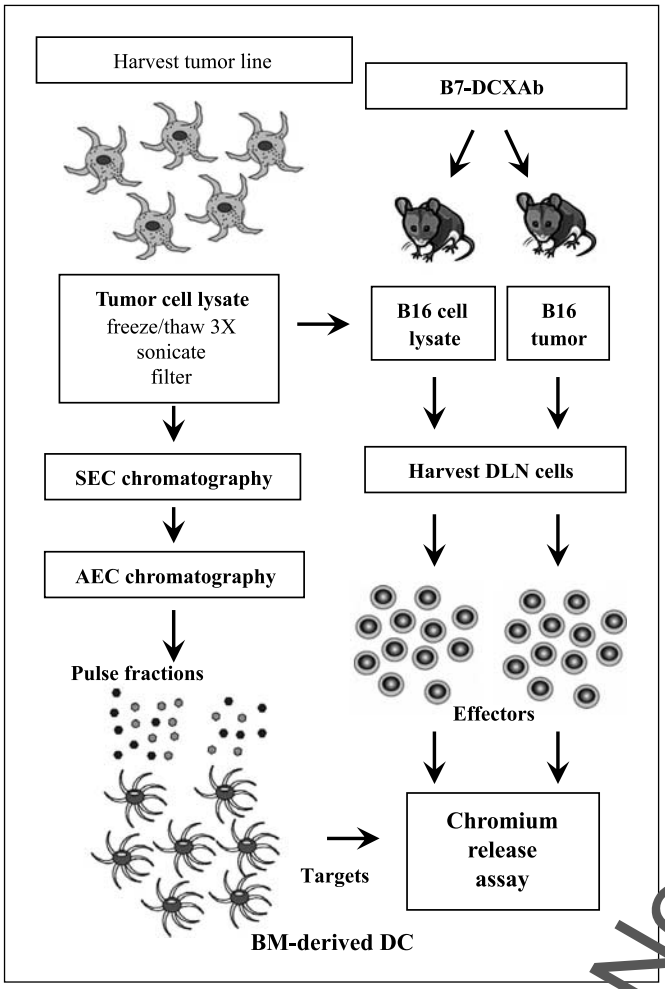

Figure 3. Strategy for fractionation of cellular lysate antigens that are targeted by CTL induced with live tumor or TCL vaccinations. B16 tumor lysates were fractionated by size-exclusion and anion exchange chromatography. These recovered fractions were pulsed onto dendritic cells to be used as targets for the two different CTL populations. CTL were generated by treating B6 mice with 30 μg of B7-DC XAb 1 day prior to tumor or B16-TCL challenge. Six days after tumor or lysate challenge, CTL from draining lymph nodes were tested for recognition of dendritic cell targets pulsed with the fractionated lysate antigens.

Fig. S1), but not the syngeneic control tumor line EL4. Remarkably, these CTL also failed to recognize the closely related tumor line B16-OVA (Fig. 1*A*). This result was unexpected because the B16-OVA line was derived from the B16 line. To evaluate whether the expression of the ovalbumen gene in the tumor may have contributed to the lack of recognition of B16-OVA by the B16-generated CTL, we transfected a subclone of B16 with the ovalbumen gene. In contrast, CTL generated using the original B16 line as a source of antigen recognized both the new subclone B16-N as well as the OVA-transfected line B16-N-OVA (Fig. 1*A*), demonstrating that expression of OVA is not the factor that discriminates the melanoma variant B16-OVA from B16.

B16 and B16-OVA are unique melanoma lines that show differential MHC class I expression and melanin synthesis. We next sought to define the basis for this unexpected specificity of tumor immunity induced against live tumor cells with the B7-DC XAb immunomodulator. One approach was to assess gene expression differences quantitatively using the Illumina Beadchip platform. We analyzed 46,120 transcripts and detected the expression of 12,518 transcripts in either the B16 or B16-OVA lines. Comparative analysis found differences in the expression of 518 transcripts (4-fold or more) confirming that the B16 and B16-OVA cell lines were in fact closely related. In contrast, the EL4 thymic lymphoma line and B16 line differed by 3,096 transcripts. The difference between B16 and B16-OVA was not due solely to ovalbumen expression because the difference between the subclone B16-N and B16-N-OVA was only 80 transcripts. The differences in gene expression between the B16 and variant B16-OVA could explain the unique antigen specificity between the lines, suggesting that an antigen subset varies between these tumor variants. The magnitude of these differences, however, is not as large as the respective differences with EL4 accounting for the antigenic distinctions between the melanomas and the EL4 lymphoma.

Because previous work has identified differences in MHC expression among sublines of B16 melanoma (23), we evaluated whether such differences might account for the observed pattern of CTL specificity. We assessed the expression of the H-2D^b and H-2K^b molecules on the surface of B16 and B16-OVA sublines used in our studies and found similar basal levels of H-2D^b expression as shown by flow cytometry (Fig. 1*B*). However, basal expression of H-2K^b seemed to be slightly higher in the B16-OVA line (mean fluorescent intensity, 5.8) compared with B16 (mean fluorescent intensity, 3.5) and this effect was further amplified in the presence of IFN-γ (Fig. 1*B*), demonstrating differential expression of H-2K^b in these two tumor lines. To verify this difference in MHC class I expression, we isolated RNA from B16 and B16-OVA in the presence or absence of IFN-γ and assessed the relative expression levels of MHC class I transcripts by real-time reverse transcription-PCR. In parallel with the flow cytometry data, no H-2K^b transcripts were detected at basal levels in the B16 line, whereas the B16-OVA line had basal levels of H-2K^b transcripts that were as high as those induced with IFN-γ in the B16 line (Fig. 1*C*). As a control, we looked at H-2D^b expression in the two lines and verified that there were no differences in noninduced or IFN-γ–induced expression of H-2D^b transcripts (Fig. 1*C*). These findings are consistent with the possibility that MHC class I expression differences contribute to the differential recognition of CTL raised using either of these lines.

Differences in class I gene expressions are not the only differences we have found in B16 and B16-OVA cell lines. A large number of B16-OVA cells express dark pigment–filled melanosomes (Fig. 1*D*), in contrast with the B16 line which express few melanosomes (Fig. 1*D*). This finding suggests that differences in the melanin synthesis pathway may be differentially regulated between these two lines. The observed differences in gene expression, as well as in melanin synthesis, provide a basis for understanding the antigenic differences that can be determined by tumor-specific CTL generated using live tumor cells as a vaccine.

Cytotoxic lymphocyte responses generated with TCL kill melanoma lines in spite of differences in MHC class I and melanin gene expression. To determine which antigens were recognized by this melanoma differentiating CTL, we generated cell lysates from the tumor lines, in anticipation of fractionating them by chromatographic methods. First, we sought to verify that the specificity of the response could be maintained with TCL. Mice were immunized with either live tumor or TCL from B16, B16-OVA, or the control line EL4 1 day after administration of i.v. B7-DC XAb. As seen previously, live tumor immunization with B16, B16-OVA, or EL4 resulted in CTL that were specific to the challenge tumor

(Fig. 2*A–C*). Immunization with B16-TCL and B7-DC XAb led to a CD8-dependent killing of B16 (Figs. S1 and S2*A*). Remarkably, these CTL also killed the B16-OVA line but did not kill the control tumor line EL4 (Fig. 2*A*). A similar result was seen when lysates of B16-OVA were used with B7-DC XAb as a vaccine (Fig. 2*B*). When EL4 lysate was used to generate CTL, both EL4 and its antigenic variant EG.7 were killed, but not B16 (Fig. 2*C*), generalizing the observation that when tumor lysate and B7-DC XAb are used as a vaccine, the generated CTL recognized related sets of tumors, but not disparate syngeneic tumor types. Antigens released by a live tumor may not be as effective at cross-protection against evolving tumor variants as are antigens present in tumor lysates, a very important finding regarding vaccine development, as tumor escape by antigenic variation is a significant problem in cancer immunotherapy (24).

Cytotoxic lymphocytes generated with live tumor or TCL recognize unique antigens. To determine the nature of the antigens recognized during live B16 or B16-TCL immunization with B7-DC XAb, we employed a fractionation strategy to identify unique lysate antigens recognized by B7-DC XAb induced CTL. This involved the development of a novel strategy using dendritic cells pulsed with TCLs as a way to monitor tumor-specific, CD8-dependent CTL responses (Fig. S2*A–D*). As outlined in Fig. 3 and in Materials and Methods, we generated TCL and fractionated them by size-exclusion chromatography and anion exchange chromatography before pulsing fractions onto dendritic cell targets. First, the 23 protein fractions were electrophoresed and stained for protein to determine whether each fraction was representative of a unique set of proteins (Fig. 4*A*). Live B16 and B16-TCL CTL were tested for their ability to lyse fraction-pulsed dendritic cells. CTL generated using live B16 recognized a unique set of antigens characterized by net negative charge (Fig. 4*B*), whereas CTL generated by B16-TCL immunization recognized a greater number of protein fractions containing broader specificity (Fig. 4*C*). The analysis revealed that antigens recognized by T cells sensitized with live tumor cells have distinct molecular properties from antigens recognized by T cells sensitized with TCL. A similar pattern was

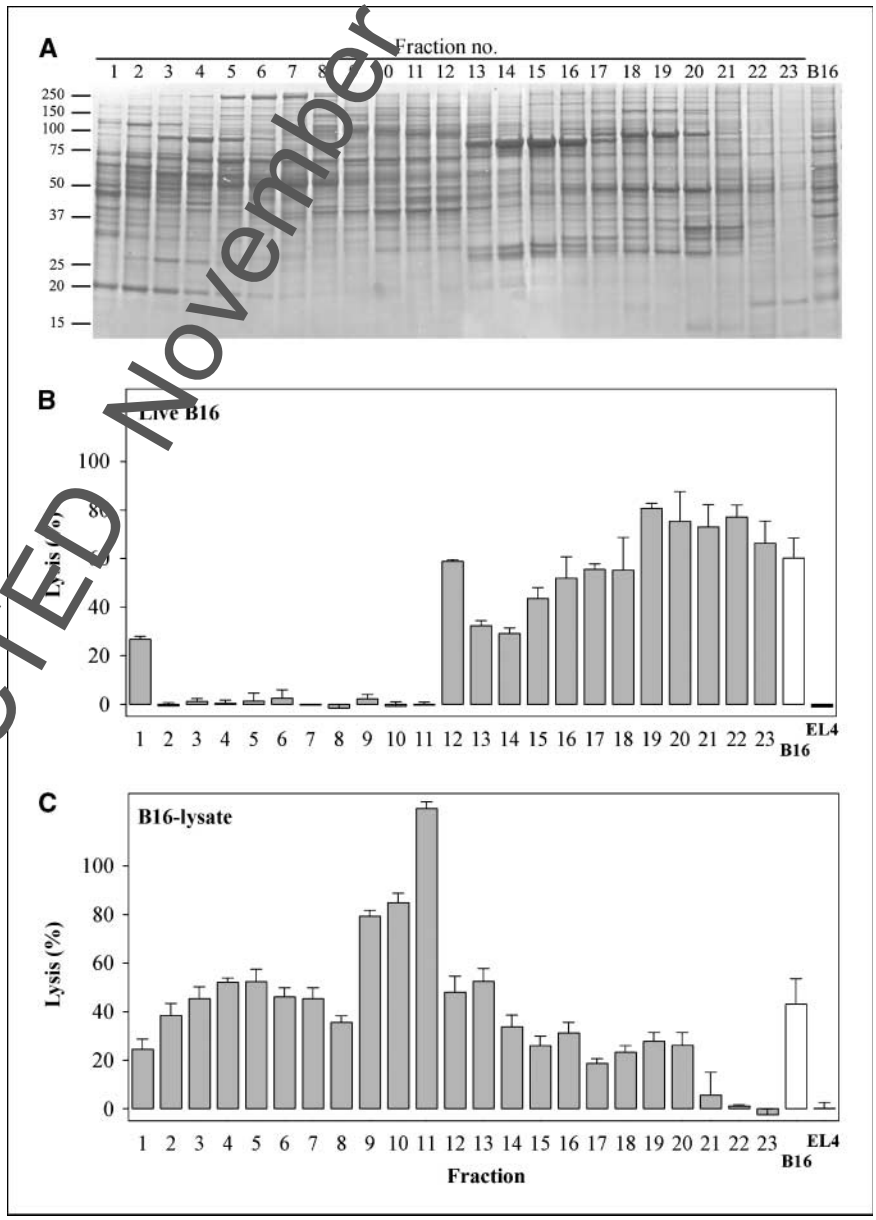

Figure 4. CTL generated with live B16 or with B16-TCL recognize different subsets of the anion exchange fractions. Dendritic cells pulsed with anion exchange fractions from B16-TCL were used as targets for live B16 or B16-TCL generated CTL. *A,* 23 distinct protein fractions from TCLs were visualized on 12% SDS-PAGE. All fractions show the presence of recovered proteins. *B,* immunization with live B16 tumor promoted CTL that recognized fractions 12 to 23. *C,* immunization with B16-TCL yielded CTL that recognized a broad spectrum of fractions ranging from 2 to 20. The results presented are representative of two independent experiments.

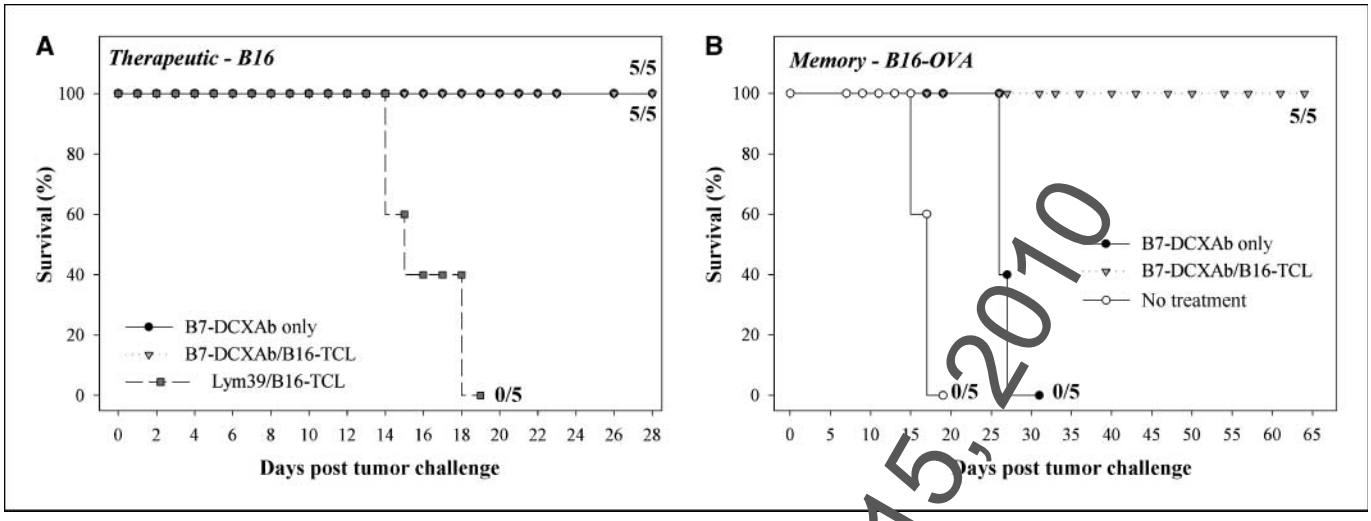

Figure 5. Immunization with B16-TCL leads to increased protection from *in vivo* challenge with B16 or B16-OVA. *A,* mice were challenged with 5×10^5 B16 6 d prior to treatment with B7-DC XAb with or without an s.c. injection of B16-TCL in the same flank. Both the live B16 (●) and the B16-TCL (▲) groups treated with B7-DC XAb remained tumor-free. Tumors did grow out of the control IgM-treated group (■), demonstrating the viability of the tumor grafts ($P < 0.05$ for both groups compared with IgM control). *B,* mice surviving initial B16 challenge (from *A*) received a secondary challenge with 5×10^5 B16-OVA cells. Survivors that were treated with B7-DC XAb alone (●) failed to clear the second challenge with B16-OVA ($P < 0.05$ compared with B7-DC XAb with TCL), although this clearance was delayed when compared with nontreated mice (○; $P < 0.05$). Mice previously treated with B7-DC XAb and B16-TCL (▲) completely cleared the secondary challenge with B16-OVA ($P < 0.05$).

seen when CTL were elicited using live EL4 and EL4 cell lysate vaccines. CTL derived from animals with live tumor grafts recognized biochemically distinct antigens in comparison to CTL derived from animals vaccinated with EL4 lysate (data not shown).

The identity of the B16-TCL–derived antigens driving the broad CTL response capable of protecting animals from the distinct melanoma variants is not known. However, CTL experiments using a panel of previously defined peptides from the B16 model ruled out any of the defined tumor antigens as relevant targets (data not shown). These included peptides derived from the melanoma differentiation antigens tyrosinase-related protein 2 ($TRP2_{180–188}$) and gp100 ($gp100_{25–33}$), as well as epitopes from the p53-interacting protein mdm-2 ($mdm2_{100–107}$ and $mdm2_{439–447}$) and the retroviral gene product p15E ($p15E_{604–611}$).

TCLs show enhanced immunotherapeutic potential against melanoma. Because B16-TCL immunization using B7-DC XAb generated CTL capable of killing B16 and B16-OVA, we wanted to verify whether this therapeutic strategy would protect against tumor growth. C57BL/6 mice were challenged with B16 6 days prior to treatment with immunomodulating antibody with or without B16-TCL. All animals in both B7-DC XAb treatment groups cleared their tumors, whereas none of the control IgM–treated animals survived past day 19 (Fig. 5*A*).

We had previously shown that mice surviving a live B16 tumor challenge in response to immunomodulation with B7-DC XAb were protected from re-challenge with B16 but not from re-challenge with B16-OVA (19). This re-challenge strategy was used to evaluate whether immunization with TCL would broaden the protective response. The two surviving B7-DC XAb–treated groups (from Fig. 5*A*) and a group of naïve mice were then challenged with B16-OVA on day 30. Tumors in the naïve group grew out rapidly with no animals surviving past 20 days (Fig. 5*B*). The animals that were challenged with live B16 and treated with the immunomodulating antibody were weakly protected from B16-OVA; tumors grew out displaying delayed growth kinetics but no animals survived past 32 days post-challenge. All tumors that grew out were verified to be OVA positive by PCR, demonstrating the absence of protective immunity against the B16-OVA variant. In contrast, mice that had been treated with B16-TCL and B7-DC XAb cleared the second B16-OVA challenge with all mice surviving past the 65-day observation period (Fig. 5*B*), demonstrating a broader protective response following treatment with the TCL and B7-DC XAb vaccine. The first B16 graft did not grow out in any of the animals treated with B7-DC XAb.

Vaccination with B16 TCL and B7-DC XAb confers extended protection against the B16 melanoma variant B78H1. To test whether treatment with TCL could protect against a more distant B16 variant, we challenged mice with the B78H1 melanoma cell line. This variant of B16 has very low MHC class I expression (Fig. 6*A*) and has lost the expression of some melanoma differentiation antigens (25). As shown previously, immunization with live B16 and B7-DC XAb generates CTL that recognize B16, however, these CTL recognize B78H1 very weakly (Fig. 6*B*). Immunization with B16-TCL generated a CTL response that recognized B78H1 with a frequency that was 8-fold lower than recognition of the B16 tumor (Fig. 6*B*). Nevertheless, we challenged mice with B78H1 that were previously immunized with either live B16 or B16-TCL. The B78H1 challenge tumor grew out of the live B16 immunized animals and no animals survived past 30 days after challenge (Fig. 6*C*). In contrast, five of five mice that were previously immunized with B16-TCL were protected from outgrowth of the B78H1 tumor. This finding shows that the antigens targeted using TCL can generate a memory response that confers increased protection even from low MHC I–expressing and antigen loss variants.

Discussion

The immune system can determine the fate of an evolving tumor. When the immune response is not sufficient to completely eradicate a developing cancer, tumor variants presenting unique sets of antigens emerge (26, 27). We find that living tumor cells interact with the immune system in a way that involves the

activation of CTL by only a subset of antigens, and that a second set of antigens displayed by the tumors does not elicit protective cellular immunity. This second set of antigens, however, can be targeted efficiently by activated T cells in an immunotherapy setting when the antigens are presented in an alternative form such as using TCLs. Our findings show that immune responses focused on these shared antigens provide effective immunotherapeutic benefit.

A protective immune response against both melanoma and lymphoma can be generated rapidly with the immunomodulator B7-DC XAb (19). In control-treated groups, these tumors progressively grow and overwhelm the animals; sufficient antigen and immune repertoire are available for the eradication of these tumors, but only following effective immunomodulation. The protective response elicited by three B16 melanoma variants was distinct because the CTL generated could only kill the live challenge tumor and the memory response in protected animals was specific for the eliminated tumor variant. Remarkably, treatment of 6-day established tumor grafts of this very aggressive tumor led to tumor regression and clearance, demonstrating the potency of the available immune repertoire against these tumors. These findings show that an immune response capable of clearing the tumor can be primed in a relatively short time and that this response is fully capable of clearing the tumor when appropriate modulation of the immune system occurs. The ability to observe rapidly generated tumor-specific CTL provided by the use of the immunomodulator B7-DC XAb in these experiments raises the question of whether the observed specificity is the result of using this new reagent or whether an underlying fundamentally important relationship between the tumor and host is being revealed. Our view is that B7-DC XAb licenses CTL activity in T cells that are available at the time of treatment, without inducing substantial expansion of the T cell repertoire (19). Therefore, our bias is that we are revealing a fundamental relationship between tumor and host and have shown that some available tumor antigens are naturally recognized by the immune system, whereas others are ignored.

Because antigenically distinct tumor variants evolve in individuals with cancer, it would be useful to identify which antigens are targets mediating selection and which antigens are less likely to change in response to immune selection by the host immunity. As the melanoma and lymphoma tumor variants show a unique antigenic profile when used as live tumor vaccines, we chose to investigate TCLs as potential sources of antigen for therapy. We found that immunization with TCL could be used to target sets of antigenically related tumors. Furthermore, our *in vivo* studies show an increased efficacy of B7-DC XAb therapy with the use of tumor lysates to prime the immune system. Efficient priming occurs even in the presence of an established tumor because tumor variants could be cleared by a recall response after eradication of the antigenically distinct line. This shows that antigens found in a TCL generate a response that is effective against tumors that have different antigenic profiles.

There is a dichotomy in the antigens recognized by the immune system when they derive from live tumor or from TCL.

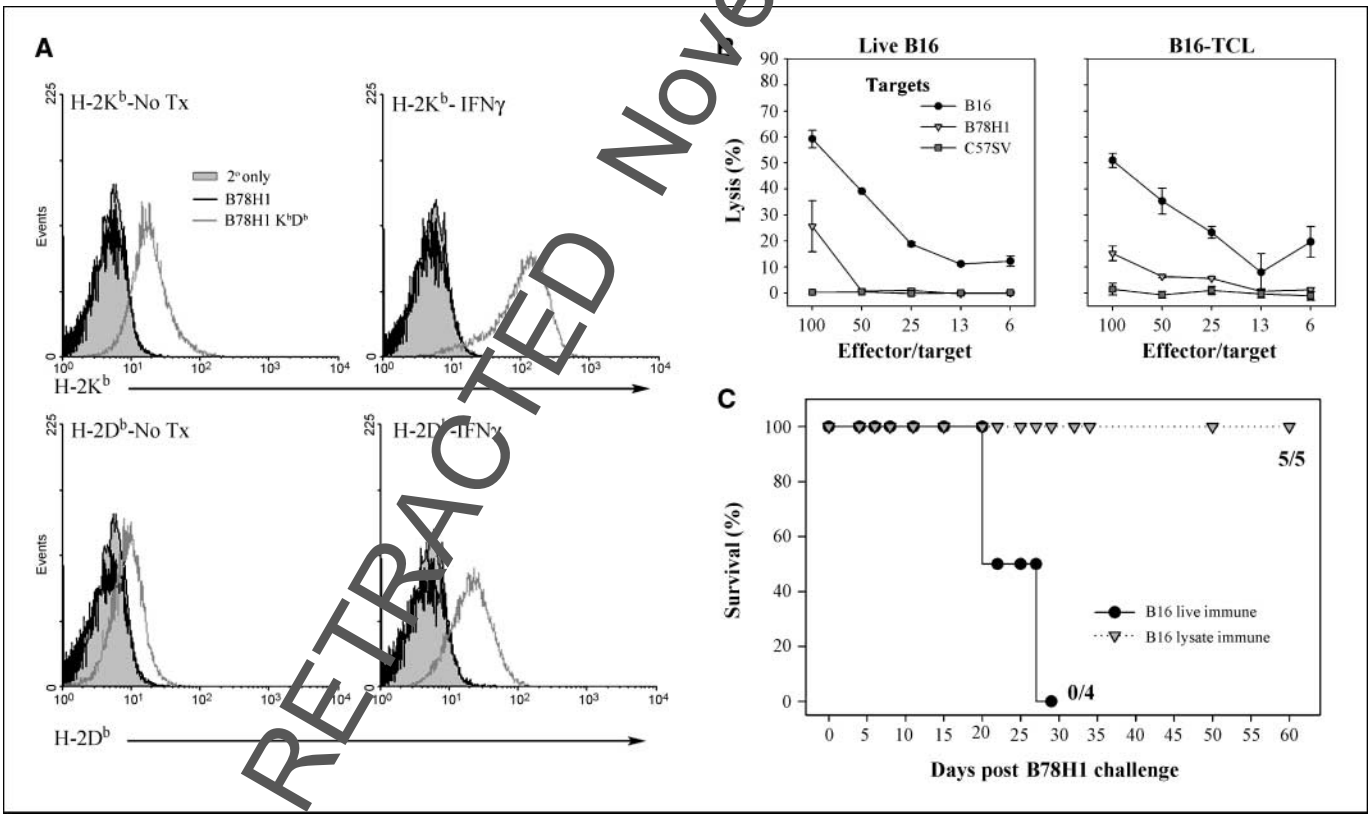

Figure 6. The low MHC I–expressing B16 variant B78H1 is cleared from mice vaccinated with B16-TCL and B7-DC XAb. H-2K^b and H-2D^b expression in the B78H1 and the MHC I–expressing cell line B78H1 K^{b}D^b was assessed by flow cytometry. *A,* H-2K^b and H-2D^b expression was absent from the B78H1 cell line as compared with secondary challenge alone and the B78H1 K^{b}D^b line. MHC I expression was up-regulated in response to IFN-γ in the B78H1K^{b}D^b line; in contrast, the B78H1 line does not express either H-2K^b or H-2D^b under these conditions. *B,* CTL generated with live B16 and B7-DC XAb recognizes B16, but has limited recognition of B78H1. B16-TCL– and B7-DC XAb–generated CTL recognize B16 and reduced the recognition of B78H1. *C,* vaccination with B16-TCL and B7-DC XAb protects against challenge with the B16 variant B78H1 ($P < 0.05$).

Fractionation of TCL provides the opportunity to study both classes of antigen. Biochemical fractionation of lysates by charge for use as cross-presented antigen on dendritic cell targets shows two key points. First, the antigens that prime immune responses to live tumor are present in the TCL in sufficient quantities to be processed and presented by dendritic cells. Second, the antigens recognized by live tumor–primed T cells are restricted to a subset of antigens that elutes from anion exchange with characteristics of molecules having increased negative charge. In contrast, TCL-primed T cells recognize a broader repertoire of antigens demonstrating recruitment of a broad spectrum of T cell clones. Furthermore, some of these antigens are recognized exclusively by TCL-primed T cells. These antigens represent a class of antigens that did not sensitize the immune system during live tumor growth but could be used to convey protection across a broad spectrum of antigen variant tumors.

An additional explanation for the increased repertoire of CTL induced with TCL is that TCL antigens are processed and presented on MHC class II elicit an increased CD4 T helper cell response. We have not analyzed this question directly. Our previous findings using live tumor vaccines showed that tumor clearance and CTL induction (18, 19) are both CD4-dependent. Because the CD4-dependent response to live vaccine is the more antigenically focused response, we do not think that the major differences determining the broad CTL repertoire induced by TCL vaccines is the availability of CD4 help. However, at this point, differences in the quality of CD4 help cannot be ruled out.

These findings provide insight into how the immune system interacts with tumors or TCL. Little is known about what antigens are available from a live tumor and how these tumor antigens access the immune system. Dendritic cells at the site of the tumor may interact directly with a tumor and acquire antigen locally before migration to the lymph node and development of antitumor immunity. The characteristics of these antigens are unknown. However, our data suggest that there are biochemically distinct entities that preferentially gain access to the immune system. The fact that negatively charged molecules are targeted suggests that heavily glycosylated molecules that may be secreted by the tumor could serve as antigens or as suppressors of the immune system and in the right context could become immune-targeting antigens. Alternatively, tumor constituents released in the form of membrane-bound exosomes (28) or secreted molecules (29, 30) may preferentially gain access to the lymphatic system, in which they could drain directly to the lymph node and interact with T cells in a tolerogenic or proinflammatory environment. These findings will be important in future considerations for the design of vaccines used for immunotherapy. Vaccine design must not only focus on the ability of an antigen to be processed for presentation to MHC class I but must also consider the biochemical makeup and function of the antigen in the tumor as these properties may determine their ability to be recognized by the immune system.

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