Cross-presentation of glycolipid from tumor cells loaded with α-galactosylceramide leads to potent and long-lived T cell–mediated immunity via dendritic cells

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We report a mechanism to induce combined and long-lived CD4$^+$ and CD8$^+$ T cell immunity to several mouse tumors. Surprisingly, the initial source of antigen is a single low dose of tumor cells loaded with α-galactosylceramide (α-GalCer) glycolipid (tumor/Gal) but lacking co-stimulatory molecules. After tumor/Gal injection intravenously (i.v.), innate NKT and NK cells reject the tumor cells, some of which are taken up by dendritic cells (DCs). The DCs in turn cross-present glycolipid on CD1d molecules to NKT cells and undergo maturation. For B16 melanoma cells loaded with α-GalCer (B16/Gal), interferon γ–producing CD8$^+$ T cells develop toward several melanoma peptides, again after a single low i.v. dose of B16/Gal. In all four poorly immunogenic tumors tested, a single dose of tumor/Gal i.v. allows mice to become resistant to tumors given subcutaneously. Resistance requires CD4$^+$ and CD8$^+$ cells, as well as DCs, and persists for 6–12 mo. Therefore, several immunogenic features of DCs are engaged by the CD1d-mediated cross-presentation of glycolipid-loaded tumor cells, leading to particularly strong and long-lived adaptive immunity.

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Abbreviations used: α-GalCer, α-galactosylceramide; B16/Gal, B16 melanoma cells loaded with α-GalCer; DC/Gal, DCs loaded with α-GalCer; DCT, dopachrome tautomerase; DTR, diphtheria toxin receptor; MNC, mononuclear cell; TAP, transporter associated with antigen presentation; TRP-2, tyrosinase-related protein 2; tumor/Gal, tumor cells loaded with α-GalCer.

A major challenge to tumor immunology is to identify and elicit protective mechanisms that parallel the more powerful immune responses that are seen in other settings, such as resistance to infection and rejection of transplants (1). However, it has been difficult to induce strong immunity to tumors, particularly T cells that recognize multiple antigens in tumor cells and differentiate to express helper and killer functions, as well as memory.

One way to improve T cell–based resistance to tumors is to expand tumor-reactive lymphocytes to large numbers ex vivo and then reinfuse them back into patients. Measurable resistance develops after such passive immunotherapy with CD8$^+$ T cells in humans (2–4). Another approach is the active induction of resistance to tumors in vivo. Certain chemotherapies can elicit some immunity to tumors, because the dying tumor cell is both recognized by DCs and matures the DCs to induce immunity, although the tumor resistance is partial (5, 6). Stronger resistance can be induced when DCs take up irradiated tumor cells injected i.v.; then, the DCs are matured through activated NKT lymphocytes or by a combination of an agonistic anti-CD40 antibody and a ligand for Toll-like receptors (7). However, this pathway requires large numbers of tumor cells, which for some tumors like the B16 melanoma, is toxic. It is important to identify pathways that allow tumor cells to more efficiently induce long-lived, specific, T cell–mediated immunity.

In this paper, we report a mechanism that induces potent T cell immunity to several poorly immunogenic, transplantable mouse tumors, including the B16 melanoma. The mechanism depends on the capacity of DCs in the spleen to take up and cross-present dying cells (8). Previous research has emphasized the cross-presentation of protein antigens onto MHC class I products permitting the generation of CD8$^+$ killer T cells (7, 9). We now describe the cross-presentation of glycolipids on CD1d molecules

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in vivo. Cross-presentation of glycolipids has been noted in one previous study in tissue culture (10), but in vivo consequences could not be assessed. We find that the i.v. administration of either live or irradiated tumor cells, which have been loaded with the agonistic glycolipid α-galactosylceramide (α-GalCer), first elicits an innate NKT and NK cell response that rejects the tumor, including cells that have been administered 3 d previously (11). This approach, even though it involves a single low dose of tumor cells lacking in co-stimulatory molecules, leads to a second NKT cell response to glycolipid cross-presented by DCs. The DCs then mature and induce combined $CD4^+$ and $CD8^+$ protective T cell immunity to the captured tumor cells.

RESULTS

Vaccination with B16 melanoma cells loaded with α-GalCer (B16/Gal) induces T cell–dependent antitumor resistance

The mouse B16 melanoma expresses little or no MHC products or co-stimulatory molecules (e.g., CD40 and CD86) and is poorly immunogenic. When injected s.c. into syngeneic C57BL/6 mice, it grows rapidly and overcomes all animals (Fig. 1 A). We evaluated whether protection against B16 could be induced by an injection of DCs loaded with α-GalCer (DC/Gal), because we had reported that DC/Gal can activate NK and NKT cells and protect against metastasis of i.v.-injected B16 to the lung (12, 13). However, mice given DC/Gal did not protect against B16 challenge s.c. (Fig. 1 A) or challenge with EL4 thymoma s.c. (not depicted). This was also the case for s.c.-injected B16/Gal and with tumor cells that had been transfected to express high levels of CD1d and loaded with α-GalCer ($CD1d^{hi}$-B16/Gal; Fig. 1 B).

Unexpectedly, if we first injected mice with live B16/Gal i.v. and then challenged the mice with B16 tumor cells s.c. 2 wk later, 6 out of 10 animals now showed resistance (Fig. 1 C). If we injected transfected $CD1d^{hi}$-B16/Gal i.v., all the mice became resistant to a challenge with B16 tumor s.c (Fig. 1 D, left). This resistance proved to be T cell mediated. Thus, $CD4^{-/-}$ and $CD8^{-/-}$ mice did not develop resistance to a subsequent s.c. challenge with B16 tumor cells (Fig. 1 D, right), although the knockout mice effectively resisted the growth of the initial inoculum of living $CD1d^{hi}$-B16/Gal tumor cells given i.v. by innate mechanisms, as shown previously (11). The finding that lung metastases were still inhibited when B16/Gal or $CD1d^{hi}$-B16/Gal were administered

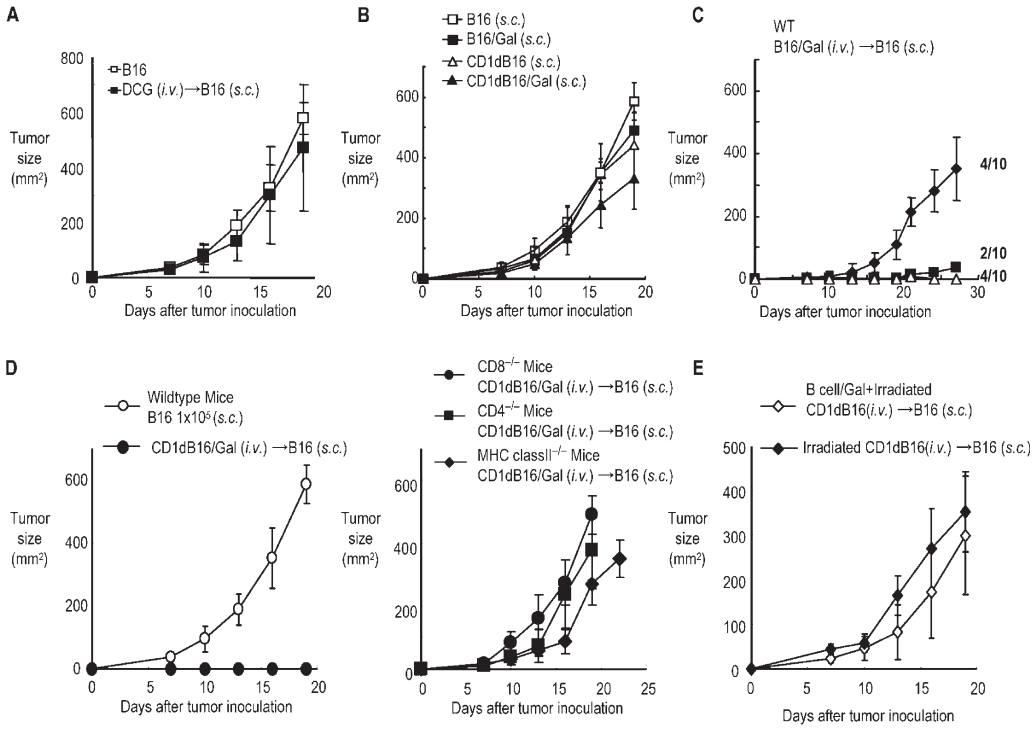

Figure 1. Vaccination with B16/Gal induces T cell–dependent antitumor resistance. (A) 10^6 α-GalCer–loaded DCs per mouse were administered to mice. 10^5 B16 melanoma tumor cells were injected s.c. 2 wk later. Measurement of tumor size was done at the indicated time points ($n = 5$ per group). Similar results were obtained in two independent experiments. (B) 10^5 B16/Gal or nonloaded B16 melanoma cells per mouse were administered s.c., and tumor size was followed ($n = 5$ per group). Similar results were obtained in two independent experiments. (C and D, left) 5×10^5 α-GalCer–loaded B16 or $CD1d^{hi}$-B16 tumor cells per mouse were given i.v., and 2 wk later mice were challenged with 10^5 parental B16 tumors s.c. ($n = 10$ per group). Similar results were obtained in two independent experiments. (D, right) At 2 wk after vaccination of $CD4^{-/-}$, $CD8^{-/-}$, and MHC $II^{-/-}$ mice with 5×10^5 $CD1d^{hi}$-B16/Gal i.v., the mice were challenged s.c. with B16 tumor cells ($n = 5$ per group). Similar results were obtained in two independent experiments. (E) $CD19^+$ B cells were isolated with magnetic beads from the spleen, coated with α-GalCer, and injected together with irradiated $CD1d^{hi}$-B16 cells ($n = 5$ per group). Data are means ± SEM. Similar results were obtained in two independent experiments.

to $CD4^{-/-}$ mice was surprising, in that $CD4^+$ NKT cells seemed dispensable for providing innate immunity. The data may be consistent with Crowe et al., who reported that $CD4^-$ NKT cells showed stronger antitumor immunity than $CD4^+$ NKT cells (14). However, $CD4^-$ NKT cells in $CD4^{-/-}$ mice may include cells that are equivalent to $CD4^+$ NKT cells in WT mice. Therefore, we also tested whether antitumor effects develop in MHC $II^{-/-}$ mice that would not have conventional $CD4^+$ T cells. Fig. S1 (available at http://www.jem.org/cgi/content/full/jem.20070458/DC1) shows that MHC $II^{-/-}$ mice had $CD4^+$ CD1d-dimer$^+$ NKT cells and some $CD4^+$ CD1d-dimer$^-$ T cells (15, 16). The latter may include T cells that are not MHC II restricted, as well as some type 2 NKT cells or MR1-restricted cells. When we gave tumor cells loaded with α-GalCer (tumor/Gal) to MHC $II^{-/-}$ mice, resistance to B16 melanoma did not develop (Fig. 1 D, right), although the mice showed delayed rejection of ∼1 wk, presumably caused by the residual $CD4^+$ T cells (Fig. S1). In contrast, as shown in Fig. 1 D, $CD4^+$ T cells were required to induce adaptive antitumor immunity to s.c. challenge with tumor. The lack of adaptive resistance of $CD4^{-/-}$ mice to tumor administered s.c. differs from our previous study showing innate resistance to α-GalCer–loaded tumor cells given i.v. (11). With the i.v. route, transplantable tumors, including B16 melanoma, are rejected by innate NKT cells (especially $CD4^-$ NKT cells) and NK cells without the need for adaptive $CD4^+$ or $CD8^+$ T cell immunity (11). Collectively, the results, which show the induction of protective T cell immunity to B16 melanoma, were surprising because it has been so difficult to raise T cell responses to this tumor, especially with a single and low dose of tumor cells.

Finally, to demonstrate that the α-GalCer needed to be loaded on the tumor cells and could not be transferred from or induced by α-GalCer on other cells, we injected a mixture of α-GalCer–loaded, splenic B cells plus irradiated $CD1d^{hi}$-B16 cells that were not loaded with α-GalCer. Mice given irradiated $CD1d^{hi}$-B16 cells alone did not develop resistance, and this was also the case when mice were given α-GalCer–loaded B cells plus irradiated $CD1d^{hi}$-B16 cells (Fig. 1 E). The latter data indicate that α-GalCer does not elute in sufficient amounts to coat $CD1d^{hi}$-B16 cells, in contrast to tumor

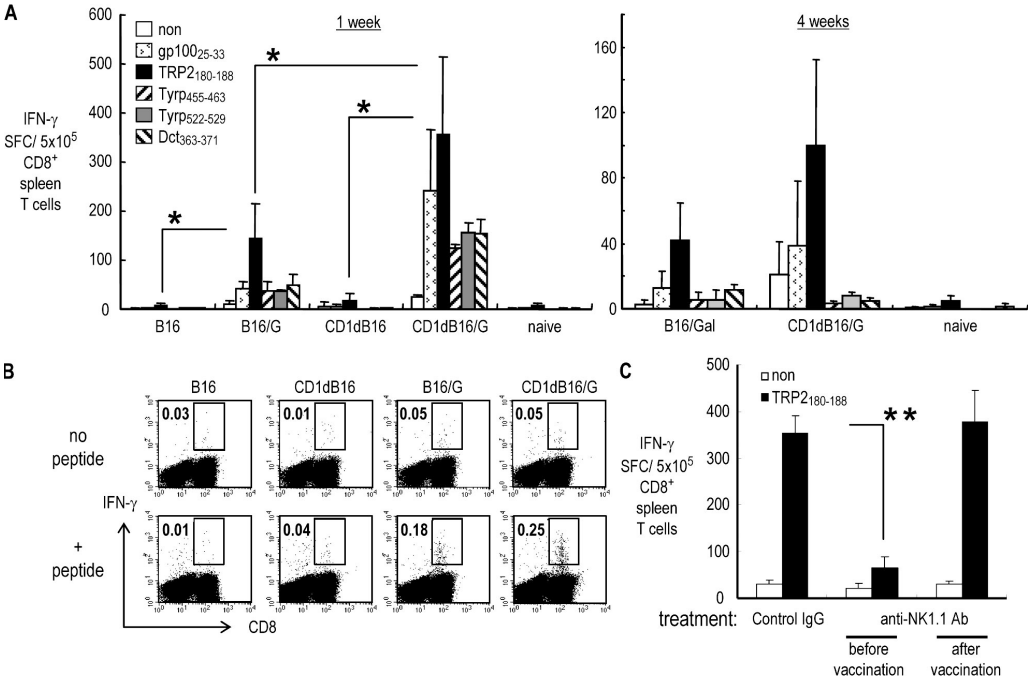

Figure 2. Vaccination with B16-GalCer tumor cells i.v. leads to T cell immunity to several defined melanoma differentiation antigens. (A) Mice were injected i.v. with 5×10^5 B16, B16/Gal, $CD1d^{hi}$-B16, or $CD1d^{hi}$-B16/Gal. To monitor antigen-specific T cell responses 1 and 4 wk later, $CD8^+$ T cells were positively selected from the spleen and were cultured with splenic $CD11c^+$ DCs from naive mice for 36 h. The DCs had been cultured for 2 h in the presence or absence of 10 μM $gp100_{25-33}$, $Trp2_{180-188}$, $Tyrp_{455-463}$, $Tyrp_{522-529}$, or $Dct_{363-371}$ peptides. T cells responding to the DCs were detected by ELISPOT assay for IFN-γ production. All data are means ± SEM obtained from three independent experiments with two mice per group. *, $P < 0.05$ for B16/G versus $CD1d^{hi}$-B16/G. (B) As in A, but the immune responses were monitored with intracellular cytokine staining. $CD8^+$ T cells were magnetically isolated from the spleen 7 d after i.v. $CD1d^{hi}$-B16/Gal. The $CD8^+$ T cells were co-cultured for 16 h with splenic $CD11c^+$ DCs from naive mice in the presence of brefeldin A, which had been pulsed with 10 μM $Trp2_{180-188}$ peptide for 2 h, for the last 10 h. Numbers indicate the percentage of total $CD8^+$ cells. The data are representative of three independent experiments ($n = 4$ per group). (C) Mice were immunized with 5×10^5 $CD1d^{hi}$-B16/Gal and depleted $NK1.1^+$ cells by injections of 300 μg of anti-NK1.1(PK136) antibody per mouse at days −1, 3, and 5 (before vaccination), or days 3 and 5 (after vaccination). As in A, the TRP2-specific $CD8^+$ T cell response was detected by ELISPOT assay for IFN-γ at day 7. Data are means ± SEM obtained from three independent experiments ($n = 4$ per group). **, $P < 0.01$.

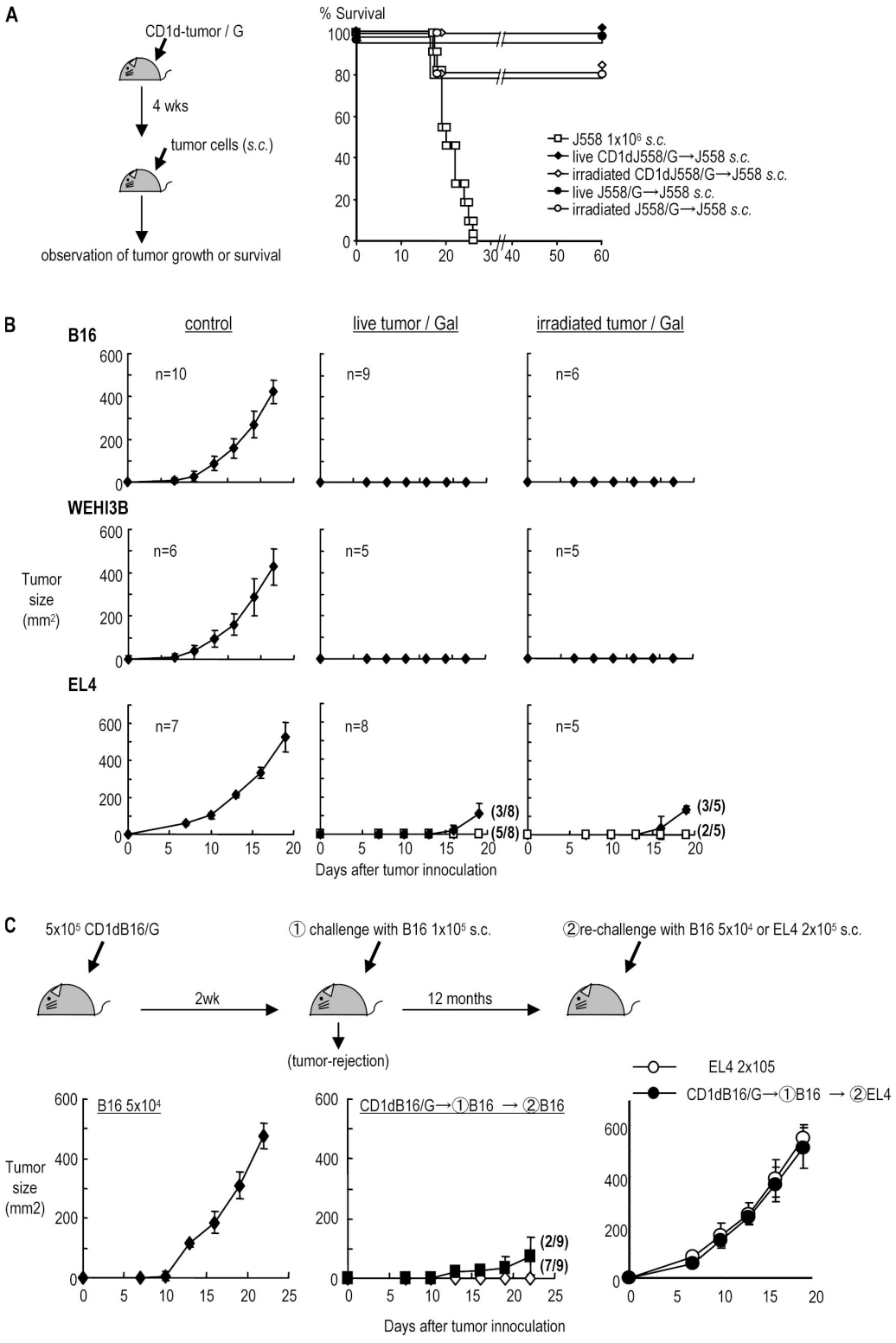

Figure 3. Vaccination with live and irradiated tumor/Gal induces antitumor protection against several mouse tumors. (A, left) Immunization experiments were set up as shown; i.e., mice were vaccinated with 5×10^6 of different forms of J558 myeloma cells, challenged at 4 wk with 10^6 live J558 s.c., and evaluated for their survival ($n = 10$ per group). Similar results were obtained in two independent experiments. (B) Mice were injected i.v. with 5×10^5 live and irradiated CD1d^{hi}-B16/Gal ($n = 9$ and 6 per group, respectively), 2×10^6 CD1d^{hi}-WEHI-3B/Gal ($n = 5$ per group), or 5×10^5 CD1d^{hi}-EL4/Gal ($n = 8$ and 5 per group, respectively). The mice were challenged s.c. with parental tumor cells (5×10^4 B16, 2×10^5 WEHI-3B, or 2×10^5 EL4) 4 wk later. Tumor sizes were measured at the indicated time points. Similar results were obtained in two independent experiments. (C) Mice were

cells directly loaded with α-GalCer (Fig. 1 D). Collectively, the results in Fig. 1 indicate that α-GalCer–loaded tumor cells in relatively small numbers are able to induce T cell resistance to an s.c. challenge with B16 melanoma.

Vaccination with B16/Gal i.v. induces T cells specific for defined melanoma differentiation antigens

Another manifestation of the weak immunogenicity of B16 melanoma is the difficulty in detecting responses to defined melanoma antigens by ELISPOT and intracellular cytokine staining assays. However, when we injected CD1d^{hi}-B16/Gal tumor cells and looked for CD8$^+$ T cells specific for melanoma antigens 1 wk later, we could detect IFN-γ–producing cells responsive to several different MHC class I–binding, nonamer peptides; these included peptides from the gp100, tryrosinase-related, and dopachrome tautomerase (DCT)/tyrosinase-related protein 2 (TRP-2) antigens (Fig. 2 A, left) (17, 18). Some of these cytokine-producing T cells persisted for 4 wk (Fig. 2 A, right). After immunization with B16/Gal, we also detected IFN-γ–producing CD8$^+$ cells, but in smaller numbers than CD1d^{hi}-B16/Gal (Fig. 2 A). We additionally documented TRP-2–specific, CD8$^+$ T cells using an intracellular cytokine staining assay after injection of B16/Gal or CD1d^{hi}-B16/Gal but not tumor cells lacking α-GalCer (0.16 ± 0.04, 0.22 ± 0.06, and ≤0.05 in B16/Gal, CD1d^{hi}-B16/Gal, and others, respectively; Fig. 2 B). Again, even if we completely deleted NK1.1$^+$ cells by anti-NK1.1 antibody treatment 3 and 5 d after vaccination, T cell immunity developed to tumor/Gal; however, if we deleted NK1.1$^+$ cells before immunization, T cell immunity was almost abolished (Fig. 2 C). These results indicate that once T cell immunity was initiated, the mice no longer needed NK1.1$^+$ cells. The detection of melanoma-specific CD8$^+$ T cell responses with a single dose of α-GalCer–modified tumor cells was of some interest given previous difficulties in eliciting immunity to this particular tumor.

Several α-GalCer–loaded tumors injected i.v. establish resistance to native tumor

To extend the analysis to other tumors, we studied the J558 plasmacytoma. J558 also grew rapidly when injected s.c., and the mice did not survive (Fig. 3 A, open squares). However, if we first injected the tumor or its CD1d transfectant i.v. (each loaded with α-GalCer), then the animals developed resistance to an otherwise lethal s.c. challenge with J558 (Fig. 3 A). We next compared the B16 melanoma in Figs. 1 and 2 with the EL4 thymoma and WEHI-3B acute myelomonocytic leukemia, each of which grew aggressively in mice after s.c. inoculation (Fig. 3 B). We had previously shown that these α-GalCer–coated tumors elicited strong NKT cell–dependent innate resistance (11). 4 wk after injecting α-GalCer–loaded, live, or irradiated CD1d^{hi} tumors i.v., we challenged the mice with tumor cells injected s.c. but without any loading with α-GalCer. In each case, the mice were resistant to s.c. challenge of the original tumor, which was otherwise lethal (Fig. 3 B). We also vaccinated WT, CD4$^{-/-}$, and CD8$^{-/-}$ mice with 5×10^5 CD1d^{hi}-EL4/Gal i.v. and challenged the mice 2 wk later s.c. with 2×10^5 parental EL4 tumor cells; the WT mice were immune, but the T cell–depleted mice were not (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20070458/DC1). In the case of B16, we rechallenged the mice with B16 or EL4 at 12 mo after rejecting tumor. Again, such mice showed the protection against B16 tumors but not EL4 tumors, indicating the establishment of protective long-term memory in a tumor-specific manner (Fig. 3 C). We likewise observed protection for 1 yr when we vaccinated with CD1d^{hi}-J558/Gal tumor (Fig. S3). Therefore, several tumors can induce resistance when exposed to α-GalCer and injected i.v. in relatively low doses into mice.

α-GalCer–loaded tumor cells are superior inducers of T cell immunity to DCs injected i.v.

Because the immune response to a single dose of co-stimulator–poor tumor/Gal was strong relative to the existing literature, we further compared tumor/Gal with DC/Gal. OVA was used as a surrogate antigen, because this provided a highly sensitive readout for CD8$^+$ T cell immunity caused by the efficacy with which the SIINFEKL peptide from OVA is presented on H-2K^b MHC class I molecules. EL4 or CD1d^{hi}-EL4 tumor cells were exposed to an osmotic shock in the presence of 10 mg/ml OVA protein (resulting in ∼100 ng OVA protein per 10^6 cells, as measured by ELISA; Morinaga Institute of Biological Science, Inc.). To monitor the processing and presentation of the tumor cells on MHC class I and II, we first adoptively transferred CFSE-labeled OT-I and OT-II OVA-specific TCR transgenic T cells as reporters, and then injected 2×10^6 α-GalCer–loaded EL4(OVA) or CD1d^{hi}-EL4(OVA) cells (Fig. 4 A, top). The injection of tumor cells induced strong proliferation in both CD4$^+$ and CD8$^+$ T cells, and the up-regulation of the CD25 high affinity receptor for IL-2 (mean of CD25$^+$-proliferated T cells = 1.65, 22.65, 2.74, and 59.8% in EL4(OVA), EL4(OVA)/Gal, CD1d^{hi}-EL4(OVA), and CD1d^{hi}-EL4(OVA)/Gal, respectively; Fig. 4 A, top). For CD8$^+$ OT-I cells, stimulation of cell division was dependent on the presence of transporter associated with antigen presentation (TAP) molecules in the recipient mice (Fig. 4 A, bottom). This indicates that injected EL4(OVA) cells were cross-presented by TAP-expressing antigen-presenting cells in the host. To implicate DCs during cross-presentation, we depleted DCs using a transgenic mouse in which the CD11c promoter drives expression of the diphtheria toxin receptor (DTR); this allows depletion of CD11c-rich DCs after injection of DT and eliminates cross-presentation

immunized and challenged as indicated in the diagram (n = 5 B16 or EL4; n = 9 tumor/Gal-B16 –EL4). Data are means ± SEM. Similar results were obtained in two independent experiments.

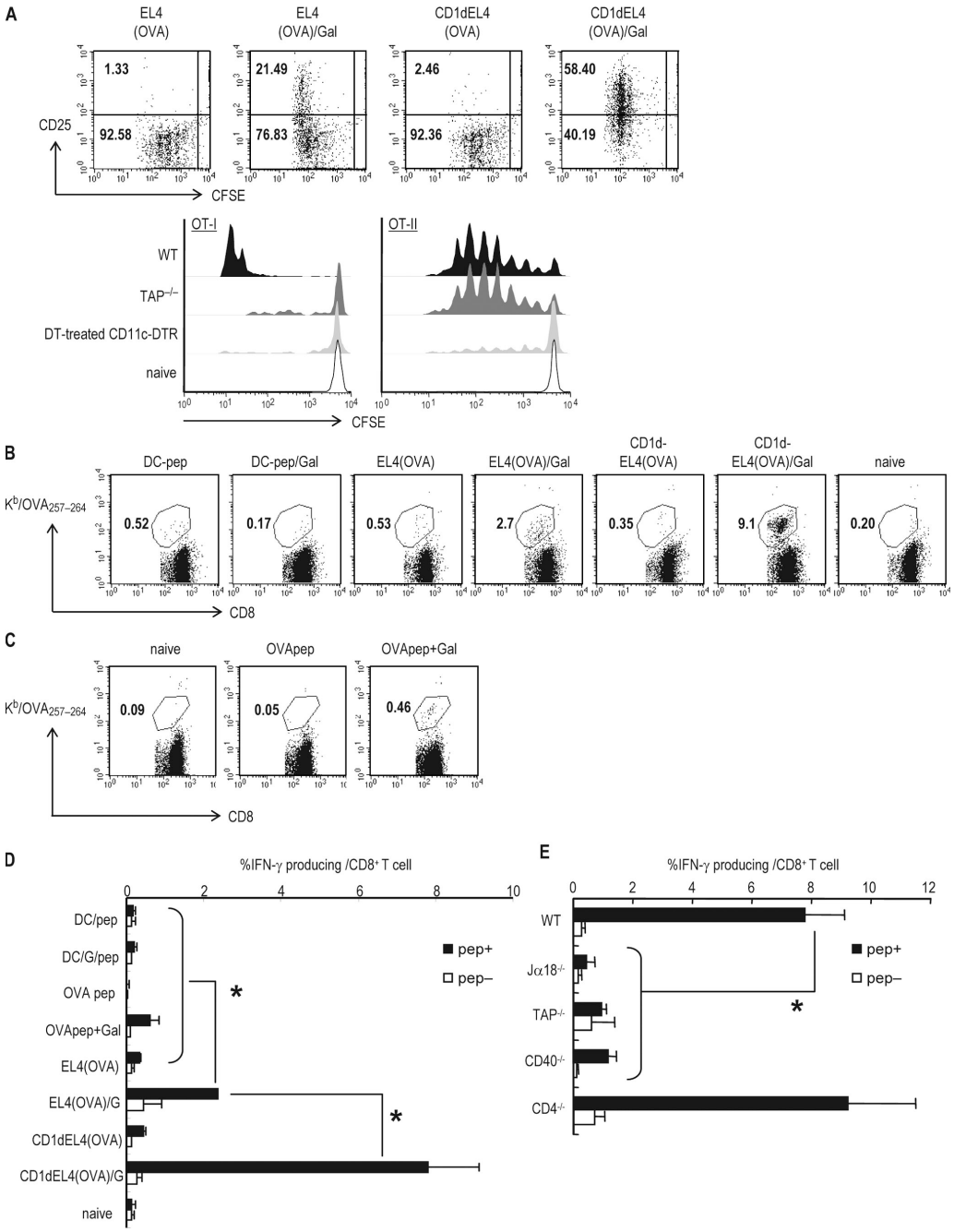

Figure 4. DCs are less effective than tumor/Gal in presenting OVA antigen when injected via the i.v. route. (A) Mice were adoptively transferred with 2×10^6 CFSE-labeled OT-I cells and were immunized the next day with EL4(OVA) or CD1d^{hi}-EL4(OVA) ± Gal. 3 d later, the animals were tested for proliferation of transferred OT-I cells as well as CD25 (top). Data are representative of two separate experiments ($n = 2$ per group). Different strains of C57BL/6 mice (bottom) were adoptively transferred with 2×10^6 CFSE-labeled OT-I cells or OT-II T cells and then challenged with 2×10^6 CD1d^{hi}-EL4(OVA)/Gal cells i.v. 3 d later, cell proliferation was monitored in the spleen by CFSE dilution (bottom). Naive indicates OT-I or OT-II cell-transferred, but not immunized, mice. The numbers indicate CD25$^+$ (top) or CD25$^-$ (bottom) differentiated OT-I cells. Data are representative of three separate experiments ($n = 3$ per group). (B) As in A, but now the immune response was measured in naive and immunized mice using H-2K^b/OVA tetramers 7 d later. Mice were injected i.v. with 2×10^6 BM-derived DCs that had been pulsed with 1 μM $OVA_{257-264}$ peptide in the absence or presence of 100 ng/ml α-GalCer. The expansion of OVA-specific CD8$^+$ T cells was compared with different forms of 2×10^6 EL4 tumors that were loaded with OVA (see Materials and methods) in the absence or presence of α-GalCer. Data are representative of four separate experiments ($n = 4$ per group). (C) The expansion of OVA-specific CD8$^+$ T cells was shown. Mice were immunized with 100 μg OVA peptide per mouse with or without 1 μg of free α-GalCer i.v. per mouse (reference 20). Data are representative of four separate experiments ($n = 4$ per group). Gated cells in B and C indicate CD8$^+$ K^b/$OVA_{257-264}^+$ double-positive cells using K^b/$OVA_{257-264}$ tetramer–PE and CD8-FITC. (D) As in B, but the immune response was measured with an intracellular cytokine staining. 7 d after immunization, spleen cells were cultured with or without $OVA_{257-264}$ peptide to stimulate OVA-specific CD8$^+$ T cells for 6 h, and T cell production of IFN-γ was

of many forms of antigen (19). Treatment with DT totally ablated the presentation of OVA from the injected tumor cells to OT-I and OT-II transgenic T cells (Fig. 4 A). These results indicate that DCs cross-present antigens from α-GalCer–loaded EL4 thymoma cells.

We next studied the $CD8^+$ T cell response in naive mice, i.e., in the absence of transferred transgenic T cells. Much stronger responses to DCs were noted when we used the more standard s.c. route of DC administration (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20070458/DC1). However, DCs coated with peptide with or without α-GalCer were poorly immunogenic when given by the i.v. as well as the s.c. route (Fig. 4, B and D; and Fig. S4). In contrast, EL4(OVA) tumor cells induced stronger immunity if the cells were loaded with α-GalCer and injected i.v. (Fig. 4, B and D), but not when given s.c. The OVA immune responses could be observed by measuring the numbers of OVA-specific $CD8^+$ T cells with H-$2K^b$/OVA tetramers (tetramer-positive $CD8^+$ T cells = 0.59 ± 0.16, 0.38 ± 0.21, 0.52 ± 0.01, 3.03 ± 0.61, 0.44 ± 0.16, 7.71 ± 1.62, and 0.11 ± 0.06 in DC-pep–, DC-pep/G–, EL4(OVA)-, EL4(OVA)/Gal-, $CD1d^{hi}$-EL4(OVA)–, and $CD1d^{hi}$-EL4(OVA)/Gal–injected mice and naive C57BL/6 mice, respectively; Fig. 4 B) or with intracellular cytokine staining for IFN-γ production (Fig. 4 D). The response to tumor/Gal was entirely dependent on the presence of $V\alpha14^+$ NKT cells, TAP, and CD40, as indicated with the appropriate knockout mice (Fig. 4 E). Silk et al. previously reported on immune responses to the combination of peptide and α-GalCer given i.v. (20), so we repeated their protocol with 100 μg OVA peptide as a model antigen plus α-GalCer. We detected just 0.49 ± 0.19% tetramer-positive $CD8^+$ T cells in the spleen, which was 20 times smaller than we observed when we injected $CD1d^{hi}$-EL4(OVA)/Gal into mice i.v. (tetramer-positive $CD8^+$ T cells = 0.03 ± 0.02 and 0.49 ± 0.19 in OVApep- and OVApep plus Gal–injected mice, respectively; Fig. 4 C). These data indicate that tumor/Gal are more immunogenic than DCs if the i.v. route of immunization is used, but that immunity requires $V\alpha14^+$NKT cells and TAP^+ DCs in the recipient mice.

DCs capture tumor cells after i.v. injection of tumor/Gal

To begin to understand the immunogenicity of co-stimulator–poor tumor/Gal, we pursued previous findings that $CD8\alpha^+$ DCs in mouse spleen captured dying cells when the latter were administered by the i.v. route (8). We reasoned that killing of the injected tumor/Gal tumor by NKT and NK cells would provide a source of dying tumor for DCs. We first verified that $CD8\alpha^+$ DCs were able to capture allogeneic spleen cells injected i.v., because allogeneic cells are killed by NK cells (8) (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20070458/DC1). Then, to follow uptake of the tumor cells, we performed experiments with CFSE-labeled EL4 tumor rather than B16 at first, because higher doses of tumor cells are needed to detect tumor cell uptake by FACS and B16 is toxic when ~10^6 cells are given i.v. By flow cytometry and confocal microscopy, we observed that $CD8\alpha^+$ DCs picked up the injected CFSE-labeled live $CD1d^{hi}$-EL4/Gal 10 h later (CFSE-positive cells from total $CD11c^+$ DCs = 3.38 ± 0.39% and <0.01% in $CD1d^{hi}$-EL4/Gal–injected mice and naive C57BL/6 mice, respectively; Fig. 5, A–C). When uptake kinetics were followed, the DCs in WT mice had $CFSE^+$ debris for at least 24 h after tumor/Gal administration, but not in $J\alpha18^{-/-}$ mice (Fig. 5, C and D). We interpret these findings to indicate that the presentation of α-GalCer on tumor cells leads to killing by NKT and/or NK cells, followed by uptake by $CD8\alpha^+$ DCs.

DC maturation in response to α-GalCer–loaded tumor cells i.v.

To explain the T cell response to injected tumor/Gal, we reasoned that the DCs capturing tumor had to be undergoing differentiation or maturation. We initially examined several cell-surface molecules after injecting tumor/Gal (Fig. 6 and Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20070458/DC1). CD86 expression on both $CD8\alpha^+$ and $CD8\alpha^-$ subsets of DCs began to increase within hours of injecting $CD1d^{hi}$-B16/Gal (tumor/Gal; Fig. 6 A). We also observed up-regulation of CD40 and CD80 and down-regulation of CD119 (Fig. S6), which are characteristic changes for DCs undergoing maturation in vivo (9). To assess DCs in other tissues, we compared spleen with lung and liver, and again there was a clear increase in CD86 expression if we injected tumor/Gal as opposed to native tumor (Fig. 6 B, compare filled and open tracings). The increase in CD86 expression after the injection of α-GalCer–loaded tumor was similar to that seen with free α-GalCer (Fig. 6 B), a particularly powerful stimulus for DC maturation (9, 21, 22), indicating that tumor/Gal were surprisingly able to mimic the agonistic glycolipid itself.

Another manifestation of maturation is that DCs start to produce protective cytokines like IL-12. In fact, IL-12 was induced within 4 h of giving mice B16/Gal or $CD1d^{hi}$-B16/Gal i.v. but not tumor cells without α-GalCer (Fig. 6 C, compare left and right panels). To establish the need for NKT cells to bring about DC maturation, we assessed cell-surface markers and IL-12 production by DCs in $J\alpha18^{-/-}$ mice given tumor/Gal. Both signs of maturation were ablated in the absence of NKT cells (IL-12p40/p70–positive $CD8^+$ cells from

measured by intracellular cytokine staining. Data are means ± SEM obtained from five mice per group in three independent experiments. *, $P < 0.05$ for EL4(OVA)/G or $CD1d^{hi}$-EL4(OVA)/G versus the other groups and EL4(OVA)/G versus $CD1d^{hi}$-EL4(OVA)/G). (E) $J\alpha18^{-/-}$, $CD4^{-/-}$, $TAP^{-/-}$, or $CD40^{-/-}$ mice were used as recipients for $CD1d^{hi}$-EL4(OVA)/Gal, and the immune responses were tested by intracellular cytokine staining in the absence or presence of OVA peptide 1 wk later. Data are means ± SEM obtained from five mice per group in three independent experiments. *, $P < 0.05$ for WT versus $J\alpha18^{-/-}$, $TAP^{-/-}$, or $CD40^{-/-}$; $P > 0.05$ for WT versus $CD4^{-/-}$.

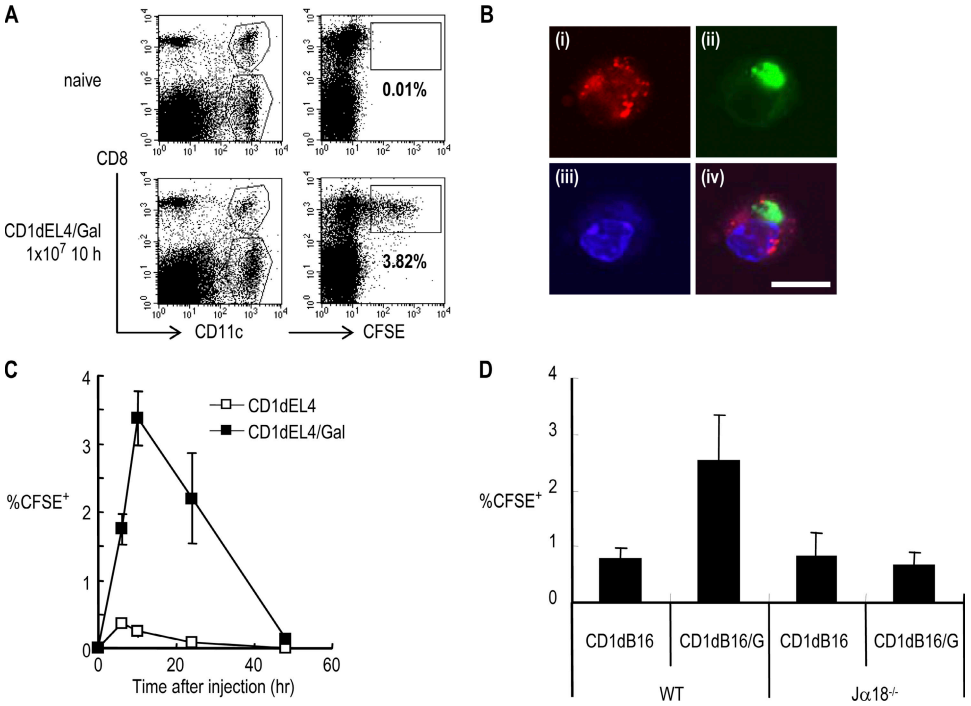

Figure 5. Maturing DCs capture tumor/Gal after i.v. injection. (A–C) 10 million CFSE-labeled CD1d^{hi}-EL4 with or without loading with α-GalCer were injected into C57BL/6 mice. (A, left) Gated areas indicate subsets of CD11c$^+$ DCs, CD8α$^+$ DCs, and CD8α$^-$ DCs. (right) The frequency of uptake of CFSE$^+$ tumor cells by CD11c$^+$ splenic DCs was measured by flow cytometry. (B) CD11c cells were enriched by MACS for analysis by confocal microscopy after injection of CFSE-labeled live CD1d^{hi}-EL4/Gal cells. (i) Tumor fragments (green), (ii) CD86 to detect mature DCs (red), (iii) DAPI nuclear stain (blue), and (iv) a merged image are shown. Bar, 10 μm. The data in A and B are representative of three independent experiments ($n = 3$ per group). (C) The mean percentage of DCs taking up CFSE-labeled tumor debris at the indicated time points is shown. (D) As in C, but antigen capture by mature DCs at 10 h was quantified in CD1d^{hi}-B16 ± Gal injected with WT C57BL/6 or Jα18$^{-/-}$ mice. All data in C and D are means ± SEM obtained from three mice per indicated time point in two independent experiments per each group.

CD11c$^+$ DCs = 1.96 ± 0.23, 2.7 ± 0.26, 2.58 ± 0.23, 5.4 ± 0.36, 7.9 ± 0.39, and 8.14 ± 0.5 in naive C57BL/6 and B16-, CD1d^{hi}-B16–, Gal-, B16/Gal-, and CD1d^{hi}-B16/Gal–injected mice, respectively, and 1.95 ± 0.73, 2.43 ± 0.31, and 2.37 ± 0.21 in naive Jα18$^{-/-}$ and B16/Gal- and CD1d^{hi}-B16/Gal–injected Jα18$^{-/-}$ mice, respectively; Fig. 6, C–E). These data indicate that DCs begin to mature quickly upon injection of tumor/Gal and suggest that DCs must be capturing glycolipid for presentation to NKT cells, a known mediator of DC maturation (9, 21).

To understand how NKT cells might be mobilized to mature the splenic DCs, we considered the possibility that DCs were cross-presenting glycolipid from phagocytosed tumor/Gal. We therefore isolated CD11c$^+$ DCs and CD11c$^-$ non-DCs from mice injected with tumor/Gal. The CD11c$^+$ DCs were selectively able to elicit IFN-γ secretion from liver mononuclear cells (MNCs), an enriched source of primary NKT cells (Fig. 6 F). In fact, liver MNCs from Jα18$^{-/-}$ mice that lacked NKT cells did not respond to the DCs (Fig. 6 F, compare shaded and open bars). To document cross-presentation of α-GalCer from the injected tumor cells, we repeated the experiments with CD1d$^{-/-}$ mice in which only the injected tumor cells expressed CD1d. Now the DCs were unable to cross-present the glycolipid to NKT cells (Fig. 6 F). Collectively, these results indicate that DCs cross-present glycolipid after i.v. injection of tumor/Gal in a CD1d-dependent manner.

DCs are needed to elicit adaptive immunity to an injection of tumor/Gal

The data in Figs. 5 and 6 indicated that DCs were both taking up tumor/Gal and undergoing NKT cell–dependent maturation in vivo, but to prove that these two events were responsible for adaptive T cell immunity, we did two additional experiments. First, we injected CD1d^{hi}-B16/Gal tumor cells and isolated CD11c$^+$ and CD11c$^-$ cells from the mice 10 h later. We then transferred each population to naive mice and looked for CD8$^+$ T cells specific for melanoma antigens 1 wk later. We were able to detect IFN-γ–producing CD8$^+$ T cells in response to trp2 and gp100 peptide in CD11c$^+$ cell–transferred mice but not CD11c$^-$ cell–transferred mice (Fig. 7 A). We also injected CD1d^{hi}-B16/Gal i.v. into CD11c-DTR transgenic mice, which allowed for the depletion of the antigen-capturing DCs after the injection of DT 1 d later, as previously described (19). Treatment with DT totally ablated presentation of trp2 or gp100 peptides from the injected tumor cells to the T cells (Fig. 7 B). These experiments indicate that DCs play a major role in the capture and presentation of tumor/Gal to T cells.

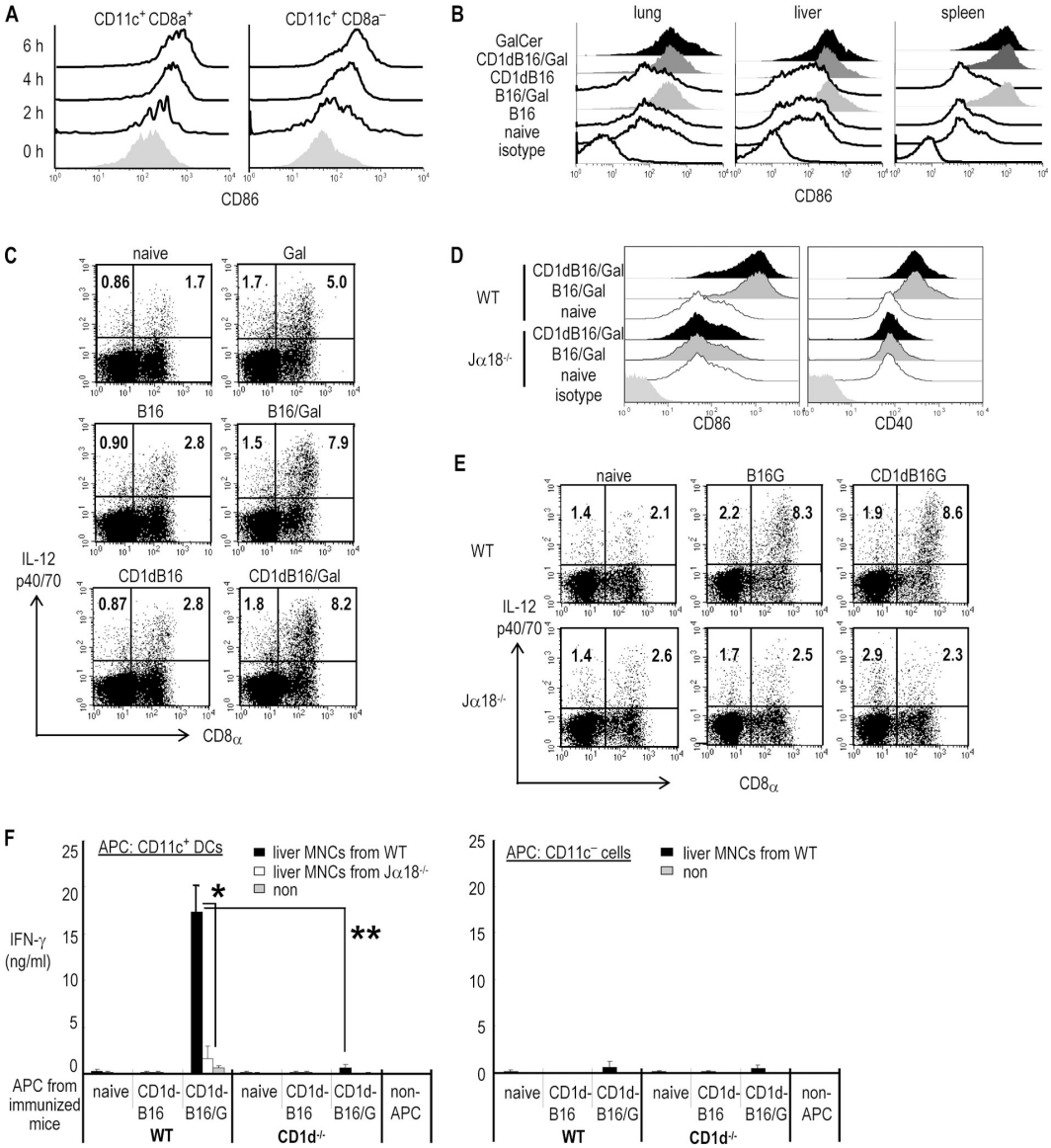

Figure 6. In vivo maturation of DCs and cross-presentation of α-GalCer in DCs after administration of tumor/Gal. (A) Spleen cells were obtained at 0, 2, 4, and 6 h after administration of CD1d^{hi}-B16/Gal. Low density spleen cells were stained with anti-CD11c-PE and CD8α-FITC (to identify DCs and their CD8$^+$ or CD8$^-$ subsets) and with biotinylated isotype control or CD86 mAb, followed by streptavidin-allophycocyanin. (B) As in A, but DCs were tested in three different organs for CD86 expression 6 h after injecting the tumor cells indicated on the left. All data in A, B, and D are representative of two independent experiments ($n = 2$ per group). (C) Splenic DCs were analyzed by intracellular cytokine staining for IL-12p40 4 h after immunization with tumor/Gal, followed by 4 h of culture in brefeldin A. DCs were identified with CD11c-allophycocyanin and CD8α-FITC and were subsequently fixed and stained with PE-conjugated anti–IL-12p40 mAb. (D and E) DC maturation studies, as in A and C, were performed in WT and Jα18$^{-/-}$ mice to show the NKT cell dependence. Numbers in C and E indicate the percentage of IL-12–producing cells in CD8$^+$ or CD8$^-$ subsets of CD11c$^+$ DCs, and all data are representative of three independent experiments ($n = 3$ per group). (F) To evaluate the cross-presentation of α-GalCer derived from tumor/Gal, DCs or non-DCs were isolated by CD11c magnetic beads at 10 h after injecting 5×10^5 CD1d^{hi}-B16/Gal into WT C57BL/6 or CD1d$^{-/-}$ mice. 10^5 APCs per well were co-cultured with 10^5 liver MNCs per well from C57BL/6 or Jα18$^{-/-}$ mice for 48 h. Then, the IFN-γ from the supernatants was measured by ELISA. All data are means ± SEM obtained from three independent experiments ($n = 5$ per group). *, $P < 0.05$ for WT versus Jα18$^{-/-}$ for liver MNCs; **, $P < 0.01$ for WT versus CD1d$^{-/-}$ for APCs.

DISCUSSION

Tumors are typically poorly immunogenic, in part because most tumors fail to express co-stimulatory molecules that are needed to induce adaptive T cell immunity. Tumor cells provide an impetus to identify principles that lead to stronger adaptive immunity in vivo. The new approach in this study surprisingly begins with several different mouse tumors as the only source of tumor antigen, but the tumor cells are loaded with an agonistic glycolipid for NKT cells, α-GalCer (tumor/Gal). When relatively low doses of tumor/Gal are injected i.v., we

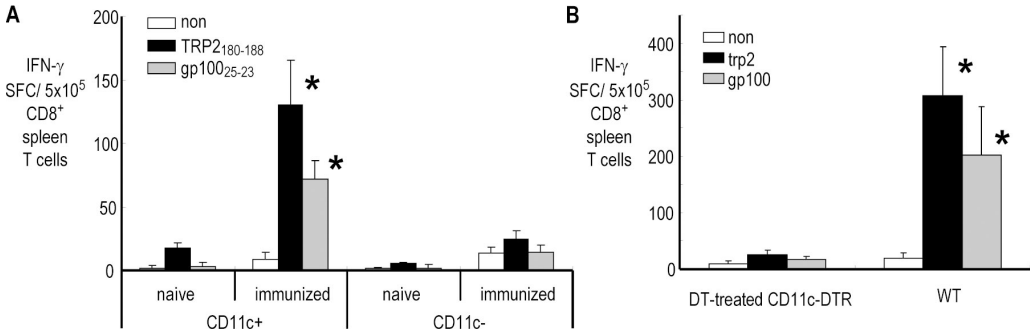

Figure 7. DCs are required for inducing immunity to tumor/Gal. (A) 10 h after injecting CD1d^{hi}-B16/Gal tumor cells, CD11c$^+$ and CD11c$^-$ cells were isolated, and 3×10^6 CD11c$^+$ and 5×10^6 CD11c$^-$ cells were transferred to naive C57BL/6 mice; spleen cells from uninjected mice served as the negative control. 1 wk later, we measured CD8$^+$ T cells specific for melanoma antigens (trp2 and gp100) by ELISPOT assay. (B) 1 d after injecting CD1d^{hi}-B16/Gal i.v. into CD11c-DTR transgenic mice, CD11c$^+$ DCs were depleted with DT. 7 d later, we analyzed antigen-specific T cell responses to trp2 or gp100 peptides by ELISPOT assays. All data are means obtained from two independent experiments ($n = 5$ per group). *, $P < 0.05$ for CD11c$^+$ transferred mice versus others (left) and DT-treated CD11c-DTR mice versus immunized WT mice (right). SFC, spot-forming cells.

find that it becomes possible to harness many of the different types of lymphocytes that can resist cancer: innate NKT and NK cells and adaptive CD4$^+$ and CD8$^+$ T cells.

The use of tumor/Gal (either live or irradiated tumor cells) leads to antitumor resistance that seems more effective than previous methods (6, 7, 23). First, immunity is induced to four different poorly immunogenic tumors that we have tested. Second, we only administer a single low dose of <1 million tumor cells. Third, we measure CD8$^+$ T cell immunity to several melanocyte differentiation antigens, again with a single dose of B16 tumor. Fourth, the immunity is long lived and requires both CD4$^+$ and CD8$^+$ T cells.

A critical feature of tumor cells coated with glycolipid is that they are active in stimulating NKT and NK lymphocytes if given by the i.v. route (11). As previously noted, NKT cells are able to recognize and resist tumor/Gal that are injected i.v., even tumors lacking co-stimulatory molecules such as CD40, CD80, or CD86. In contrast, these molecules are required to initiate responses by polyclonal T cells that recognize peptides presented on MHC products. After the resistance of tumor/Gal by NKT and NK cells, some of the killed tumor cells are captured by DCs, and glycolipids also are presented on CD1d molecules to additional NKT cells. This leads to IL-12 production from DCs, most likely through the expression of CD40L that occurs within 4 h of activation of NKT cells (22). The IL-12 further mobilizes NK cells in vivo (24). Importantly, the maturing DCs are then able to mobilize the adaptive T cell limb of immunity, resulting in long-term T cell resistance to the tumor.

The pivotal step in inducing adaptive immunity to tumor/Gal is the capture and cross-presentation of glycolipid by DCs in vivo. We have observed that DCs from CD1d$^{-/-}$ mice are unable to present tumor/Gal to NKT cells, indicating that DCs have to cross-present glycolipid from the tumor cells on CD1d, a process that has been reported by Wu et al. in tissue culture (10). The ensuing presentation of glycolipid by DCs to NKT cells is one of the most potent known means to induce DC differentiation or maturation (9, 21, 22), and it is more effective in vivo than other stimuli such as Toll-like receptor ligands and CD40 ligation (7).

This new pathway to enhanced antitumor immunity differs from immunogenic cell death that takes place with certain chemotherapies (6). In the latter instance, dying tumor cells are recognized by DCs and also provide high mobility group box 1 to mature the DCs, whereas in the system described in this paper, the tumor cells become immunogenic because of cross-presentation of glycolipid to NKT cells, which in turn leads to DC maturation. NKT cell–based DC maturation seems more potent than high mobility group box 1–based maturation, because higher percentages of animals resist tumor challenge, the resistance is longer lived, and protection can be exerted on highly tumorigenic transplantable tumors.

In effect, the injection of glycolipid-loaded tumor cells harnesses four of the major components responsible for the immunogenicity of antigen-capturing DCs: (a) antigens gain access to DCs for antigen processing to take place; (b) the DCs mature, including heightened expression of co-stimulatory molecules and production of IL-12; (c) the DCs are localized in lymphoid tissues in a position to select antigen-reactive T cells; and (d) the DEC$^+$ CD8$^+$ subset of DCs that cross-presents peptides to CD8$^+$ T cells is engaged. We have confirmed, using EL4 tumor cells, that the DEC-205$^+$ CD8$^+$ subset of DCs cross-presents antigens on MHC class I products (25, 26).

Previous observations have shown that innate NK cells can amplify MuLV antigen-specific T cell immunity using CD70 gene–transduced RMA tumor cells (27). It is possible that the NK cells were being mobilized by the CD70-transduced tumor cells, leading to the killing, uptake, and maturation of DCs, as shown in this study.

We would like to propose that the experiments in this paper should be extended into patients with malignancy, particularly hematologic malignancies or malignancies with

tumor-rich pleural effusions and ascites, in which tumor cells are readily available for loading with appropriate glycolipids. Some human tumor cells express CD1d and are able to present endogenous glycolipids as well as α-GalCer to NKT cells, eliciting IFN-γ release and cytolysis (28, 29). The glycolipid α-GalCer, although it has not yet been used extensively, has shown safety in humans (30–32). Irradiated tumor cells, when coated with glycolipid, were active in our experiments, so that a patient's tumor cells could be subject to ionizing irradiation to block tumor growth. A critical unknown is the type of tumor cells that should be injected, because cancer stem cells or cancer-initiating cells (33) may well represent the pivotal targets for immune-based resistance to malignancy. Research in patients will be required to test tumor cells that have been coated with a glycolipid agonist for NKT cells as a means to energize several types of tumor-reactive lymphocytes and several fundamental immunogenic features of DCs.

MATERIALS AND METHODS

Reagents. α−GalCer was synthesized at the Institute of Physical and Chemical Research. α−GalCer and vehicle (0.4% DMSO) were diluted in PBS. This concentration of DMSO by itself did not elicit innate or adaptive immunity to tumors. LPS-free OVA was obtained from Seikagaku Corp. The following mAbs were purchased from BD Biosciences: anti–mouse CD1d (1B1), CD8α (53-6.7), CD11c (HL3), CD19 (1D3), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD40 (3/23), CD70 (FR70), CD80 (16-10A1), CD86 (B7-2), CD119 (GR20), CD154 (MR1), NK1.1(PK136), TCRβ (H57-597), Vα2 TCR (B20.1), H-2K^b (AF16-88.5), I-A^b(KH74), IFN-γ (XMG1.2), IL-4 (11B11), and IL-12p40/p70 (C15.6) and mouse IgG1 (A85-1). Biotinylated mAbs were detected with streptavidin-allophycocyanin. For flow cytometry of $OVA_{257–264}$ peptide (SIINFEKL)–specific CD8 T cells, we used H-2K^b OVA tetramer–SIINFEKL–PE (Beckman Coulter). For analysis, we used a flow cytometer (FACSCalibur; BD Biosceinces) and CELLQuest (BD Biosciences) or FlowJo (Tree Star, Inc.) software.

Cell preparation. DCs were generated from bone marrow progenitors, as previously described (34). On day 6, 100 ng/ml α-GalCer was added to DCs for 40 h, and 100 ng/ml LPS was added for the last 16 h to mature the DCs. Tumor cells were also cultured for 48 h with 500 ng/ml α-GalCer to load them with glycolipid. These α-GalCer–loaded cells were washed three times before injection. To isolate NKT cell–enriched MNCs, livers were teased into a single suspension, resuspended in a 40/60% Percoll solution (GE Healthcare), and centrifuged for 20 min at 900 *g* to float the MNCs. To enrich DCs from liver and lung, we digested them with collagenase and again floated the DCs on Percoll gradients (35). Before collagenase treatement, lungs were perfused with 5 ml of ice-cold PBS and removed. In some experiments, splenic DCs were isolated using CD11c magnetic beads (Miltenyi Biotec).

Mice and cell lines. Pathogen-free C57BL/6 (B6) and BALB/c female mice at 6–8 wk old were purchased from CLEA Japan, and B6 CD4$^{-/-}$, CD8$^{-/-}$, CD40$^{-/-}$, TAP$^{-/-}$, and MHC II$^{-/-}$ (B6.129-H2$^{dlAbl\text{-}Ea}$) mice were purchased from the Jackson Laboratory. B6 CD1d$^{-/-}$ and Jα18$^{-/-}$ mice have been previously described (11, 36). OT-I and OT-II TCR transgenic mice (B6 background) were provided by W. Heath (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). B6 CD11c-DTR/GFP transgenic mice were a gift of D. Littman (New York University, New York, NY) and were backcrossed 12 generations to C57BL/6. All mice were maintained under specific pathogen-free conditions and studied in compliance with institutional guidelines. B16, EL4, and J558 cell lines were obtained from the American Type Culture Collection, and WEHI-3B cells were obtained from the Institute for Fermentation. As previously described, for stable introduction of CD1d, pMX-mCD1d-IRES-GFP–carrying mCD1d was retrovirally transduced into tumor cells by lipofection, and cells were subsequently sorted based on the expression of GFP by FACS-Vantage (BD Biosciences) (11).

Immune assays and in vivo tumor studies. Mice were immunized i.v. with tumor/Gal. In experiments for evaluation of adaptive immunity, immunized mice were challenged with parental tumor cells s.c. For immune assays, we used 2 mice per group and performed two to four experiments; for tumor growth curves, we used 5–10 mice per group and performed two experiments. Tumor size was measured every 2 d.

Assays for priming of antigen-specific T cell immunity. To identify antigen-specific T cells for OVA, spleen cells were isolated 7 d after immunization with OVA carrying tumor cells or OVA peptide–pulsed cells and challenged in culture for 6 h in the presence or absence of 1 μM $OVA_{257–264}$ peptide (SIINFEKL, for CD8$^+$ T cells) or 10 μM $OVA_{323–339}$ peptide (ISQAVHAAHAEINEAGR, for CD4$^+$ T cells) with brefeldin A. The cells were then stained for cell-surface markers, FITC-conjugated anti-CD4 or anti-CD8 mAb, fixed with Cytofix/Cytoperm Plus permeabilization (BD Biosciences), and stained with PE-conjugated anti–IFN-γ mAb. ELISPOT assays for melanoma antigen–specific IFN-γ–secreting cells were performed on 96-well filtration plates (Millipore) coated with rat anti–mouse IFN-γ capture antibody at 10 μg/ml (BD Biosciences), as previously described (17). CD8$^+$ T cells from spleen cells were purified by positive selection and were incubated with $Trp2_{180–188}$ (SVYDFFVWL; Kb) or $gp100_{25–33}$ (EGSRNQDWL; Db), $Tyrp_{455–463}$ (TAPDNLGYA; Db), $Tyrp_{522–529}$ (YAEDYEEL; Kb), or $Dct_{363–371}$(SQVMNLHNL; Db) peptide-pulsed CD11c$^+$ spleen DCs for 36 h. Biotinylated anti–mouse IFN-γ detection antibody was added at 2 μg/ml (BD Biosciences) for 2 h, and spots were developed with an avidin–peroxidase complex (Vectastain Elite Kit; Vector Laboratories) and stable diaminobenzidine substrate (Research Genetics), and were counted microscopically.

Assays for capture of tumor antigens by DCs in vivo. DC capture of CFSE-labeled tumor cells in vivo was assessed directly by FACS by gating on CD11c$^+$ spleen cells. We assessed CFSE labeling of CD11c$^+$CD8α$^+$ and CD11c$^+$CD8α$^-$ DC subsets, as well as reactivity with antibodies to DC maturation markers like CD86. Results were verified by confocal microscopy to look for uptake of CFSE-labeled fragments into the DCs. The cytosmears were fixed for 10 min at room temperature in 4% paraformaldehyde in PBS. Cells were stained with anti-biotin–CD86 mAb and streptavidin–Alexa Fluor 547 (Invitrogen), and DNA was labeled with DAPI. Cells were visualized by confocal microscopy (Leica).

Assays for antigen presentation in vivo using OVA as a surrogate antigen. To assess the capacity of tumor/Gal to induce T cell–mediated immunity, we treated tumor cells with osmotic shock in the presence of 10 mg/ml OVA protein in hypertonic medium and subsequently treated with hypotonic medium; such tumor cells, when injected i.v., are actively captured and processed by DCs (8, 9). Using this approach, T cell responses were monitored with 2×10^6 CFSE-labeled, CD8$^+$ (OT-I), and CD4$^+$ (OT-II) OVA-specific TCR transgenic T cells. The EL4(OVA) tumor cells were loaded with α-GalCer for 2 d before osmotic shock with OVA protein. 2×10^6 EL4(OVA)/Gal or other controls were given i.v. 1 d after adoptive transfer of the OVA-specific TCR transgenic T cells. Spleen cells were tested 3 d later to monitor T cell proliferation based on dilution of the CFSE signal. OT-I and OT-II T cells were identified by the expression of CD8 or CD4 and Vα2 and evaluated for the up-regulation of CD25 or CD44 and the down-regulation of CD62L. In some experiments, to assess host DC presentation of cell-associated antigens to T cells, CD11c-DTR mice after treatment with DT (Sigma-Aldrich) (19) and TAP$^{-/-}$ and CD40$^{-/-}$ mice were used as recipients.

Statistical analysis. Differences in survival of treatment groups were analyzed using the log-rank test. Differences were analyzed using the Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Online supplemental materials. Fig. S1 shows $CD4^+$ T cells and $CD4^+$ NKT cells in $CD4^{-/-}$ and MHC class II $^{-/-}$ mice by flow cytometry. Fig. S2 demonstrates $CD4^+$ T and $CD8^+$ T cell–dependent antitumor resistance after vaccination with EL4 thymoma cells loaded with α-GalCer. Fig. S3 shows the long-term protection against J558 plasmacytoma induced by tumor/Gal. Fig. S4 is the induction of T cell response by peptide-pulsed DCs via the s.c. route. Fig. S5 shows the uptake of allogeneic spleen cells by the $CD8^+$ DC subset in the spleen. Fig. S6 shows the maturation of splenic DCs in mice given EL4/Gal or $CD1d^{hi}$-EL4/Gal. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070458/DC1.

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