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Tumor Cells Loaded with α-Galactosylceramide Induce Innate NKT and NK Cell-Dependent Resistance to Tumor Implantation in Mice

Kanako Shimizu,* Akira Goto,* Mikiko Fukui,* Masaru Taniguchi,† and Shin-ichiro Fujii2*

Dendritic cells (DCs) loaded with α-galactosylceramide (α-GalCer) are known to be active APCs for the stimulation of innate NKT and NK cell responses in vivo. In this study, we evaluated the capacity of non-DCs to present α-GalCer in vitro and in vivo, particularly tumor cells loaded with α-GalCer (tumor/Gal). Even though the tumor cells lacked expression of CD40, CD80, and CD86 costimulatory molecules, the i.v. injection of tumor/Gal resulted in IFN-γ secretion by NKT and NK cells. These innate responses to tumor/Gal, including the induction of IL-12p70, were comparable to or better than α-GalCer-loaded DCs. B16 melanoma cells that were stably transduced to express higher levels of CD1d showed an increased capacity relative to wild-type B16 cells to present α-GalCer in vivo. Three different tumor cell lines, when loaded with α-GalCer, failed to establish tumors upon i.v. injection, and the mice survived for at least 6 mo. The resistance against tumor cells was independent of CD4 and CD8 T cells but dependent upon NKT and NK cells. Mice were protected from the development of metastases if the administration of live B16 tumor cells was followed 3 h or 3 days later by the injection of CD1dhigh-α-GalCer-loaded B16 tumor cells with or without irradiation. Taken together, these results indicate that tumor/Gal are effective APCs for innate NKT and NK cell responses, and that these innate immune responses are able to resist the establishment of metastases in vivo. The Journal of Immunology, 2007, 178: 2853–2861.

A marine sponge-derived glycolipid, α-galactosylceramide (α-GalCer),3 has proven to be valuable for activating NKT cells in a CD1d-restricted manner. The activation of these NKT cells, which use an invariant TCR, subsequently leads to the transient bystander activation of NK, B, and T cells (1, 2). Activation of NKT cells also can lead to Ag-specific T cell responses by inducing the maturation of Ag-capturing dendritic cells (DCs), thus illustrating how α-GalCer and NKT cells play roles in linking innate and adaptive immunity (3). Invariant NKT cells have been implicated in both stimulation and inhibition of the immune response, including protective roles in microbial infection, tumor immunity, and prevention against various autoimmune diseases (4).

The immune responses of invariant NKT cells depend not only on the expression of CD1d molecules but also on the type of glycolipid that is presented. NKT cells recognize a limited number of synthetic and naturally occurring α-anomeric glycosphingolipids and, to a lesser extent, β-anomeric glycosphingolipids in association with the CD1d on APCs, such as DCs (5, 6). Different types of glycolipids can induce distinct functional NKT cells, i.e., α-GalCer or isoglobotrihexosylceramide (7) has the potential to induce suppressive or stimulatory forms of immune responses, α-C-GalCer can strongly enhance Th1 type NKT cells (8), and OCH can induce Th2 type NKT cells (9).

The CD1 family of MHC-unlinked class Ib molecules is conserved across mammalian species (10, 11). CD1d expression is heterogeneous with respect to cell type and level of expression, but CD1d is expressed by many nonhemopoietic as well as hemopoietic lineages (12, 13). It is well known that NKT cells do not develop in CD1d-deficient mice and the expression pattern of CD1d is important for NKT cell development and selection of NKT cells in the thymus (4, 11). In the periphery, CD1d expression is also responsible for NKT cell recruitment and regulation. Natural up-regulation of CD1d in inflammation has been reported in hepatic cells in hepatitis C virus-infected patients (14), cardiac endothelial cells in coxsackievirus-induced myocarditis (15), B cells in gut-associated lymphoid tissues in the intestinal inflammation (16), and tumor cells in myeloma patients (17). However, the relation of CD1d expression on APCs to the initiation of NKT cell responses has not been well studied. It is possible that any CD1d+ cell is capable of eliciting some aspects of the NKT cell response.

We previously demonstrated that α-GalCer-loaded DC induced a prolonged IFN-γ-producing NKT cell response in mice, whereas free α-GalCer induced long-term anergy of NKT cells (18). The anergy of NKT cells responding to α-GalCer has been confirmed (18–21). In this study, we have compared different cell types, particularly tumor cells that lack costimulatory molecules like CD86 and CD40, for their capacity to stimulate innate NKT and NK cell responses following loading with α-GalCer ex vivo. When we studied tumor cells that expressed low levels of endogenous CD1d, or were transduced to express higher levels of CD1d in a stable fashion, we surprisingly found that tumor cells loaded with α-GalCer (tumor/Gal) induced strong NKT and NK responses in...
vivo, and that these innate lymphocytes could provide T cell-independent protection against the establishment of growing tumors.

Materials and Methods

Mice and cell lines

Pathogen-free C57BL/6 and BALB/c female mice at 6–8 wk from CLEA Japan, and CD4<sup>−/−</sup>, CD8<sup>−/−</sup>, CD40<sup>−/−</sup>, and CD80/86<sup>−/−</sup> mice from The Jackson Laboratory were purchased. The mice listed and Jo18<sup>−/−</sup> 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 from the Institute for Fermentation. The retroviral vector pMX-ORES-GFP and a Plat-E packaging cell line were provided by Dr. T Kitamura (University of Tokyo, Tokyo, Japan). After the introduction of full-length cDNA of murine CD1d to pMX-ORES-GFP, it was retrovirally transduced into tumor cells by lipofection, and the cells were subsequently sorted based on the expression of GFP by FACSVantage.

Reagents

α-GalCer was synthesized in RIKEN, α-GalCer and vehicle (0.4% DMSO) were diluted in PBS. This concentration of DMSO had no effect for loading on tumor cells as a control in our studies. The following mAbs were purchased from BD Pharmingen: anti-mouse CD1d (1B1), anti-CD3 (145-2C11), anti-CD19 (1D3), anti-CD40 (3 of 23), anti-CD80 (16-10A1), anti-CD86 (B7-2), anti-CD154 (MR1), anti-NK1.1 (PK136), anti-TCR-β (H-57-597), anti-Vo2 TCR (B20.1), anti-H-2K<sup>k</sup> (AF16-88.5), anti-I-A<sup>d</sup> (K774), anti-IFN-γ (XM16.2), anti-IL-4 (11B11), and mouse IgG1 (A85-1). Biotinylated reagents were detected with streptavidin-allophycocyanin. For flow cytometry of invariant NKT cells, we used recombiant soluble dimeric mouse CD1d1g (BD Pharmingen). For analysis, FACS Calibur instrument and CellQuest (BD Biosciences) or FlowJo (Tree Star) software were used.

Cell preparation

Primary cells were isolated from spleen in C57BL/6 mice using magnetically (Milenyi Biotec). DCs were isolated using CD11c-magnetic beads and subsequently other cells were isolated from CD11c<sup>−</sup> fraction. Macrophages (Mφ) and NK cells were isolated using anti-biotin F4/80 Ab and anti-biotin magnetic beads or DX5-magnetic beads. T cells were isolated by negative selection using Abs and anti-biotin magnetic beads, and B cells were isolated using anti-CD19 magnetic beads. Bone marrow-derived DCs were generated in the presence of GM-CSF as previously described (22). On day 6, α-GalCer (100 ng/ml) was added to DCs for 40 h, and 100 ng/ml LPS was added for the last 16 h. For loading of α-GalCer, tumor cells were cultured for 48 h in presence of 500 ng/ml α-GalCer. These α-GalCer-loaded cells were washed three times before injection. To isolate mononuclear cells, the livers were teased into a single suspension and resuspended in a 40/60% Percoll solution (Amersham Biosciences) for centrifugation for 20 min at 900 × g.
Real-time quantitative RT-PCR

Total RNA was extracted using an RNasy mini kit (Qiagen). Random hexamer (Applied Biosystems) and Superscript II reverse transcriptase (Invitrogen Life Technologies) were used for cDNA synthesis. Predesigned TaqMan probes for murine CD1d (Mm00763541_m1) and 18 S RNA were purchased from Applied Biosystems. CD1d and 18 S transcripts were quantified by real-time quantitative PCR using TaqMan PCR Master Mix reagents and an ABI Prism 700 Sequence Detector (Applied Biosystems) according to the manufacturer’s instructions. For each sample, the mRNA abundance was normalized to the amount of 18 S rRNA.

Cytokine assays

The serum concentrations of IFN-γ, IL-4, and IL-12p70 were measured by sandwich ELISA (Opti ELA; BD Biosciences) 2, 6, 16, 24, and 48 h after administration of tumor, tumor/Gal, vehicle, or α-GalCer. ELISPOT assays for IFN-γ-secreting cells were performed by culturing with or without α-GalCer for 16 h as previously described (18). The number of ligand-dependent IFN-γ spots was analyzed with the series 3B ImmunoSpot Image Analyzer (Cellular Technology). For intracellular cytokine staining of NK or NKT cells by FACS, the cells were preincubated with 2.4G2 culture medium to block FcγR, washed, incubated with anti-CD1d monomer-Gal followed by anti-mouse IgG1-biotin and streptavidin-allophycocyanin and CD3-FITC mAb for NK cells. In some experiments, mononuclear cells from spleen were cultured with brefeldin A (BD Biosciences) with or without in vitro stimulation with α-GalCer for 6 h, and stained for cell surface markers as described. After the cell surface was labeled with mAbs, cells were permeabilized in Cytofix-Cytoperm Plus (BD Biosciences) and stained with anti-IFN-γ PE.

In vivo tumor studies

Mice were immunized i.v. with tumor/Gal (5 × 105) or 500 ng of free α-GalCer. Mice were killed 14 days after tumor inoculation, the lungs were removed and individual surface lung metastases were counted with the aid of a microscope. In some experiments, CD4+/−, CD8+/−, CD40+/−, or J11d−/− mice were used as recipient mice, or otherwise treated i.p. with 50 μl of polyclonal Ab to asialo-GM-1 (Wako Pure Chemical) 3 day before injection of B16 cells loaded with α-GalCer (B16/Gal) or CD1dhigh-B16/Gal and every other day until day 14. As previously described, to test the adjuvant effects for NK cells, we injected 2 × 106 B16 cells 3 h before administration of tumor/Gal to mice. In the tumor-bearing mouse models, mice were administered i.v. α-GalCer-loaded B16 melanoma, EL4 thymoma, or WEHI-3B leukemia cells and were evaluated for survival.

Statistical analysis

Differences in the survival of treatment groups were analyzed using the log-rank test. Differences in in vitro data were analyzed using Mann-Whitney U test. A value for p < 0.05 was considered statistically significant.

Results

Most types of leukocytes present α-GalCer to NKT cells in vitro

When we previously studied the capture of α-GalCer in vivo, we found that only CD11c+ DCs could successfully capture the glycolipid for stimulation of NKT cells to produce IFN-γ over several days (18). We repeated this experiment and confirmed that CD11c+ cells, which were isolated from mice that had been given i.v. α-GalCer 16 h earlier, could be reinfused into new mice and elicit IFN-γ-producing NKT cells in vivo, whereas CD11c− cells were inactive (Fig. 1A, right). However, when we did the same experiment by loading the CD11c+ and CD11c− ex vivo before infusion, then both cell fractions were able to elicit the NKT response (Fig. 1A, left). To pursue the capacity of different CD11c+ leukocytes to present α-GalCer, we first evaluated expression of CD1d. T cells, B cells, NK cells, and Mφ all expressed CD1d, and at substantial levels relative to DCs, both in the steady state as well as 16 h after i.v. injection of α-GalCer into mice (Fig. 1B). We then used these different cell types to stimulate liver mononuclear cells in vitro because liver is enriched in NKT cells relative to spleen. All populations (B, T, NK, Mφ, DC) could induce IFN-γ and IL-4 in a glycolipid-dependent manner, although B and T cells were less efficient (Fig. 1C). In contrast, only the DCs were active when we loaded the different cell types with α-GalCer in vivo rather than ex vivo, and then cocultured the in vivo loaded cells with liver mononuclear cells (Fig. 1D). These data suggested that as long as different types of CD1d-expressing leukocytes were able to capture glycolipid in vitro, they could stimulate NKT cells, and by extension, that the costimulatory properties of DCs were not essential. In fact, when we tested bone marrow-derived DCs from CD40 or CD80/CD86-deficient mice, we observed that these costimulatory molecules for T cell immunity were not required to
stimulate cytokine production from NKT cells in vivo (Fig. 1E). Taken together, these observations indicate that many cell types are capable of presenting α-GalCer to NKT cells and that standard costimulators like CD40 and CD80/CD86 are not essential.

**Transduction of the CD1d gene into costimulation-poor tumor cells**

The findings in Fig. 1 led us to hypothesize that tumor cells, which characteristically lack costimulatory molecules, might also be able to present α-GalCer to NKT cells. We verified that the EL4 thymoma and B16 melanoma lacked expression of CD40, CD70, CD86, and MHC class II, although EL4 cells expressed substantial levels of MHC class I (Fig. 2A). For CD1d, we examined the parental cell lines as well as stable variants that were transduced with a retrovirus to express high levels of murine CD1d. Because the retroviral vector contained both murine CD1d and GFP genes, the stable CD1dhigh-tumor cell lines were selected by sorting on a FACSVantage instrument (to a purity of >98%) (Fig. 2B). Before transduction, B16 melanoma cells and EL4 thymoma cells expressed lower levels of CD1d than J558 myeloma cells and WEHI-3B myelomonocytic leukemia cells, whereas bone marrow-derived DCs expressed the highest levels (Fig. 2C). When we quantified the expression of CD1d for all the transfectants by real-time RT-PCR and flow cytometric analysis, we found higher expression of CD1d by CD1dhigh-tumor cells than by any of the other cell lines or DCs (Fig. 2, C and D). Tumor cells were then cultured for 48 h in the presence of fluorescent α-GalCer (labeled with Cy3) or vehicle. We observed by microscopy the uptake of the glycolipid into the tumor cells (Fig. 2E). These observations set the stage to test different costimulator-poor, CD1d low and high expressing tumor cells as APCs for innate immune responses to α-GalCer.

**α-GalCer-loaded tumor cells stimulate innate lymphocytes in vitro**

To test the ability of tumor/Gal to activate primary NKT cells and NK cells, we cocultured liver mononuclear cells with tumor/Gal or tumor alone for 48 h and measured the supernatants for IFN-γ and IL-4 production in comparison to cytokine levels induced by α-GalCer-loaded DCs. The liver lymphocytes were activated by tumor/Gal to produce both IFN-γ and IL-4 (Fig. 3A). The use of CD1d-transfected tumor/Gal cells resulted in a modest increase in IFN-γ but not the IL-4 response, and the responses to tumor/Gal were similar to DCs loaded with α-GalCer (DC/Gal) (Fig. 3A). The production of cytokines was entirely dependent upon the presentation of α-GalCer and the presence of NKT cells. Cytokines were not produced when the tumor cells were not exposed to α-GalCer, or when the responding liver mononuclear cells were from NKT-deficient α18L−/− mice (Fig. 3A). Also, as shown in Fig. 3A, when NK cell depletion was induced in mice by injections of anti-asialo GM-1 Ab, IFN-γ production (but not IL-4 after 48 h. Murine IFN-γ (upper) or IL-4 (lower) was measured by ELISA. B, To block the function of CD1d, anti-mouse CD1d Ab or isotype control IgG (BD Pharmingen) were used. Tumor/Gal cells were incubated in the presence of 20 μg/ml anti-CD1d mAb or isotype control IgG for 2 h before coculturing with liver mononuclear cells. At 48 h later, the supernatants were collected. C, A human NKT-B1 NKT cell line was established from a healthy donor. A total of 1 × 10^4 NKT-B1 cells were cocultured with 1 × 10^4 tumor/Gal or DC/Gal cells in 96-well round-bottom plates, and the supernatants were collected after 48 h. Human IFN-γ was measured by ELISA. D, 51Cr-labeled tumor cells as targets were mixed with liver mononuclear cells at various E:T ratios for 20 h (24, 25). Data represent mean ± SD of triplicate wells from three independent experiments.
production) by liver mononuclear cells was reduced, indicating the involvement of NK cells, which are known to be recruited when /H9251-GalCer stimulates NKT cells (23). These results indicate that CD1dhigh-B16/Gal activated not only NKT cells but NK cells as well. Blocking experiments with anti-CD1d Ab were also conducted (Fig. 3B) and confirmed that /H9251-GalCer presentation on CD1d was required for tumor cells to trigger innate NKT responses. To further demonstrate glycolipid-dependent NKT cell responses without the need for costimulatory molecules that are species restricted, the mouse tumor cells or tumor/Gal were cocultured with an established human NKT cell line. As shown in Fig. 3C, human NKT cells specifically responded to tumor/Gal, with more IFN-γ being induced by CD1d-transfected tumor cells. To evaluate whether tumor/Gal also served as targets for innate killer cells, we cultured bulk liver mononuclear cells with /H9251-GalCer loaded tumor cells for 20 h (Fig. 3D) (24, 25). The glycolipid loaded tumor cells were targets, with CD1dhigh-B16/Gal being slightly better targets of activated innate lymphocytes than B16/Gal. There was no significant specific cell lysis seen in the control groups of B16 and GFP-transfected B16 cells (data not shown). Together, these findings indicate that tumor cells are capable of presenting /H9251-GalCer on CD1d molecules and elicit combined NKT and NK responses.

**Tumor/Gal activate both NK and NKT cells in vivo, including prolonged expansion of IFN-γ-producing NKT lymphocytes**

Mice were then i.v. injected with either free /H9251-GalCer or live tumor/Gal cells. Serum was collected at different intervals and evaluated for IFN-γ, IL-12p70, and IL-4. We found higher serum levels of IL-12p70 in mice after injection with tumor/Gal, but lower levels of IFN-γ and IL-12p70 in mice injected with tumor cells alone.
levels of IL-4 and IFN-γ as compared with mice injected with α-GalCer alone (Fig. 4A). The parental cell lines and CD1d transfected tumor cells were comparable (Fig. 4A). No cytokines were elicited in control groups administered B16 or CD1d<sup>transf</sup>-B16 cells without α-GalCer (data not shown). Because we had previously shown that the expression of CD40L is an early activation marker of NKT cells (26), we evaluated up-regulation of this costimulatory molecule at 2 and 6 h after injection of tumor/Gal. Already at 2 h, we detected CD40L expression on NKT cells activated by tumor/Gal (Fig. 4B). At 6 h, we also used FACS assays to measure IFN-γ production by both NKT cells and NK cells at the single cell level, and we noted comparable induction of IFN-γ to that seen after injection of α-GalCer alone (Fig. 4B). These data indicate that tumor/Gal stimulate innate NKT and NK immunity in vivo.

To further evaluate the response of NKT cells in vivo, we administered DC/Gal or tumor/Gal, and 2 days later, we restimulated spleen cells in an IFN-γ ELISPOT assay without or with α-GalCer in the assay (Fig. 4C). To determine the optimal dose of α-GalCer for loading tumor cells, we loaded various doses of α-GalCer on B16 and EL4 tumor cells and administered them to mice. The optimal dose of α-GalCer required to load onto the tumor cells ex vivo was 500 ng/ml. To more stringently compare the function of various APCs, graded cell doses of APCs loaded with α-GalCer were i.v. administered and then we assayed IFN-γ production 2 days later with an ELISPOT assay of spleen cells. The optimal cell number to induce this response was 5 × 10<sup>5</sup> in tumor/Gal injected mice and DC/Gal injected mice, but again, both ex vivo loaded DC/Gal and tumor/Gal were able to stimulate an innate NKT cell response (Fig. 4D). To verify that the IFN-γ-producing cells were NKT cells, we cultured the spleen cells from the immunized mice in the absence or presence of α-GalCer and performed intracellular staining rather than ELISPOT assays, after gating for cells that were CD19<sup>−</sup> and were able to bind α-GalCer-loaded CD1d dimers. IFN-γ producing invariant NKT cells were readily detected in mice given CD1d<sup>transf</sup>-B16/Gal, and to a lesser extent in mice given B16/Gal at day 5 after immunization (Fig. 4E). These data further indicate that tumor/Gal act as APCs for innate NKT cell responses in vivo.

**FIGURE 5.** Metastases on the surface of the lungs after injection of tumor/Gal. A total of 5 × 10<sup>5</sup> B16 melanoma tumor cells or B16/Gal or CD1d<sup>transf</sup>-B16/Gal cells were i.v. administered. The number of B16 melanoma metastases in the lung was evaluated 14 days later. CD4<sup>−</sup>/−, CD8<sup>−</sup>/−, Jα18<sup>−</sup>/−, CD40<sup>−</sup>/−, and wild-type (WT) mice were used as recipients. One group of mice was injected every other day with anti-asialo GM-1 mAb to deplete NK cells. The data are representative of five mice in each group.

**FIGURE 6.** Survival rates in animals injected with tumor/Gal. A–D, α-GalCer-loaded melanoma (B16), lymphoma (EL4), or leukemia (WEHI-3B) cells (5 × 10<sup>5</sup> cells/mouse) were i.v. administered. Survival rates in each group of mice were evaluated. D, CD4<sup>−</sup>/− and CD8<sup>−</sup>/− mice were used as recipients. Data represent a minimum of two experiments, each with five mice per group. *, p < 0.001 (B16/Gal vs B16, CD1d<sup>transf</sup>-B16/Gal vs CD1d<sup>transf</sup>-B16) in A. **, p < 0.005 (WEHI-3B/Gal vs WEHI-3B, CD1d<sup>transf</sup>-WEHI-3B/Gal vs CD1d<sup>transf</sup>-WEHI-3B) in B. *, p < 0.001 (CD1d<sup>transf</sup>-EL4/Gal vs CD1d<sup>transf</sup>-EL4) and **, p < 0.01 (EL4/Gal vs EL4) in C.
Antitumor effects of innate lymphocytes responding to α-GalCer-loaded tumor cells

We then assessed the antitumor effects of the innate immune response to injected tumor/Gal. We first used a lung metastasis model in which mice were i.v. injected with live tumor/Gal. Without exposure to α-GalCer, the tumor cells readily established metastases, but this did not occur if we used either live B16/Gal or CD1d<sup>high</sup>-B16/Gal (Fig. 5, first two rows). This resistance to the establishment of metastases did not require T cells because metastasis formation was largely resisted in CD4 and CD8 knockout mice (Fig. 5, third row). However resistance was reduced partially when we removed NK cells with anti-asialo GM-1 Ab, and largely abolished when NKT cells were absent in Jα<sub>H9251</sub>18/H11002/Jα<sub>H9251</sub>18/H11002/Jα<sub>11002</sub> recipient mice (Fig. 5, fourth row). There was no contribution of CD40 in the recipient mice, and there was no resistance when we administered B16 tumor cells, followed by free α-GalCer (Fig. 5, fifth row). These results indicate that tumor/Gal activate innate lymphocytes in vivo sufficiently to block the establishment of lung metastases.

Improved survival of mice to several tumors following vaccination with tumor cells loaded with α-GalCer

To extend the analysis of tumor resistance, and to consider other tumors than B16 melanoma, we conducted survival studies of mice injected with B16 melanoma, WEHI-3B myelomonocytic leukemia, and EL4 thymoma tumor cells. For each tumor, CD1d transfecteds that were loaded with α-GalCer were resisted for over 6 mo (Fig. 6, A–C). In the case of EL4, it was necessary to use CD1d transfecteds rather than native EL4 cells loaded with α-GalCer to observe such resistance, but CD1d transfecteds were not required for B16/Gal and WEHI-3B/Gal (Fig. 6C vs A and B). The types of tumor cells in addition to the levels of CD1d expression, i.e., susceptibility to NK cells, may contribute to efficacy at the level of innate immune cell activation in vitro and in vivo. No significant survival was seen in the control groups, i.e., GFP-transfected tumor cells (data not shown). For CD1d transfected EL4, we verified that neither CD4<sup>+</sup> nor CD8<sup>+</sup> T cells were required for resistance (Fig. 6D). These results further demonstrate protection against...
α-GalCer-loaded tumor cells by innate immunity, but there is the value in CD1d transfection for some tumors like EL4.

α-GalCer-loaded tumor cells induce innate resistance to native tumor cells given 3 h to 3 days earlier

In contrast to the experiments described in this study in which we followed the development of metastases by tumor/Gal themselves, we turned to the capacity of tumor/Gal to provide a therapeutic effect on native tumor cells that were not loaded with glycolipid. We also compared tumor/Gal to DC/Gal, using bone marrow-derived DCs. We first i.v. injected live B16 melanoma cells, and 3 h later, we injected tumor/Gal (B16/Gal or CD1dhigh-B16/Gal) at low (5 × 10^5) and high (5 × 10^6) doses. We found that CD1dhigh-B16/Gal induced stronger antitumor effects than DC/Gal or B16/Gal against B16 melanoma cells in a cell dose-dependent manner (Fig. 7A). To assess the potential contribution of NK cells, we depleted mice of NK cells with anti-asialo GM-1 Ab and found that resistance was markedly reduced (Fig. 7B). In addition, we verified that irradiated tumor/Gal, which would be feasible to administer in clinical trials, also induced resistance to the establishment of metastases by live B16 melanoma (Fig. 7C). When the NK cell responses were assessed directly by FACS at 6 days after immunization with B16/Gal, CD1dhigh-B16/Gal cells, or DC/Gal, IFN-γ production by CD3−NK1.1+ NK cells was greatest in CD1dhigh-B16/Gal immunized mice, relative to B16/Gal and DC/Gal (Fig. 7D). To extend the therapeutic model further, mice were injected with 1 × 10^5 live B16 cells, and 3 days later they were treated with a single dose of tumor/Gal or DC/Gal (Fig. 7E) or irradiated tumor/Gal (Fig. 7F). All forms of α-GalCer-loaded APC provided resistance to metastasis when live APCs were used (Fig. 7E), and likewise, irradiated α-GalCer-loaded tumor cells, especially CD1d transfectants, exerted a therapeutic effect (Fig. 7F). These results indicate the potential of tumor/Gal to elicit innate resistance to native tumor cells.

Discussion

In the current study, we demonstrate the capacity of tumor cells loaded with α-GalCer ex vivo (tumor/Gal) to induce strong innate immunity in a CD1d and NK cell-dependent manner in mice. DCs are strong APCs for activating NKT cells. However, constitutive CD1d expression is found on many types of parenchymal cells, especially intestinal epithelial cells, hepatocytes and hematopoietic cells (11), and tumor cells (Figs. 1B and 2, C and D). Although these cells are not as potent as DCs, they are nonetheless active. We proved in this study that tumor/Gal-induced IFN-γ production by both NKT and NK cells and that this was associated with significant innate resistance to the implantation of several tumors.

Once loaded onto CD1d, the binding of CD1d-glycolipid complexes to the αβ TCR has substantial affinity and is stable (27). However, it can take 12 h to load α-GalCer onto DCs (28). Also, as shown in Fig. 5, if we coinjected tumor cells with unbound α-GalCer, we could not protect against B16 metastases in the lung, whereas an injection of B16/Gal or CD1dhigh-B16/Gal was protective. Therefore, there is little possibility that α-GalCer-loaded tumor cells significantly transfer α-GalCer to unloaded tumor cells. In the case of the EL4 tumor, which required CD1d transduction to bind sufficient α-GalCer to elicit protective innate immunity, we were unable to induce resistance if we injected CD1dhigh-EL4 cells 3 h before injection with EL4 cells loaded with α-GalCer. In other words, the α-GalCer could not transfer from the EL4 cells to the CD1dhigh-EL4. In contrast, α-GalCer loaded CD1dhigh-EL4 induced strong resistance. These findings make it unlikely that elution of α-GalCer from the tumor/Gal could charge other tumor cells effectively in vivo. Instead the α-GalCer-loaded tumor needs to directly induce protective innate immunity.

Our data indicate that costimulatory molecules are not required on APCs to activate NKT cells in vivo. Previously Matsuda et al. (29) showed that IFN-γ secretion by NKT cells is induced in CD40−/− mice after injection of α-GalCer. We also have previously shown that DC surface remodeling occurs in both CD80/CD86−/− and CD40−/− mice after administration of α-GalCer to the same extent as in wild-type mice (3, 26). Thus, CD40 and CD80/86 molecules are not essential in activating NKT cells in the primary response, but may act as supporting costimulators (20). In the current study, we demonstrated prominent innate immunity to APCs that lacked costimulatory molecules, particularly tumor cells (Fig. 2A).

As we discussed in Figs. 3D and 5, the tumor/Gal would be a target for killing by activated NKT cells soon after the NKT cells had encountered the injected tumor/Gal. Thus, both activated Th1 type NKT cells, as well as the inflammatory events that may take place following the killing of tumor cells, may enhance IL-12 production by DCs or Mφ. The different kinetics and amounts of cytokines in the serum of mice given tumor/Gal compared with mice given free α-GalCer (Fig. 4A) could in part depend on the timing of NKT cell activation, that is, free α-GalCer can rapidly and systemically activate NKT cells, whereas in contrast, tumor/Gal requires more time to migrate and activate NKT cells in various organs. One critical variable that we are now assessing is that NKT activation by tumor/Gal may in turn lead to activation of DCs, including IL-12 production, followed by IFN-γ production by NK cells. The kinetics of the two cytokines, IL-12 and IFN-γ, may not be coordinated given the multiple pathways that can take place in vivo.

By several assays of the innate response, we assessed the value of CD1d transfection of tumor cells in presenting α-GalCer to NKT cells. As shown in Fig. 3B, anti-CD1d mAb blocks responses to α-GalCer-loaded tumor cells, indicating that NKT cells recognize the glycolipid on the CD1d of tumor cells. Increased numbers of NKT (Fig. 4E) and NK cells (Fig. 7D) capable of IFN-γ production were detected by intracellular staining assays in response to CD1dhigh-B16/Gal when compared with B16/Gal cells. When we compared tumor cells to DCs, we found that tumor/Gal were more effective in inducing innate resistance, as long as we loaded the tumor cells ex vivo (Fig. 7). For example, even at 6 days after injection, NK production of IFN-γ was much greater in mice treated with tumor/Gal than DC/Gal (Fig. 7D). The innate immunity including the adjunct effects were apparently correlated with antitumor effects. The strategy using tumor/Gal as APCs would appear to be an approach to mobilize both NKT and NK cells in resistance to tumors.

We therefore undertook experiments to use tumor/Gal to provide therapeutic innate resistance to tumors. Here CD1dhigh-B16/Gal cells were more effective than B16/Gal, but both induced significant antitumor effects when given 3 h or 3 days after an injection of tumor cells that were not loaded with α-GalCer (Fig. 7, A, E, and F). Because DC/Gal therapy was shown to be safe in recent clinical trials (30–32), the successful innate effects induced by irradiated tumor/Gal suggests the feasibility of our strategy for an immunotherapy. Our strategy might be pursued for example with hematological malignancies, such as acute myelogenous leukemia and acute lymphocyte leukemia. Because one could harvest many tumor cells at the onset of the diseases, the tumor cells could be loaded with α-GalCer and used as APCs in patients to activate NKT and NK cell-based resistance.
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References


