

Molecular Identification of GD3 as a Suppressor of the Innate Immune Response in Ovarian Cancer

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Abstract

Tumors often display mechanisms to avoid or suppress immune recognition. One such mechanism is the shedding of gangliosides into the local tumor microenvironment, and a high concentration of circulating gangliosides is associated with poor prognosis. In this study, we identify ganglioside GD3, which was isolated from the polar lipid fraction of ovarian cancer-associated ascites, as an inhibitory factor that prevents innate immune activation of natural killer T (NKT) cells. Purified GD3 displayed a high affinity for both human and mouse CD1d, a molecule involved in the presentation of lipid antigens to T cells. Purified GD3, as well as substances within the ascites, bound to the CD1d antigenic-binding site and did not require additional processing for its inhibitory effect on NKT cells. Importantly, *in vivo* administration of GD3 inhibited α -galactosylceramide (α -GalCer)-induced NKT cell activation in a dose-dependent manner. These data therefore indicate that ovarian cancer tumors may use GD3 to inhibit the antitumor NKT cell response as an early mechanism of tumor immune evasion. *Cancer Res*; 72(15); 3744–52. ©2012 AACR.

Introduction

In the United States, ovarian cancer ranks fifth as a cause of cancer-related deaths among women (1). Unfortunately, the majority of cases are diagnosed at an advanced stage, leading to poor overall survival. By the time of presentation, ovarian cancer has often undergone successive accumulation of multiple molecular alterations. Therefore, each tumor tends to be molecularly distinct, increasing the difficulty of identifying a common molecular target with prognostic or therapeutic potential.

Patients with ovarian cancer often present with ovarian cancer-associated ascites, which contains cellular compo-

nents of the immune system such as lymphocytes and natural killer T (NKT) cells, regulatory factors, such as cytokines, and potential immune inhibitory factors. It has been reported that patients with advanced ovarian cancer have higher levels of gangliosides in their plasma and ascites than plasma ganglioside levels in controls (2). Furthermore, abnormal ganglioside expression is strongly associated with clinically aggressive malignancies. Thus patients with cancer who have high circulating ganglioside levels at the time of clinical diagnosis exhibit a faster rate of disease progression and a decreased survival rate (3).

One of the earliest pathways in immune activation is the presentation of phospho/glycolipid antigens on CD1d molecules to NKT cells. NKT cells are primed cells that have large reservoirs of cytokines such as IFN- γ and TNF- α . These cells can, if appropriately activated, induce the development of a robust adaptive immune response. Many studies have characterized the adaptive T-cell immune response in ovarian cancer (4–8). However, mechanisms of immune evasion by ovarian cancers, specifically those affecting the NKT cell/CD1d system, remain to be elucidated.

Here, we identified the ganglioside GD3 as a major factor in ovarian cancer ascites fluid that inhibited NKT cell activation. Mechanistically, we found that antigen processing was not required as CD1d-Ig dimers loaded with α -galactosylceramide (α -GalCer) were no longer recognized by NKT cells following treatment with GD3 and that pulsing CD1d-Ig-based artificial antigen-presenting cells (aAPC) with GD3 or ascites fluid led to inhibition of NKT activation. Furthermore, GD3 bound with high affinity to both human and mouse CD1d, and *in vivo* treatment with GD3 inhibited α -GalCer-mediated NKT cell activation. These data indicate that ganglioside shedding may be an early mechanism of

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immune evasion used by ovarian cancer and indicate that GD3 could be an important diagnostic and/or therapeutic target in the treatment of ovarian cancer.

Materials and Methods

Tumor-associated ascites

Ovarian cancer-associated ascites was collected from patients undergoing primary cytoreductive surgery by the Kelly Gynecologic Oncology Service at Johns Hopkins Medical Institutions (Baltimore, MD). All donors gave written informed consent before enrolling in the study. The Institutional Review Board of Johns Hopkins Medical Institutions approved this investigation.

Mice

Six- to eight-week-old C57BL/6 mice were purchased from the Jackson Laboratory or Janvier and were maintained in the animal facility at New York University School of Medicine (New York, NY) and at the University of Bonn (Bonn, Germany) under pathogen-free conditions.

Cell lines

Murine L cells transfected with wild-type *cd1d1* cDNA (LCD1dwt) were kindly provided by R.R. Brutkiewicz (Indiana University School of Medicine, Indianapolis, IN; ref. 9) in 2005. The cell lines used have been tested and authenticated routinely by staining for stable cell surface expression of CD1d, compared with isotype control staining, and also compared with control cells stably transfected with the empty control vector.

NKT cells

The V α 14J α 18⁺V β 8.2⁺ NKT cell hybridoma cell lines DN32.D3, D3 and N38-3C3, and the CD1d-specific NKT cell hybridoma N37-1A12 (V α 5⁺V β 7⁺) have all been described (10–12) and were graciously provided by R.R. Brutkiewicz. A mouse *i*NKT hybridoma, 1.2, which coexpresses mouse invariant V α 14-J α 18 and V β 8.2 chains, was kindly provided by Dr. Mitchell Kronenberg (La Jolla Institute of Allergy and Immunology, San Diego, CA). The NKT cells are tested for specificity to CD1d in each experiment via functional T assay.

Lipid antigens

18:1 Biotinyl PE [1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(biotinyl)] and 18:1 caproylamine PE [1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(hexanoylamine)] were purchased from Avanti Polar Lipids. α -GalCer was purchased from Enzo/Axxora.

Generation of human *i*NKT cell lines

Human *i*NKT cell lines expressing V α 24 T cell receptor were generated, as previously reported with a slight modification (13). Briefly, V α 24⁺ cells were isolated from peripheral blood mononuclear cells (PBMC) by using anti-mouse IgG magnetic beads (Miltenyi Biotec) coupled to a mouse anti-human V α 24 TCR antibody (Beckman Coulter). V α 24⁺ cells were then cocultured with mitomycin-C (Sigma-Aldrich)-treated autol-

ogous immature dendritic cells (DC) for 24 hours in the presence of 100 ng/mL of α -GalCer and 10 IU/mL of a recombinant human interleukin (IL-2) (R&D System) and further cultured for 7 to 10 days in the presence of 10 IU/mL of human IL-2 alone. After 2 cycles of stimulations, more than 95% of NKT cells were shown to be V α 24⁺ cells by fluorescence-activated cell sorting (FACS).

Generation of mouse *i*NKT cell lines

Mouse *i*NKT cell lines were generated as described with some modifications (14, 15). Thymocytes were cocultured with autologous immature DCs in the presence of 100 μ g/mL

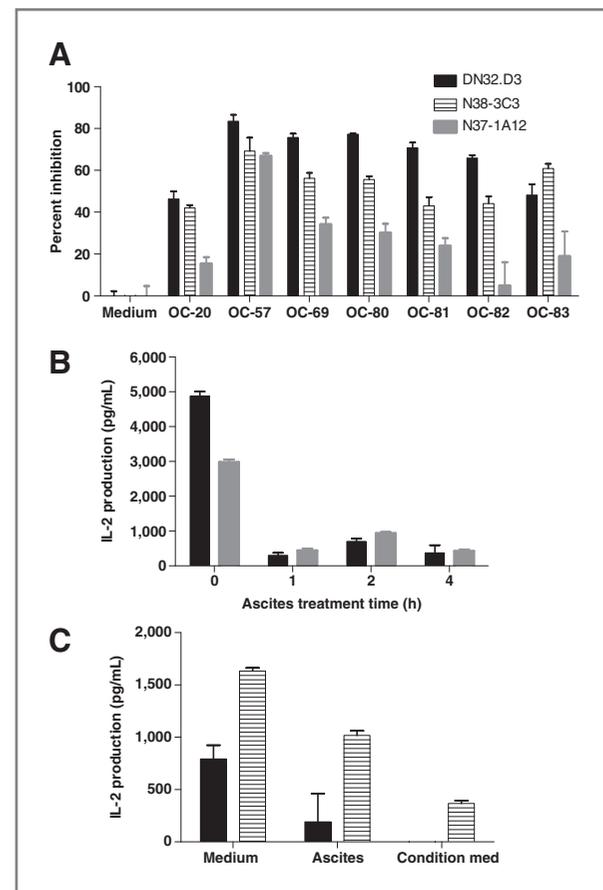


Figure 1. Tumor ascites inhibits CD1d-mediated NKT cell activation. A, LCD1dwt cells were treated with control medium or with ovarian cancer ascites fluid from patients for 4 hours, then washed extensively and cocultured with a panel of NKT cell hybridomas (DN32.D3, N38-3C3, and N37-1A12). After 20 to 24 hours, IL-2 was measured as an indication of NKT cell activation using standard cytokine ELISA. B, the effect of ascites on NKT cell recognition of CD1d1 molecules was rapid. LCD1d wt cells were incubated with ascites for the indicated times. The cells were then cocultured with NKT cell hybridomas and IL-2 release was measured. C, conditioned medium from ascites-derived cells was inhibitory. LCD1dwt cells were incubated for 4 hours with fresh cell culture medium, ascites fluid, or conditioned medium from ascites-derived cells from patient OC-95. Treated APCs were then cocultured with NKT cell hybridomas and IL-2 release was measured. Data are shown as mean \pm SEM of 1 experiment set up in triplicate. The experiments were carried out at least twice with each ascites sample.

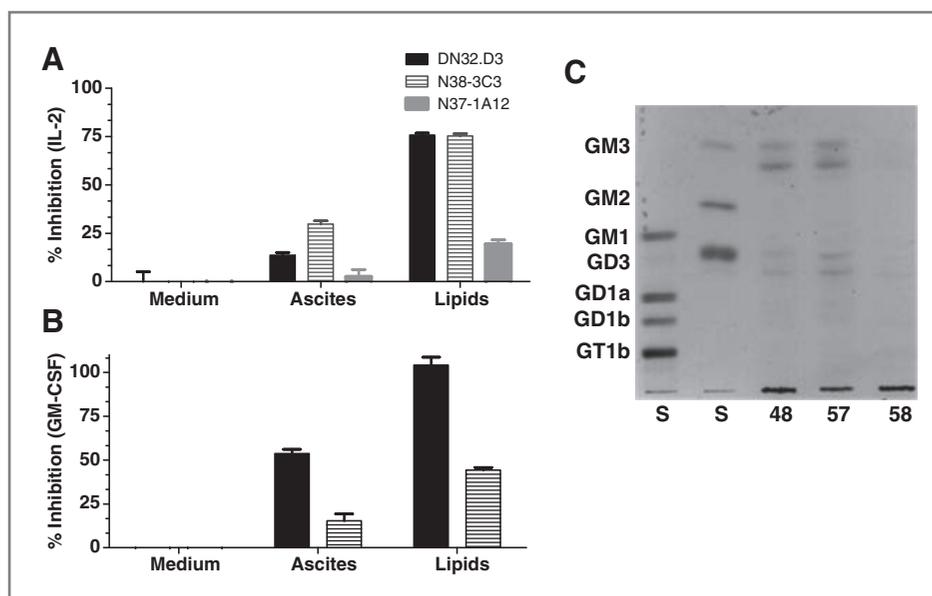


Figure 2. The polar lipid fraction of ascites inhibits NKT cell activation. The polar lipid fraction was isolated from the ascites of patients with ovarian cancer. LCD1dwt cells were treated with ascites or the polar lipid fraction of the ascites for 4 hours, then washed extensively and cocultured with a panel of NKT cell hybridomas. A, IL-2 production was measured as an indication of NKT cell activation. Data are shown as mean \pm SEM. In addition, treatment with the polar lipid fraction significantly inhibited all hybridomas examined ($P < 0.001$). B, GM-CSF production was measured as an indication of NKT cell activation. Data are shown as mean \pm SEM. C, TLC of the polar lipid fraction. The extracted lipids were analyzed by TLC. Gangliosides were detected with a resorcinol-HCl Cu_2^+ reagent. Bovine brain gangliosides were used as standards as indicated at the left. Tumor gangliosides typically appear as doublets due to heterogeneity in their ceramide lipid moieties.

α -GalCer for 10 days. After being purified with lympholyte-M (Accurate Chemical), cells were restimulated with immature DCs in the presence of 100 $\mu\text{g}/\text{mL}$ α -GalCer and 10 $\mu\text{g}/\text{mL}$ mouse IL-2 (Cell Sciences) for 1 week. After 3 cycles of stimulations, more than 95% of NKT cells were shown to react with mouse CD1d dimer loaded with α -GalCer by FACS.

Generation of artificial APC

The preparation of CD1d-Ig-based aAPC was carried out according to the previously described method (16). The hCD1d-aAPCs were loaded with lipid antigen and α -GalCer (5 $\mu\text{g}/\text{mL}$ in 1 mL PBS containing 5×10^7 beads; Axxora, LLC).

Treatment of cells with tumor-associated ascites

The ascites was cleared of cellular debris by centrifugation as previously described (17). The APCs were treated with the clarified supernatants for 4 hours at 37°C, unless otherwise indicated. The APCs were subsequently washed extensively with PBS, and cocultured with NKT hybridomas for 20 to 24 hours at 37°C. IL-2 was measured as an indication of NKT cell activation and was measured by standard sandwich ELISA. For the conditioned medium from ascites derived cells, 50 mL of ascites was centrifuged at 1,500 rpm for 5 minutes. The ascites fluid was removed and the remaining cell pellet was cultured in 50 mL of RPMI supplemented with 2 mmol/L L-glutamine (BioWhittaker), 10% FBS (HyClone), and ciprofloxacin (SeroLogic Proteins) in a T-175 flask for 4 to 7 days, until the adherent cells reached confluency, then the cultured supernatant was

harvested, centrifuged to remove cellular debris, and used for experiments.

Extraction of the polar lipid fraction from the ascites

The polar lipid fraction was isolated from the ascites of the indicated patients with ovarian cancer. Methanol and then chloroform were added to give chloroform-methanol-water (4:8:3), and the samples were extracted by stirring the mixture at ambient temperature. Insoluble material was removed by centrifugation, and water was then added to the supernatant to give chloroform-methanol-water (4:8:5.6). The resulting phases were separated by centrifugation and the upper polar lipid phase was desalted using a Sep-Pak C18 cartridge (18). The extracted lipids were analyzed by thin-layer chromatography (TLC; HPTLC Silica Gel 60; Merck) with chloroform-methanol-0.25% aqueous KCl (60:35:8) as running solvent. Gangliosides were detected with a resorcinol-HCl Cu^{2+} reagent. Bovine brain gangliosides were used as standards. The commercially available, purified gangliosides used in the NKT cell assays: Gg3Cer, GM2, GM3, GD3 (Matreya), and GD2 (Calbiochem) were reconstituted in either methanol or chloroform-methanol as suggested by the manufacturer.

Determination of serum cytokine concentrations

The serum concentrations of IFN- γ , IL-12 p70, and IL-4 were measured 2, 6, 12, 24, 48, and 72 hours after treatment with α -GalCer and different concentrations of GD3 by standard sandwich ELISA as previously described (19).

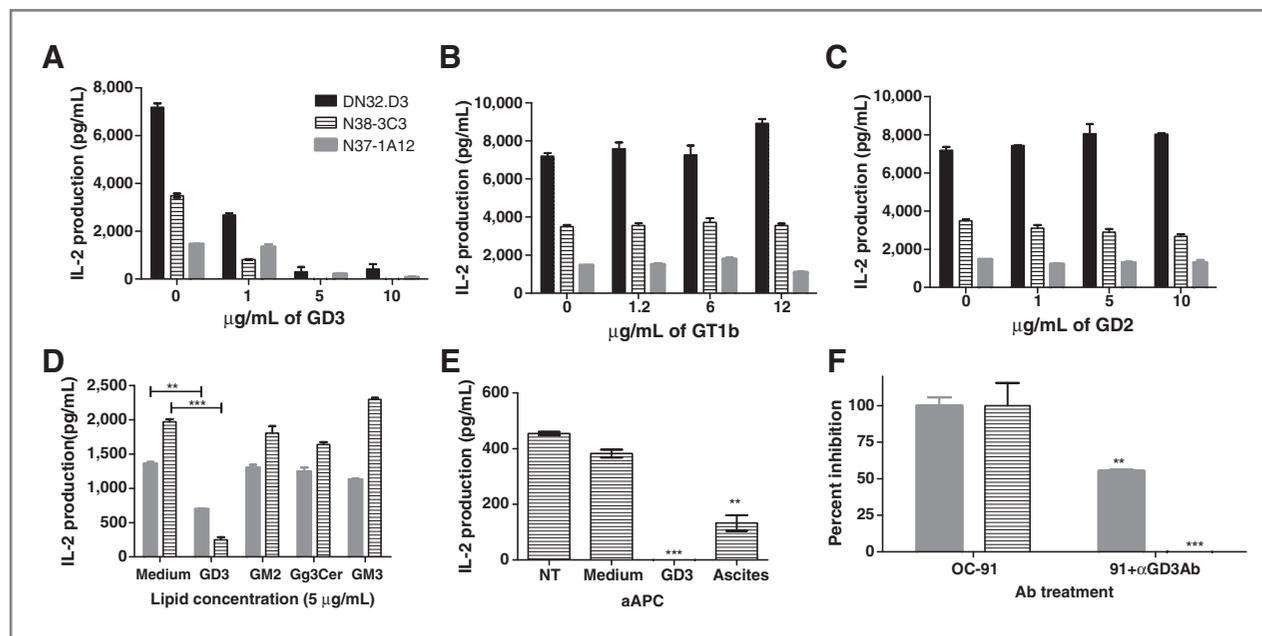


Figure 3. Ganglioside treatment alters CD1d-mediated NKT cell activation. LCD1dwt cells were treated with GD3 (A), GT1b (B), or GD2 (C) at the indicated concentrations for 4 hours, washed extensively, and cocultured with NKT cell hybridomas, DN32.D3, N37-1A12, and N38-3C3 overnight. D, LCD1dwt cells were treated with the indicated gangliosides (5 $\mu\text{g/mL}$) for 4 hours, washed, and cocultured with NKT cell hybridomas. Pretreatment with the indicated purified gangliosides altered CD1d-mediated NKT cell activation. E, treatment of α -GalCer-loaded CD1d-Ig aAPC with GD3 inhibits antigen presentation. α -GalCer-loaded aAPC were incubated for 4 hours with either medium, GD3 (5 mg/mL), or ascites fluid. The aAPC were washed extensively and then cocultured with the Va14⁺ NKT cell hybridoma, N38-3C3. F, addition of anti-GD3 mAb to tumor-associated ascites restores NKT cell recognition of CD1d molecules. LCD1dwt cells were treated with ascites (OC-91) or the ascites pretreated with an antibody specific for GD3 (clone R24) for 4 hours, then washed extensively and cocultured with a panel of NKT cell hybridomas. IL-2 was measured, as an indication of NKT cell activation, by standard cytokine ELISA. Data are shown as mean \pm SEM of 1 experiment set up in triplicate. **, $P \leq 0.01$; ***, $P \leq 0.001$. The experiments were carried out 3 times.

Staining of human *i*NKT cells with CD1d-mIgG dimers

One microgram of human or mouse CD1d-mouse IgG dimer was incubated with 1.4 μg α -GalCer, GD3, or ascites in 50 μL of PBS at 37°C overnight to load the lipid onto hCD1d-mIgG dimer and used to stain 2×10^5 Human *i*NKT cells on ice for 30 minutes. Then the cells were washed with PBS containing 5% FBS twice and incubated with phycoerythrin (PE)-labeled rat anti-mouse IgG1 antibody (A85-1) and allophycocyanin-labeled anti-human CD3 ϵ antibody (BD Biosciences) on ice for 30 minutes. After washing, the stained cells were analyzed with FACSCalibur System (BD Biosciences). Flow cytometric data were analyzed with FlowJo v8.8 software (Tree Star, Inc.).

Competitive ELISA assay

The assay was conducted as previously described (20). Briefly, Nunc MaxiSorp flat-bottom 96-well plates (Thermo Fisher Scientific) were coated with 100 μL of goat anti-mouse IgG Fc gamma antibody (Biomedica). The plates were washed 3 times with PBS/Tween and 100 μL of lipid-CD1 dimer mixture was added to the plates immediately after washing. The mixture solution was prepared by mixing CD1dimer (5 $\mu\text{g/mL}$) and lipids in the presence of biotinyl PE (2 $\mu\text{g/mL}$) in PBS. The CD1-biotinyl PE complex was detected with horseradish peroxidase (HRP)-labeled Avidin (eBioscience). K_d of biotinyl PE was determined by titrating the amount of biotinyl PE to reach the maximum binding in the absence of competitors and applying the following equation to the data; $Y = (B_{\text{max}} \times X) / (K_d + X)$. K_d of biotinyl PE and K_i of PE and α -GalCer were

calculated using GraphPad Prism (Ver.4.02; GraphPad Software, Inc.).

Intracellular cytokine staining

Two hours after the injection of lipids, liver mononuclear cells were isolated (21) and cultured *ex vivo* in the presence of GolgiStop (BD Pharmingen) for 4 hours. Cells were stained with anti-TCR β -FITC, anti-NK1.1-PE, anti-CD45-APC-Cy7 (eBioscience), fixed, permeabilized (PermWash, BD), and stained intracellularly for IFN- γ . Dead cells were excluded (LIVE/DEAD Fixable Violet Dead Cell Stain Kit, Invitrogen).

Statistical analysis

Data analysis was conducted by Prism software (version 5.02 for Windows; GraphPad). Parametric statistics were used to analyze differences between experimental groups, when needed. Where multiple groups existed within a single experiment, multiple between-group comparisons were made by ANOVA with the Bonferroni posttest. A P value less than 0.05 was considered significant. The error bars in the bar graphs show the SEM.

Results

Pretreatment with tumor-associated ascites rapidly inhibits CD1d-mediated activation of NKT cells

Incubation of CD1d-expressing L cells with ovarian cancer ascites fluid inhibited stimulation of NKT

hybridomas, as measured by the release of IL-2 (Fig. 1A). Treatment with ascites for 1 hour maximally inhibited the ability of LCD1dwt to stimulate NKT cells (Fig. 1B). Conditioned medium from cultured cells isolated from ascites also inhibited antigen presentation to both canonical ($V\alpha 14^+$, DN32.D3) and noncanonical ($V\alpha 5^+$, N37-1A12) NKT cell hybridomas (Fig. 1C). These studies suggest that cells present in the ascites actively produce substances that abrogate CD1d-mediated activation of NKT cells. To determine whether incubation with ascites-treated cells resulted in a permanent functional defect in NKT cell activation, fresh untreated LCD1dwt cells were added to NKT cells previously exposed to ascites-pretreated APCs. Addition of untreated cells activated NKT cells to produce cytokines (data not shown), showing that stimulation of NKT cells with ascites-treated LCD1dwt cells did not permanently impair NKT cell function.

Polar lipids present in ascites inhibit NKT cell recognition of endogenous antigen

Because ascites of patients with ovarian cancer is rich in gangliosides, we hypothesized that these lipids are responsible for the inhibition of NKT cell activation. The polar lipid fraction was extracted from the ascites and found to significantly reduce the ability of the APC to stimulate NKT cells (Figs. 2A and B). In fact, the reduction by the polar lipid fraction was greater than the inhibition caused by ascites pretreatment. Analysis of the polar lipid fraction by TLC showed that the majority of patient samples contained the gangliosides GM3, GD3 and minor amounts of more complex gangliosides (Fig. 2C).

Identification of GD3 as an inhibitory lipid in ovarian cancer ascites

To test which of the observed gangliosides is responsible for the inhibition associated with ascites, APCs were incubated with increasing doses of purified GD3 and the related gangliosides including GT1b and GD2 (Supplementary Fig. S1 for structures; refs. 22, 23), washed and cocultured with NKT cells (Fig 3A–C). Only GD3 inhibited CD1d-mediated activation of NKT cells in a dose-dependent fashion. Other tumor-associated glycolipids, such as GM3 (the biosynthetic precursor of GD3) and the neutral glycosphingolipid gangliotriaosylceramide (Gg3Cer), have been reported to inhibit canonical NKT cell activation when used at high concentrations (9, 24). Therefore, we also compared the ability of GD3, GM3, Gg3Cer, and GM2 to inhibit NKT cells (Fig. 3D). We found that only incubation with GD3 resulted in a significant reduction in NKT cell activation.

To determine whether the inhibition observed following pretreatment with GD3 required antigen processing, we used CD1d-Ig-based aAPC. CD1d-Ig-based aAPC were loaded with α -GalCer (16) and the loaded aAPCs were then treated with control medium, ascites fluid, or purified gangliosides. Treatment with GD3 completely inhibited α -GalCer presentation by aAPCs whereas treatment with control medium had only minimal effects on aAPC-mediated NKT cell activation

(Fig. 3E). To further show that the presence of GD3 in ovarian cancer-associated ascites was responsible for the loss of NKT cell activation, we pretreated ascites with a monoclonal Ab specific for GD3. The presence of antibodies against GD3 significantly blocked the ascites-mediated NKT cell inhibition (Fig. 3F). Taken together, these data show that GD3 in ovarian cancer ascites fluid inhibits NKT activation and does not require further processing for its inhibitory effects.

In vivo treatment with GD3 abrogates α -GalCer-induced cytokine secretion by NKT cells

To test the effects of GD3 *in vivo*, we used an *in vivo* NKT cell activation model in which α -GalCer injection results in cytokine release by NKT cells. Mice were injected with α -GalCer, in the presence or absence of different concentrations of GD3. Blood samples were obtained at various time points, and serum cytokine levels were assessed. IFN- γ production was detectable at 2 hours, with the peak production at 24 hours after treatment and returned to baseline by 48 hours (Fig. 4A). Treatment with GD3 resulted in a dose-dependent inhibition of

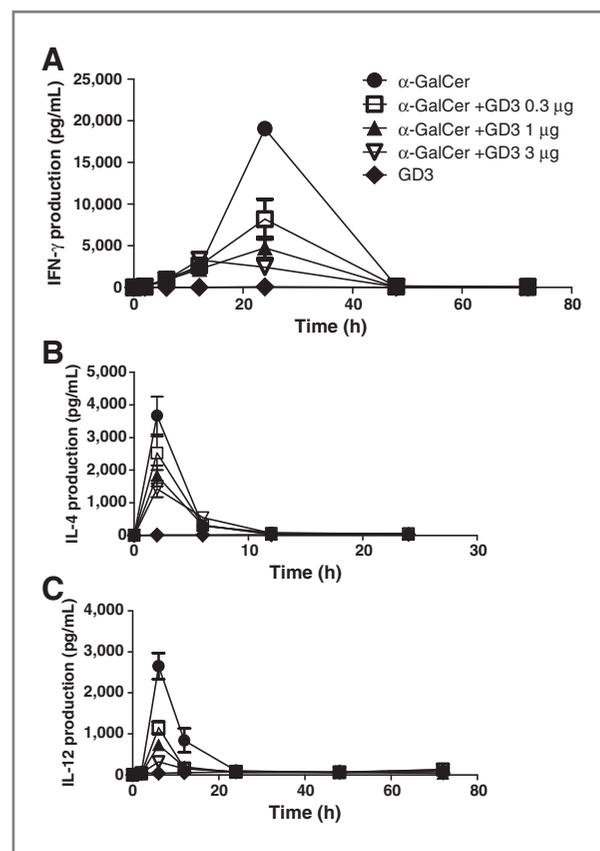


Figure 4. GD3 inhibits α -GalCer-induced cytokine production *in vivo*. A, wild-type (WT) C57BL/6 mice were treated intravenously with 1 μ g of either α -GalCer in the presence or absence of the indicated concentrations of GD3, and serum samples were obtained at the indicated time points after injection for ELISA analyses of IFN- γ , IL-4 (B), and IL-12 (C) concentrations. These data are expressed as the mean \pm SD of 2 different dilutions of pooled sera. In all figures, these data represent 1 of 2 or more experiments with similar results.

α -GalCer-induced IFN- γ production with half-maximal inhibition seen at 0.3 μ g GD3. *In vivo* administration of GD3 also inhibited IL-4 (Fig. 4B) and IL-12 production by α -GalCer-stimulated NKT cells, as shown in Fig. 4C.

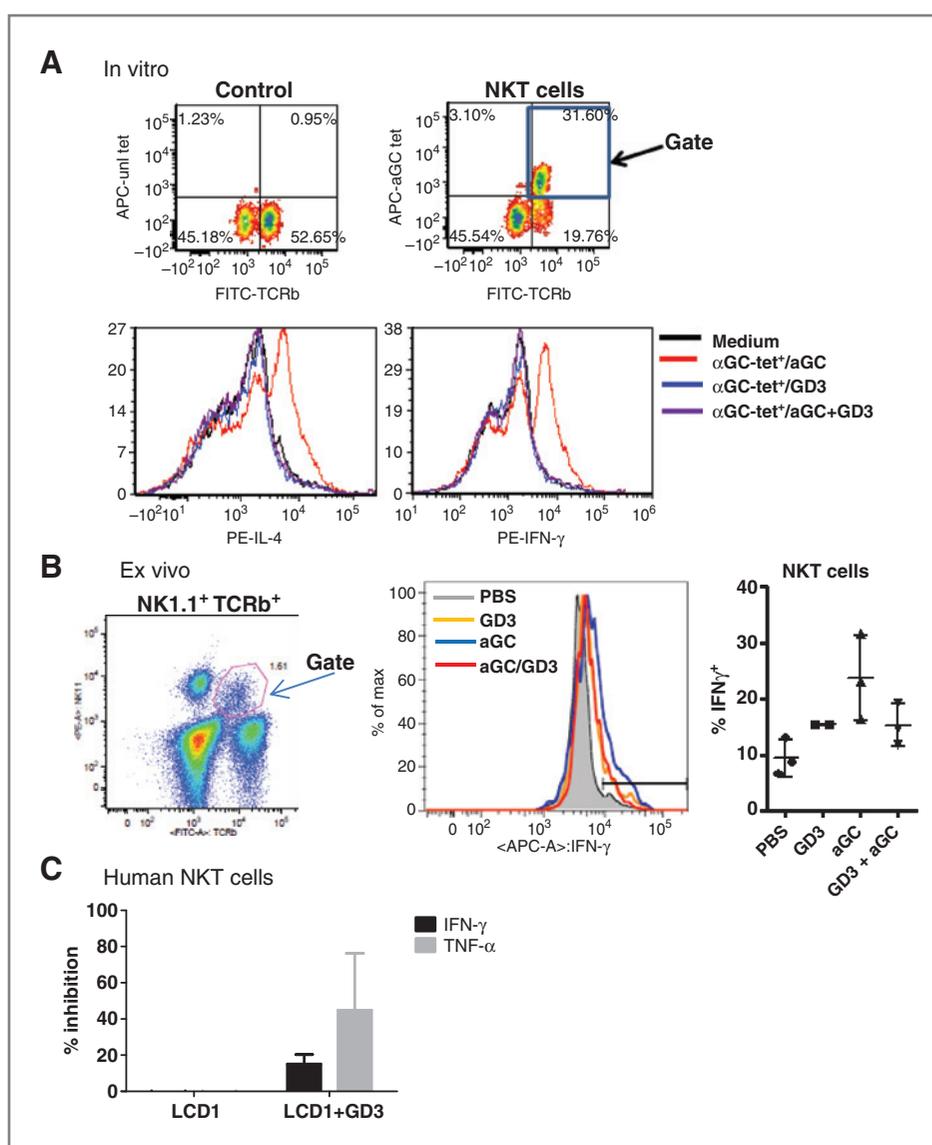
To determine whether the GD3-mediated inhibition of α -GalCer-induced cytokine production was directly due to the suppression of NKT cell function, we next sought to determine whether GD3 could inhibit freshly prepared bulk NKT cells. Liver mononuclear cells were isolated from C57BL/6 wild-type mice and pulsed with antigen or cultured in medium alone as a control and cytokine production was assessed by intracellular cytokine staining (Fig. 5A). To confirm whether reduced serum cytokine levels were indeed due to impaired production by NKT cells in the presence of GD3, liver mononuclear cells were harvested 2 hours after injection of α -GalCer and GD3 or the combination of both. Assessment of IFN- γ production by intracellular cytokine staining revealed that

coadministration of GD3 with aGC reduced IFN- γ production by NKT cells (Fig 5B). Primary human NKT cells were also cocultured with APCs, which had been pretreated with GD3 (Fig. 5C). α -GalCer-induced cytokine production was reduced in primary murine and human NKT cells following the addition of GD3.

GD3 binds with high affinity to CD1d molecules

To analyze the mechanism of the inhibition, we studied the binding of GD3 to CD1d molecules using a competitive-binding assay (20). A dose-dependent competition by GD3 to the tagged lipid 18:1 biotinyl PE was observed for both human and mouse CD1d molecules (Figs. 6A and B). The IC₅₀ values (24 nmol/L for hCD1d and 11 nmol/L for mCD1d) indicate that purified GD3 binds with significantly higher affinity to CD1d than PE (IC₅₀ > 800 nmol/L, Table 1). Notably, the apparent affinity of GD3 was higher than that

Figure 5. Ganglioside GD3 treatment inhibits the activation of primary NKT cells. **A**, liver mononuclear cells were harvested from C57BL/6 mice and were stained with either unloaded tetramer or α -GalCer-loaded tetramer and mAbs specific for TCR β (top). The CD1d-tetramer⁺TCR β ⁺ population was gated following stimulation with α -GalCer and induction of IL-4 (bottom right) or IFN- γ (bottom left) by NKT cells was assessed by flow cytometry. Data are from 1 experiment, representative of 3 independent experiments. **B**, *ex vivo* studies examining the effects of GD3 on α -GalCer-mediated NKT cell activation. C57BL/6 mice were injected with 0.1 μ g aGC, 2 μ g GD3, or both. The liver mononuclear cells were isolated and stained for intracellular IFN- γ production. **C**, primary human NKT cells were cocultured with LCD1dwt cells pretreated with GD3.



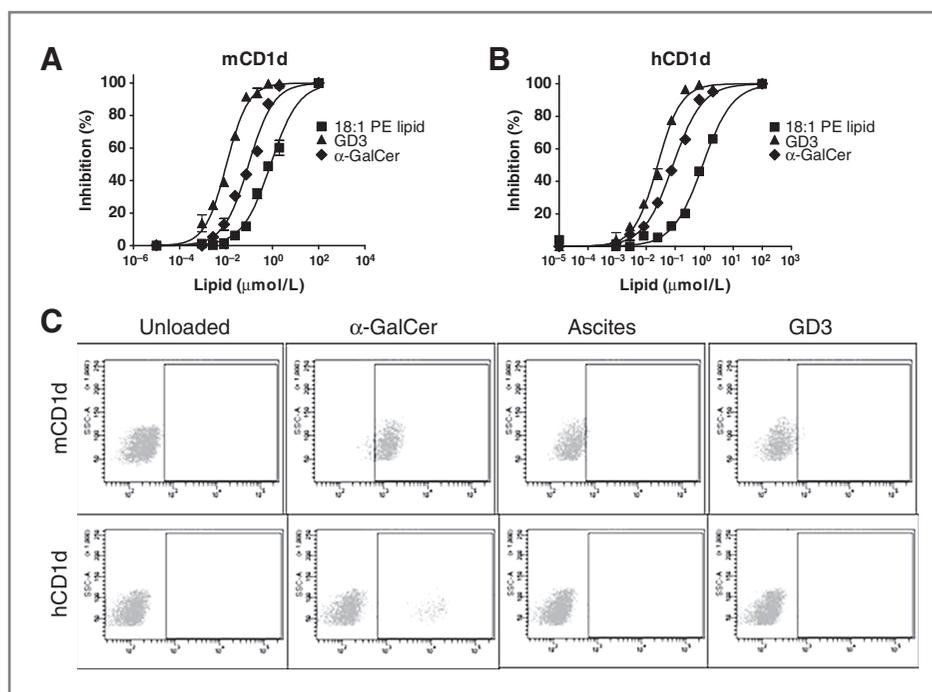


Figure 6. GD3 competes with PE for binding to CD1d. A and B, Nunc MaxiSorp flat-bottom 96-well plates were coated with goat anti-mouse IgG Fc gamma antibody and the plates were washed and blocked. Then, the wells were coated with CD1d:IgG1 dimer, after washing, serially diluted lipids were added into the wells in the presence of 2 $\mu\text{g}/\text{mL}$ biotinyl PE. The plates were then washed and the amounts of CD1d:mIgG1-biotinyl PE complex were detected by adding HRP-labeled Avidin. Comparisons were done among all of the lipids (GD3, 18:1PE, and α -GalCer) and the binding affinity between any 2 lipids is significantly different. C, GD3-loaded CD1d dimeric molecules do not stain NKT cells. Mouse CD1d:Ig dimer was incubated with α -GalCer, ascites, or GD3 to load the lipid onto the dimer, then used to stain NKT cells. The cells were washed and incubated with PE-labeled rat anti-mouse IgG1 antibody (A85-1). After washing, the stained cells were analyzed by flow cytometry. Human CD1d:Ig dimer was incubated with α -GalCer, ascites, or GD3 to load the lipid onto the dimer, then used to stain primary NKT cells. The differences observed in binding between α -GalCer-loaded mouse and human CD1d:Ig dimers may be due to the use of primary human T cells, rather than NKT cell hybridomas. In all figures, these data represent 1 of 2 or more experiments with similar results.

of α -GalCer (84 nmol/L for hCD1d and 95 nmol/L for mCD1d), this could be due to intrinsic differences in the affinities or differences in solubility. As this assay has been shown to reflect lipid selectivity by CD1 molecules, these data indicate that GD3 binds strongly to both human and mouse CD1d molecules.

We next examined whether CD1d-Ig dimers loaded with GD3 or ascites fluid could bind to NKT cells (Fig. 6C). Whereas the controls, α -GalCer-loaded human and mouse CD1d-Ig dimers, bound to NKT cells, CD1d-Ig dimers loaded with GD3 or ascites did not. These data may explain the inhibitory effect mediated by ascites treatment. While GD3 binds with high affinity to CD1d molecules, those complexes do not bind to or activate NKT cells; thus GD3 present in the ascites may compete with and displace the endogenous

stimulatory ligands for binding to CD1d molecules and thereby inhibit activation of NKT cells by their natural stimulatory ligands.

Discussion

Previous studies implicated various roles for specific T-cell subsets in ovarian cancer (4–8). Preclinical studies and clinical trials have shown that immunotherapy can effectively treat cancer; however, ovarian cancer immunotherapy needs to also focus on removing negative factors that could inhibit the immune response to maximize its therapeutic effects. In the current study, we identified an inhibitory CD1d-binding ligand present in ovarian cancer ascites as the ganglioside GD3. Mechanistically, we found that GD3 inhibited the binding of

Table 1. Relative binding affinity of GD3 to human and mouse CD1d molecules

Ligand (competitor)	IC ₅₀ in hCD1d, nmol/L	IC ₅₀ in mCD1d, nmol/L
18:1 PE lipid	882 \pm 125	843 \pm 181
GD3 (CHCl ₃ + CH ₃ OH)	24.6 \pm 3.2	10.5 \pm 1.5
α -GalCer	84.0 \pm 10.0	95.1 \pm 18.3

CD1d-Ig dimers loaded with α -GalCer to NKT cells. Furthermore, GD3 bound with an "apparent" high affinity to both human and mouse CD1d. Finally, *in vivo* treatment with GD3 inhibited α -GalCer-mediated NKT cell activation. These studies thus identify GD3 as the first high affinity, competitive ligand that inhibits the activation of NKT cells *in vitro* and *in vivo*.

Specific gangliosides, such as GD3 and GM3, have been previously suggested to play a role in regulating NKT cell function. GD3 expression is upregulated on human melanomas and when mice were immunized with GD3-loaded DC, low levels of NKT-specific GD3 response have been observed (25). A small fraction of the GD3-specific NKT cells can also crossreact with GM3. Analysis of the GM3 response showed that it partially inhibited some of the NKT-specific GD3 response, furthermore it had minimal effect on the α -GalCer-induced IL-4 response and no effect on α -GalCer-induced IFN- γ responses. In contrast, we did not find a GD3-specific NKT cell response but rather that spontaneously secreted GD3 was a natural inhibitor of canonical, as well as noncanonical NKT cells. The difference between the 2 systems could be the fact that in the previous work, the goal was to see whether GD3 could serve as a target for immunotherapy and in those experiments animals were immunized with DC-loaded GD3. In contrast, we looked at α -GalCer driven NKT cell responses *in vitro* and *in vivo* and found inhibition by GD3. This is consistent with the potential role of GD3 in the setting of ovarian cancer, where it could inhibit the endogenous NKT cell response and is consistent with the finding that ganglioside secretion is associated with decreased survival rates.

Using a quantitative binding assay, we measured the affinity of GD3 for CD1d molecules. The affinity of GD3 was high at 84 and 95 nmol/L for human and mouse, respectively. In contrast most other naturally occurring ligands such as isoglobotrihexosylceramide (iGb3) are thought to be relatively low affinity ligands for CD1d, as determined by functional assays. Even glycolipids isolated from foreign pathogenic bacteria such as *Sphingomonas* are not as active as α -GalCer and are thought to be lower affinity ligands (13, 26, 27). The high affinity of GD3 for human and mouse CD1d may enable it to displace most NKT activating ligands, making it a uniquely potent immunomodulatory substance.

The proliferative index and metastatic status of malignant melanomas, renal cell carcinoma, and head and neck tumors are correlated with excessive synthesis of GD3 (28, 29). Although GD3 is expressed at a low level on normal melanocytes and a few other cell types, its highly restricted expression select tumor type makes it an attractive therapeutic target. In fact, it has been used for passive and active immunotherapy of melanomas and small-cell lung cancers (30–32, 33). Our data suggest that therapeutic strategies targeting GD3 may also be useful for the treatment of ovarian cancer.

To our knowledge, this is the first report showing a mechanism by which ovarian cancer-derived ascites inhibits NKT cell activation. Furthermore, GD3 is the only known endogenous ligand with such a high affinity for CD1d. Therefore, the presence of GD3 in the ascites, and its impact on the activation of NKT cells, may be a prognostic factor for ovarian cancer. While additional work is necessary, these studies indicate that GD3 may be important for early detection and monitoring of ovarian cancer.

Disclosure of Potential Conflicts of Interest

R.L. Schnaar is the consultant/advisory board member for Zacharon Pharmaceuticals. The authors T.J. Webb, M. Oelke, and J.P. Schneck have filed a patent application on the use of GD3 as a biomarker or therapeutic target for ovarian cancer. No potential conflicts of interest were disclosed by the other authors.

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Molecular Identification of GD3 as a Suppressor of the Innate Immune Response in Ovarian Cancer

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