

Fine specificity of natural killer T cells against GD3 ganglioside and identification of GM3 as an inhibitory natural killer T-cell ligand

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Summary

GD3, a ganglioside expressed on melanoma, is the only tumour-associated glycolipid described to date that can induce a CD1d-restricted natural killer T (NKT)-cell response. We analysed the fine specificity of GD3-reactive NKT cells and discovered that immunization with GD3 induced two populations of GD3-reactive NKT cells. One population was CD4⁺ CD8⁻ and was specific for GD3; the other population was CD4⁻ CD8⁻ and cross-reacted with GM3 in a CD1d-restricted manner, but did not cross-react with GM2, GD2, or lactosylceramide. This indicated that the T-cell receptors reacting with GD3 recognize glucose-galactose linked to at least one *N*-acetyl-neuraminic acid but will not accommodate a terminal *N*-acetylgalactosamine. Immunization with GM2, GM3, GD2, or lactosylceramide did not induce an NKT-cell response. Coimmunization of GM3-loaded antigen-presenting cells (APCs) with GD3-loaded APCs suppressed the NKT-cell response to GD3 in a CD1d-restricted manner. This suppressive effect was specific for GM3 and was a local effect lasting 2–4 days. *In vitro*, GM3-loaded APCs also suppressed the interleukin-4 response, but not the interferon- γ response, of NKT cells to α -galactosylceramide. However, there was no effect on the T helper type 2 responses of conventional T cells. We found that this suppression was not mediated by soluble factors. We hypothesize that GM3 induces changes to the APC that lead to suppression of T helper type 2-like NKT-cell responses.

Keywords: CD1d; α -galactosylceramide; ganglioside; GM3; natural killer T cell

Introduction

Natural killer T (NKT) cells constitute a unique population of T cells that express both T-cell receptors (TCR) and C-type lectin receptors characteristic of NK cells. In mice, the TCRs of type I NKT cells express the invariant TCR-V α chain V α 14-J α 18 combined with limited TCR-V β chains.^{1–3} The antigens recognized by type I NKT cells are glycolipids presented by CD1d that share a common structural motif: a hydrophilic head group and two hydrophobic acyl chains.^{4,5} CD1d, the only CD1 isoform expressed in the mouse, has two hydrophobic pockets

that accommodate the hydrophobic acyl tails of the glycolipid molecules, allowing the hydrophilic carbohydrate portion to be presented to the TCRs.^{4,5}

Much of what is known about NKT-cell function comes from studies utilizing α -galactosylceramide,^{3,6,7} a glycolipid from the marine sponge *Agelas mauritanus* that is presented by CD1d and can activate most type I NKT cells. Among the natural glycolipids identified so far that can be recognized by NKT cells, most are from prokaryotes.^{8–13}

We have reported that GD3 ganglioside, a glycolipid that is highly expressed on human tumours of

Abbreviations: APC, antigen-presenting cell; DMSO, dimethylsulphoxide; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot-forming cell assay; α GalCer, α -galactosylceramide; GalNAc, *N*-acetylgalactosamine; IFN- γ , interferon- γ ; iGb3, isoglobotrihexosylceramide; IL-4, interleukin-4; LacCer, lactosylceramide; mAb, monoclonal antibody; MHC, major histocompatibility complex; NANA, *N*-acetyl-neuraminic acid; NKT, natural killer T; OVA, ovalbumin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TCR, T-cell receptor; TGF- β , transforming growth factor- β ; Th2, T helper type 2.

neuroectodermal origin such as melanoma and small-cell lung carcinoma, can be cross-presented by CD1d on antigen-presenting cells (APCs) and can induce a CD1d-restricted GD3-reactive NKT-cell response in the mouse.¹⁴ This is the only example to date of NKT cells recognizing a glycolipid expressed by human tumours, indicating the potential for NKT cells to recognize cancer cells.

The specificity of NKT cells against mammalian glycolipids has not been previously described. In the current study, we examined the fine specificity of the GD3-reactive NKT cells and explored the requirement of carbohydrate components for NKT-cell recognition of GD3. In these studies, we discovered that GM3, a ganglioside structurally related to GD3, suppresses the T helper type 2 (Th2) -like response of NKT cells. This is the first inhibitory NKT-cell ligand demonstrated to function *in vivo*.

Materials and methods

Mice

Female inbred C57BL/6 mice, 6–8 weeks old, purchased from the Jackson Laboratory (Bar Harbor, ME) were used throughout this study. CD1d^{-/-} mice with a C57BL/6 background were kindly provided by Dr Steven P. Balk (Harvard Medical School, Boston, MA). OT-II mice, transgenic for T cells against major histocompatibility complex (MHC) class II restricted ovalbumin peptide (OVA_{323–339}; ISQAVHAAHAEINEAGR), were purchased from the Jackson Laboratory. Mice were maintained in the Memorial Sloan-Kettering Cancer Center (MSKCC) animal facility under specific pathogen-free conditions. The studies were reviewed and approved by the Institutional Animal Care and Use Committee.

Glycolipids

GD3 [*N*-acetyl-neuraminic acid (NANA) α 2 $\rightarrow$ 8NANA α $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer], GM3 (NANA α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc-Cer), GM2 [GalNAc β 1 $\rightarrow$ 4(NANA α 2 $\rightarrow$ 3)Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer], and lactosylceramide (LacCer) (Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer) were purchased from Matreya, Inc. (Pleasant Gap, PA) and were $\geq$ 98% pure by thin-layer chromatography. GD2 [GalNAc β 1 $\rightarrow$ 4(NANA α 2 $\rightarrow$ 8NANA α 2 $\rightarrow$ 3) Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer] was purchased from Sigma (St Louis, MO). We confirmed that GD3 was negative for endotoxin by *Limulus* amoebocyte lysate assay. The α GalCer was obtained from Dr Chi-Huey Wong (Scripps Research Institute, La Jolla, CA). Direct binding of gangliosides to CD1d was measured by isoelectrofocusing (IEF) electrophoresis using previously described methods.¹⁵

To prepare GM3 and GM2 glycans, 75 μ g ganglioside was dried under N₂ and resuspended in 0.4 ml 50 mM

acetate buffer, pH 5.0 containing 0.75 mg/ml sodium cholate. Ceramide glycanase (V-Labs, Inc., Covington, LA) was added (0.4 U) and incubated at 37° for 3 hr. Undigested ganglioside was removed by extraction with chloroform : methanol (2 : 1). The aqueous-phase material was dried by vacuum centrifugation and resuspended in water. The glycan was further purified by preparative thin-layer chromatography. The purity of the preparation was confirmed by thin-layer chromatography and glycan was quantified by measuring sialic acid spectrophotometrically.

Cell preparation

A single-cell suspension of splenocytes was prepared as described previously.¹⁴ The APCs were splenocytes from mice injected subcutaneously with 10 μ g Flt3 ligand (kindly provided by Amgen, Thousand Oaks, CA) daily for 9 consecutive days. Splenocytes were washed, and suspended in complete RPMI-10. The APCs were enriched by incubating the splenocytes in plastic culture dishes for 2 hr at 37°. The adherent cells were pulsed with ganglioside antigen GD3, GM3, GM2 [1 μ g/ml in 0.1% dimethyl sulphoxide (DMSO)], GM3-glycan (5 μ g/ml), GM2-glycan (6.2 μ g/ml), α GalCer (200 ng/ml) or with control vehicle (0.1% DMSO). After overnight incubation at 37°, the non-adherent cells were collected, washed, and used as APCs.

Immunization protocol

Mice were injected subcutaneously into footpads with 10⁵ APCs loaded with GD3, GM3, GD2, LacCer (1 μ g/ml in 0.1% DMSO) or unloaded APCs. In some experiments, 10⁵ GM3-loaded APCs were mixed with 10⁵ GD3-loaded APCs, and were injected into the footpad. Seven days after the injection, splenocytes were collected and processed. To immunize against SIINFEKL peptide, mice were immunized with APCs loaded with SIINFEKL peptide injected into the footpad.

For experiments testing the time-course of the GM3-mediated inhibitory effect, mice were injected into the footpad with 10⁵ GM3-loaded APCs on day 0, then injected with 10⁵ GD3-loaded APCs on day 0, 2, 4 or 7. Seven days after injecting GD3-loaded APCs, splenocytes were collected and processed.

An anti-transforming growth factor- β (TGF- β) antibody (clone 1D11.16.8) and an isotype control antibody (clone 14C3) were obtained from the monoclonal antibody (mAb) facility of MSKCC and administered by intraperitoneal injection. In some experiments, 200 μ g antibody was injected 6 hr before injection with GM3-loaded APCs, 100 μ g 2 days after the APC injection, and also on day 4 and day 6 after the APC injection. Seven days after APC injection, mice were killed and their

splenocytes were tested by enzyme-linked immunosorbent spot-forming cell assay (ELISPOT). Binding of TGF- β antibody to TGF- β 1 was confirmed by Western blot using purified TGF- β 1 (R&D Systems, Minneapolis, MN).

ELISPOT and enzyme-linked immunosorbent (ELISA) assays

Multiscreen-IP plates (Millipore, Burlington, MA) were coated with 100 μ l anti-mouse interleukin-4 (IL-4) mAb (10 μ g/ml; clone BVD4-1D11; BD Biosciences, San Jose, CA), or anti-mouse interferon- γ (IFN- γ) mAb (10 μ g/ml; clone AN18; MabTech, Nacka Strand, Sweden) in phosphate-buffered saline (PBS), incubated overnight at 4°, washed with PBS to remove unbound antibody, and blocked with medium for 2 hr at 37°. Splenocytes were plated in triplicates at a density of 2×10^5 /well. Effector cells were stimulated with 5×10^4 APCs in 100 μ l pulsed with 1 μ g/ml ganglioside or with control vehicle (0.1% DMSO). Control wells contained effector cells alone, APCs alone, or medium alone. After incubation for 20 hr at 37°, plates were extensively washed with PBS plus 0.05% Tween-20, followed by incubation with 2 μ g/ml biotinylated rat anti-mouse IL-4 (clone BVD6-24G2, BD Biosciences) or rat anti-mouse IFN- γ (clone R4-6A2, MabTech) for 2 hr at 37°. Spot development was performed as described previously.¹⁴ Spots were counted using an automated ELISPOT reader system with KS 4.3 software (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Splenocytes were prepared from OT-II mice and plated in triplicates at a density of 1×10^5 /well. Effector cells were stimulated with 5×10^4 APCs pulsed with MHC class II-restricted OVA_{323–339} and with 5×10^4 unloaded APCs or GM3-loaded APCs. After incubation for 20 hr at 37°, ELISPOT assay was performed as mentioned above.

TGF- β and IL-13 ELISA kits were purchased from R&D Systems and IL-4 and IFN- γ ELISA kits were purchased from Pierce (Rockford, IL). The assay followed the manufacturer's instructions.

Flow cytometry

As described previously,¹⁴ splenocytes pooled from four to six immunized mice were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 (clone 17A2), phycoerythrin-conjugated anti-NK1.1 (clone PK136), cytochrome-conjugated anti-CD4 (clone RM4-5) and allophycocyanin-conjugated anti-CD8 (clone 53-6.7) mAbs (BD Biosciences), and subjected to cell sorting in a MoFlow cell sorter (DakoCytomation, Carpinteria, CA). NKT-cell subpopulations were sorted by surface expression of CD3, NK1.1, CD4, and CD8: CD4[–] CD8[–] NKT cells (CD4[–] CD8[–] CD3⁺ NK1.1⁺), and CD4⁺ CD8[–] NKT cells (CD4⁺ CD8[–] CD3⁺ NK1.1⁺).

Real-time polymerase chain reaction (PCR)

RNA was extracted using RNeasy mini kit (QIAGEN, Valencia, CA) following the manufacturer's guidelines. RNA was converted to cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems) with TaqMan[®] Gene Expression Assays as a primer and TaqMan[®] Universal PCR Master Mix as a polymerase (Applied Biosystems). The gene expression level was analysed by SEQUENCE DETECTION software version 1.2.3. GAPDH was used as endogenous control and the level of gene in unloaded APCs was set to 1.

Transwell experiments

For transwell experiments, naïve splenocytes and APCs were incubated in 24-well plates (BD Biosciences). Cell Culture Insert 1.0 μ m pore-size polyethyl terephthalate (PET) track-etched membranes (BD Biosciences) were inserted in each well. The APCs were added to well inserts as indicated and 2 days later the supernatants were collected for cytokine assay.

Statistical analysis

For statistical analysis, unpaired two-tailed Student's *t*-test was performed unless stated otherwise.

Results

A subset of NKT cells against GD3 cross-react with GM3

Mice immunized against purified GD3 developed NKT-cell responses against GD3, as we have reported previously.¹⁴ We noted that some NKT cells also reacted with GM3 by secreting IL-4 (Fig. 1a), but not IFN- γ (Fig. 1b). There was no cross-reactivity with GD2, GM2, or LacCer detected with either cytokine assay (the structures of glycolipids used in this study are shown in Fig. S1). This suggested that GM3 can be presented by CD1d and that a subset of GD3-reactive NKT cells cross-react with GM3. NKT cells from unimmunized mice do not react with any of the gangliosides tested (data not shown). To confirm that there were no contaminating gangliosides in the GD3 or GM3, we performed thin-layer chromatography with 3 μ g of each ganglioside. We could detect no contaminating gangliosides with a sensitivity < 100 ng (data not shown).

The lack of cross-reactivity with GM2, GD2 and LacCer could have been the result of an inability of CD1d to bind these glycolipids. To determine if CD1d could bind these glycolipids, we used isoelectric focusing (IEF) gel electrophoresis to detect ganglioside–CD1d binding

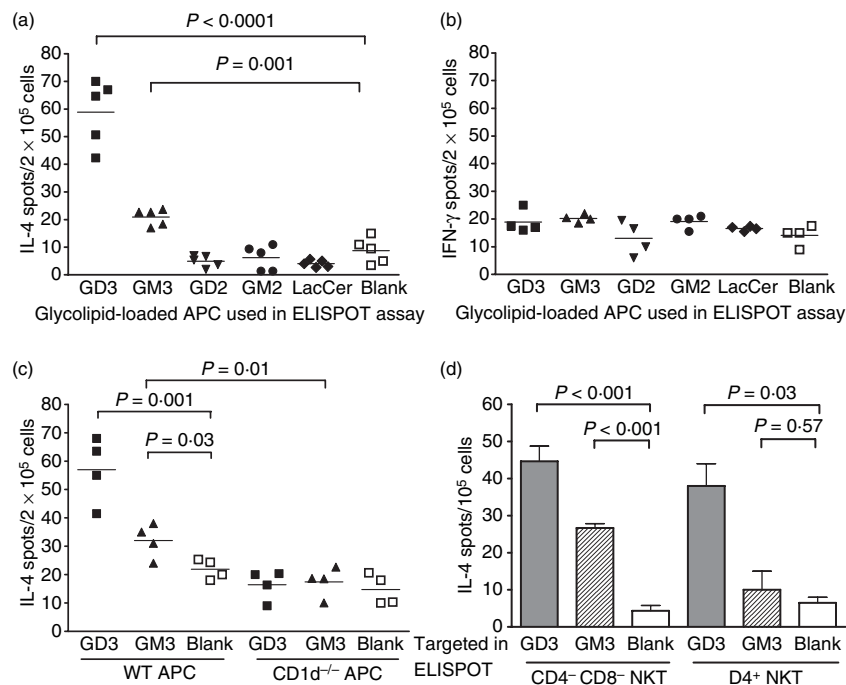


Figure 1. Immunization with GD3-loaded antigen-presenting cells (APCs) induces a subset of CD1d-restricted GM3-reactive natural killer T (NKT) cells. Mice were injected with 10⁵ GD3-loaded APCs into the footpad. Splenocytes were collected on day 7 and tested for reactivity against syngeneic APCs loaded with ganglioside antigens GD3, GM3, GD2, GM2, lactosylceramide (LacCer) or unloaded APCs ('blank'). Interleukin-4 (IL-4) production (a) or interferon-γ (IFN-γ) production (b) was detected by ELISPOT assay. Each point represents the mean of triplicate wells from an individual mouse; horizontal lines indicate mean values. For the IFN-γ ELISPOT, response to concanavalin A served as a positive control (data not shown). (c) Splenocytes were collected on day 7 and tested for reactivity against APCs from either wild-type or CD1d^{-/-} mice loaded with GD3 or GM3. Production of IL-4 was detected by ELISPOT assay. (d) Mice were injected with 10⁵ GD3-loaded APCs into the footpad. Splenocytes pooled from four to six immunized mice were separated into CD4⁻ CD8⁻ NKT (CD4⁻ CD8⁻ NK1.1⁺ CD3⁺) and CD4⁺ CD8⁻ NKT (CD4⁺ CD8⁻ NK1.1⁺ CD3⁺) subpopulations based on surface expression markers. The IL-4 production by each cell population was detected by ELISPOT assay. Sorted NKT-cell subsets were stimulated with syngeneic APCs loaded with GD3 (grey bars), GM3 (hatched bars), or unloaded (open bars) APCs for 44 hr. Error bars represent of SEM of triplicate wells from each condition. Data are representative of two separate experiments. Unsorted splenocytes (not shown) served as positive controls.

directly.¹⁵ We found that all gangliosides tested (GD3, GM3, GD2, GM2) bound to CD1d with equal avidity (Fig. S2). Binding of LacCer to CD1d could not be detected by this assay, probably because of its poor solubility under the assay conditions and its lack of negative charge.

We tested whether GD3-reactive NKT cells could recognize GM3 presented by APCs lacking CD1d. The GD3-reactive NKT cells did not react with either GD3 or GM3 pulsed on to APCs from CD1d^{-/-} mice (Fig. 1c), confirming that GM3 is presented by CD1d.

These experiments showed that glycolipids without NANA (e.g. LacCer) are not recognized by the NKT cells generated after immunization with GD3. For some of these NKT cells, a single NANA (as in GM3) can be sufficient for recognition by the TCR. That neither GM2 nor GD2 were recognized despite having one or two NANA led us to conclude that the presence of a terminal GalNAc interferes with GD3-reactive NKT-cell TCR recognition of gangliosides.

GM3-crossreactive splenocytes are a subset of GD3-reactive CD4⁻ CD8⁻ NKT cells

We reported previously that GD3-reactive NKT cells consist of two sub-populations, CD4⁻ CD8⁻ and CD4⁺ CD8⁻ NKT cells.¹⁴ We tested the hypothesis that the subpopulation of GD3-reactive NKT cells that cross-react with GM3 could be distinguished by expression of CD4. Splenocytes from mice immunized with GD3-loaded APCs were sorted into two NKT-cell subpopulations: CD4⁻ CD8⁻ NKT and CD4⁺ CD8⁻ NKT cells, then tested for recognition of GD3 or GM3 by IL-4 production by ELISPOT assay (Fig. 1d). Although these sorted populations included some NK1.1⁺ conventional T cells, our previous data showed that only αGalCer tetramer-positive CD3⁺ NK1.1⁺ NKT cells were reactive to GD3.¹⁴ As expected, we detected GD3-reactive NKT-cell activity in both CD4⁻ CD8⁻ and CD4⁺ CD8⁻ NKT-cell subsets, demonstrating that both sorted NKT-cell populations were functional. However, GM3-reactive cells were only

detected in the CD4⁺ CD8⁺ NKT-cell fraction. The percentage of GD3-reactive NKT cells measured after sorting was not increased compared with unsorted NKT cells (Fig. 1a). The process of sorting decreased the general activity of the NKT cells and required incubation for 44 hr in the ELISPOT assay (rather than the standard 20 hr), making direct quantitative comparisons of results from sorted and unsorted NKT cells difficult. No signal was observed when total or sorted cells were tested against unloaded APCs in the ELISPOT assay, confirming that the response was antigen-specific.

Based on the fine specificity observed in these experiments, we suspect that at least two TCRs are available in the mouse for recognition of GD3. The TCR on CD4⁺ CD8⁺ NKT cells requires two NANA molecules and is specific for GD3. On the other hand, a single NANA molecule is sufficient to engage the TCR on CD4⁺ CD8⁺ NKT cells against GD3 allowing cross-reactivity with GM3. Neither TCR can accommodate a terminal GalNAc.

Immunogenicity of gangliosides: structural requirements to induce antigen-specific NKT-cell responses

The above experiments did not rule out the possibility that immunizing against GD3 somehow induced a sepa-

rate set of NKT cells reactive only with GM3. To address this, and to determine if immunization with other gangliosides could induce antigen-specific NKT cells, we immunized mice with APCs pulsed with GM3, GM2, GD2 or LacCer and tested for antigen-specific production of IL-4 or interferon- γ . Mice immunized with GD3-loaded APCs served as a positive control; mice immunized with unloaded APCs served as negative controls. We found that only immunization with GD3 induced a detectable NKT-cell IL-4 response; no other ganglioside induced a response (Fig. 2). None of the gangliosides induced an IFN- γ response at the time-point tested (7 days after immunization; Fig. S3).

These data indicate that GM3, GM2, GD2 and LacCer do not readily induce NKT-cell responses in mice. Specifically, GM3-reactive NKT cells are not induced by immunizing mice with GM3 itself even though GM3 can be presented by CD1d. This supports our conclusion that GM3-reactive NKT cells detected as a result of immunization with GD3 are cross-reacting NKT cells.

GM3-loaded APCs suppress the NKT-cell response against GD3

We tested the effect of immunizing mice with both GD3 and GM3. Surprisingly, mice immunized with a mixture

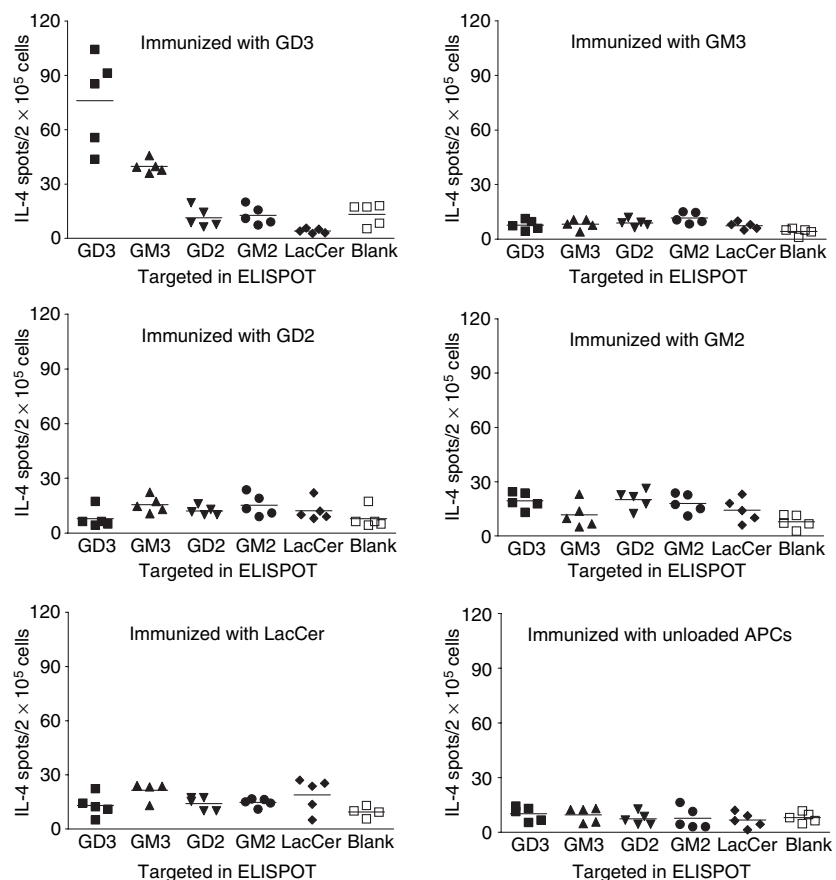


Figure 2. Immunizing with GM3, GD2, GM2 or lactosylceramide (LacCer) does not induce antigen-specific natural killer T (NKT)-cell responses. Mice were injected with 10⁵ syngeneic antigen-presenting cells (APCs) loaded with GD3, GM3, GD2, GM2, LacCer or unloaded APCs. Splenocytes were collected on day 7 and tested for reactivity against syngeneic APCs loaded with GD3, GM3, GD2, GM2, LacCer or unloaded-APC (blank), as indicated. Interleukin-4 (IL-4) production was detected by ELISPOT assay. Each point represents the mean of triplicate wells from an individual mouse; horizontal lines indicate mean values.

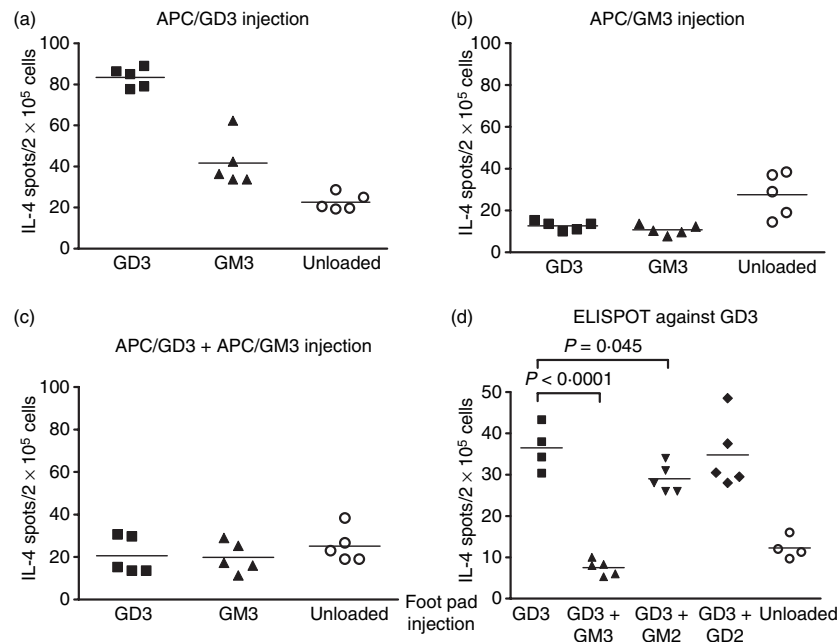


Figure 3. Immunizing with GM3-loaded antigen-presenting cells (APCs) inhibits natural killer T (NKT) cell interleukin-4 (IL-4) production in response to GD3. Mice were injected with (a) 10^5 GD3-loaded APCs, (b) 10^5 GM3-loaded APCs, or (c) a mixture of 10^5 GD3-loaded APCs and 10^5 GM3-loaded APCs into the footpads. Splenocytes were collected on day 7 and tested for reactivity against syngeneic APCs loaded with GD3, GM3, or unloaded APCs. Production of IL-4 was detected by ELISPOT assay. Each point represents the mean of triplicate wells from an individual mouse; horizontal lines indicate mean values of five mice. (d) Mice were immunized with GD3-pulsed APCs mixed with APCs pulsed with GM3, GM2, or GD2. The response to GD3 was measured by ELISPOT. Each symbol represents the mean of triplicate wells from an individual mouse. Negative control mice were immunized with unpulsed APCs; positive control mice were immunized only with GD3-pulsed APCs. Horizontal lines indicate mean values.

of GD3-loaded APCs and GM3-loaded APCs failed to develop an NKT-cell response against GD3 (Fig. 3). As previously observed, mice immunized with GD3-loaded APCs developed an NKT-cell response against GD3 with a subset cross-reacting with GM3 (Fig. 3a). As noted previously, mice immunized with GM3 did not develop a detectable response (Fig. 3b). However, when mice were immunized with both GD3 and GM3, no response to GD3 was induced (Fig. 3c). This inhibitory activity was specifically induced by GM3; APCs pulsed with structurally similar gangliosides (GM2 or GD2) did not suppress the response to GD3, although in some experiments, GM2 caused a slight suppression (Fig. 3d).

Inhibition by GM3 is CD1d-restricted

Although we knew that GM3 could be presented by CD1d (Fig. 1c), it was possible that the inhibitory effect of GM3 was the result of GM3 binding to lectin on the APCs. To test this, we prepared GM3 glycan in which the ceramide portion of GM3 was removed so that it could not bind to CD1d. Antigen-presenting cells pulsed with GM3 glycan failed to inhibit the response to GD3 (Fig. 4a), which was consistent with the model that GM3 is presented by CD1d.

To test this in another way, we examined whether GM3 loaded on to APCs derived from CD1d^{-/-} mice could inhibit the NKT-cell response to GD3. Mice were immunized with wild-type APCs loaded with GD3 mixed with GM3-loaded APCs from either wild-type mice or CD1d^{-/-} mice. Figure 4b shows that CD1d^{-/-} APCs pulsed with GM3 did not inhibit the response to GD3. These experiments prove that the inhibitory effect of GM3 requires binding to CD1d.

We next turned to whether the inhibition by GM3-loaded APCs was a local or systemic effect. Mice were immunized with GM3-loaded APCs and GD3-loaded APCs either mixed together, as in previous experiments, or injected into opposite footpads. Splenocytes were harvested at day 7 and tested for reactivity against GD3 as before. When GM3-loaded APCs were injected into the opposite footpad, the inhibitory effect was lost, indicating that the inhibitory effect was local (Fig. 4c).

To determine the time-course of inhibition by GM3, mice were injected with GM3-loaded APCs into the footpad either simultaneously with GD3-loaded APCs (Day 0), or before GD3-loaded APC injection (2 days, 4 days, 7 days before immunization with GD3-loaded APCs). One week after GD3 immunization, ELISPOT was performed. Control mice immunized with GD3-loaded APCs

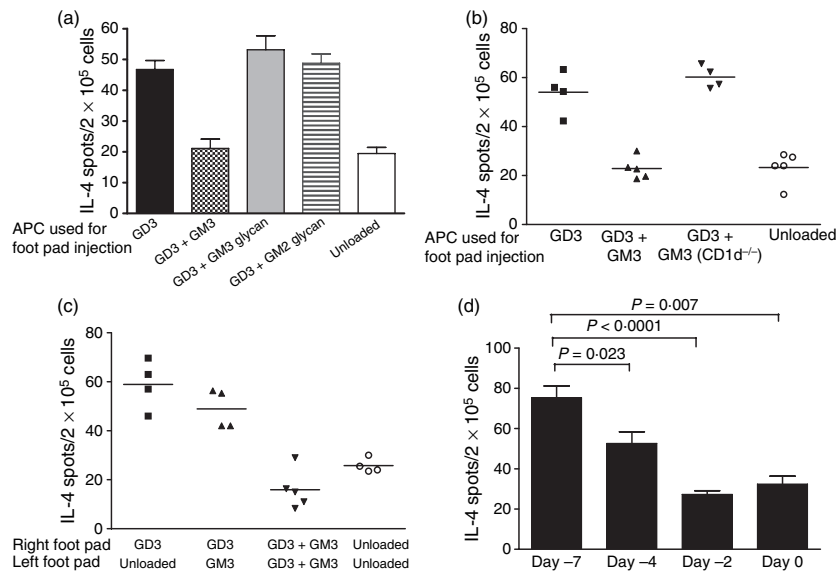


Figure 4. Characterization of GM3-mediated inhibition of the natural killer T (NKT)-cell response to GD3. (a) Mice were immunized with GD3-pulsed antigen-presenting cells (APCs) alone or mixed with APCs pulsed with GM3, GM3 glycan, or GM2 glycan. Seven days later, splenocytes were collected and interleukin-4 (IL-4) production was measured by ELISPOT assay. (b) Mice were immunized with GD3-pulsed APCs (column 1) or with a mixture GD3-pulsed and GM3-pulsed APCs. For GM3-pulsed APCs, the APCs were either from wild-type mice (column 2) or from CD1d^{-/-} mice (column 3). Unimmunized mice served as background controls (column 4). Each point represents triplicate wells from an individual mouse. (c) Mice were injected with 10⁵ GM3-loaded APCs and 10⁵ GD3-loaded APCs either mixed together into the same footpad injection or injected into opposite footpads. Control mice were injected with GD3-pulsed APCs mixed with unpulsed APCs. Splenocytes were collected and IL-4 secretion was measured by ELISPOTs; GD3-loaded APCs were used as target cells. Each symbol represents one mouse. Horizontal lines represent mean value. (d) Mice were injected with 10⁵ GM3-loaded APCs into the footpads on day 0, then injected with 10⁵ GD3-loaded APCs on day 2, 4 or 7. Some mice were injected with a mixture of 10⁵ GM3-loaded APCs and 10⁵ GD3-loaded APCs into the footpads on day 0 (as indicated). Splenocytes were collected 7 days after injection of GD3-loaded APCs and tested for reactivity against syngeneic APCs loaded with GD3. Production of IL-4 was detected by ELISPOT assay. Data represents the mean ± SEM of triplicate wells from five mice.

alone developed an NKT-cell response against GD3 (data not shown). The NKT-cell response to GD3 was inhibited by GM3-loaded APCs for 2 days (Fig. 4d). By 4 days, the inhibitory effects of the GM3-loaded APCs were decreased and by day 7 no inhibitory effects were observed.

These experiments showed that the inhibitory effect of GM3-loaded APCs was local, CD1d-restricted, and persisted for at least 2 days.

GM3 inhibits the IL-4 response of NKT cells to α GalCer

We wished to determine whether the inhibition by GM3-loaded APCs was specific for the NKT-cell response to GD3 or if it extended to other NKT-cell responses. We tested whether GM3-loaded APCs inhibit the NKT-cell response to α GalCer, a potent NKT-cell activator that stimulates naïve type I NKT cells. Splenocytes from naïve mice were incubated with α GalCer-loaded APCs mixed with increasing numbers of GM3-loaded APCs and incubated overnight. We found that GM3-loaded APCs inhibited the IL-4 response to α GalCer (Fig. 5a) although this inhibition was not complete, perhaps because of the polyclonality of the response to α GalCer. On the other hand,

GM3-loaded APCs had no effect on the IFN- γ response to α GalCer (Fig. 5b). This indicates that GM3 did not induce apoptosis, anergy, or down-regulation of TCRs in NKT cells, or block antigen-processing pathways in APCs. The specificity for inhibition of the IL-4 response to α GalCer, together with the inhibition of the IL-4 response to GD3, suggests that GM3 inhibits Th2-like NKT-cell responses. Consistent with this, we found that GM3 inhibited the basal level of IL-13 in splenocytes, another Th2 cytokine (Fig. S4).

The inhibitory effect of GM3 did not globally block antigen-presenting functions. We found that GM3-loaded APCs did not inhibit the ability of mouse APCs to present SIINFEKL peptide to CD8⁺ T-cell responses *in vivo* or to present OVA_{323–339} peptide to CD4⁺ T cells *in vitro*. (Fig. S5).

GM3 inhibition is not mediated by soluble factors

We hypothesized that soluble factors secreted from either the GM3-loaded APCs or NKT cells interacting with GM3 mediate the inhibitory effect observed. By real-time PCR, we detected no change in TGF- β or IL-10 messenger RNA levels in GM3-loaded APCs or in splenocytes

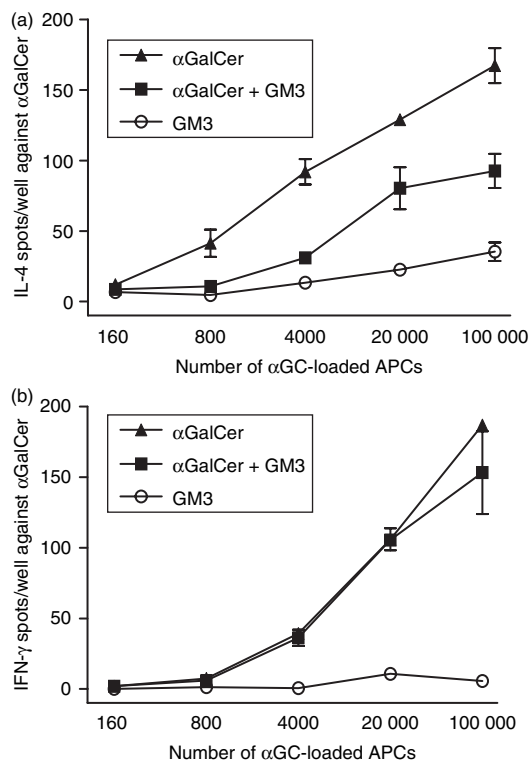


Figure 5. GM3 inhibits α -galactosylceramide (α GalCer)-induced interleukin-4 (IL-4) production by natural killer T (NKT) cell. From 160 to 10^5 (as indicated on x-axis) α GalCer-loaded antigen-presenting cells (APCs) were mixed with 10^5 GM3-loaded APCs; α GalCer-loaded APCs were mixed with 10^5 unloaded APCs (as positive control); and unloaded APCs were mixed with 10^5 GM3-loaded APCs (as negative control). Naïve splenocytes (2×10^5) were used as effector cells and IL-4 (a) or interferon- γ (IFN- γ) (b) production by splenocytes was detected by ELISPOT assay. Data represents the mean $\pm$ SEM of triplicate wells from each treatment group.

incubated with GM3-loaded APCs (Fig. S6a). Consistent with this, we detected no increase by ELISA in TGF- β protein in supernatants of APCs pulsed with GM3 either with or without added splenocytes (data not shown). *In vivo* experiments showed that the neutralizing anti-TGF- β mAb 1D11.16.8 did not reverse the inhibitory effects of GM3-loaded APCs (Fig. S6b).

It was possible that soluble factors other than TGF- β or IL-10 were important in GM3-mediated inhibition. Supernatants from GM3-loaded APCs alone or GM3-loaded APCs incubated with splenocytes were tested for the ability to inhibit response to α GalCer. We found that supernatants did not inhibit the IL-4 response to α GalCer confirming that inhibition mediated by GM3 is not mediated by soluble factors (data not shown).

The implication from these experiments was that cell-cell contact is necessary for the inhibitory effect mediated by GM3-loaded APCs. Using a transwell format, we tested whether GM3-loaded APCs can inhibit α GalCer-stimulated IL-4 secretion by NKT cells without direct cell con-

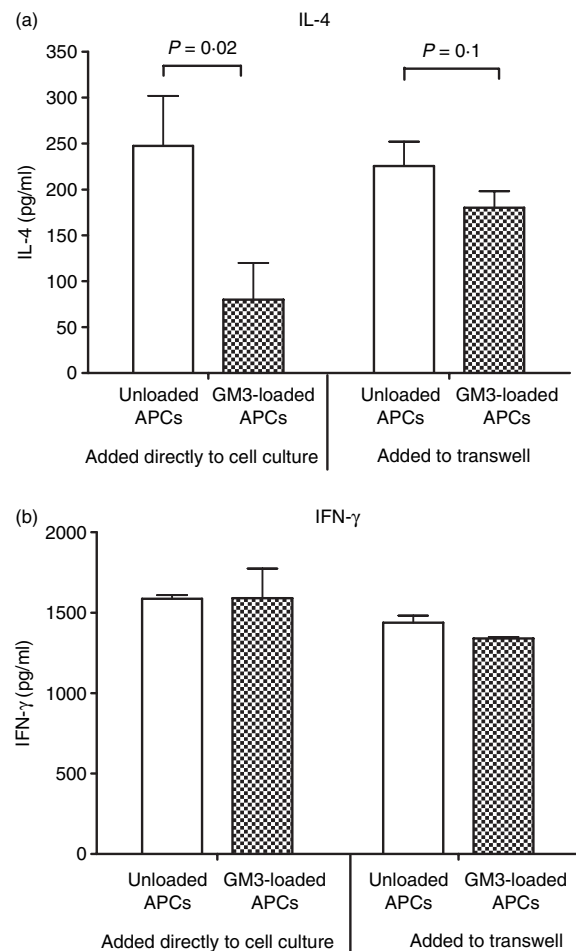


Figure 6. Cell-cell contact is necessary for GM3 inhibition. α -Galactosylceramide (α GalCer)-loaded antigen-presenting cells (APCs) (10^5) were incubated with 2×10^5 naïve splenocytes as a source of NKT cells. Using a transwell format, 10^5 GM3-loaded APCs (or unloaded APCs as controls) were added either directly, or were applied to the well insert, which prevented direct cell contact with other cells. After 2 days of incubation, the supernatant was collected and interleukin-4 (IL-4) (a) and interferon- γ (IFN- γ) (b) ELISA was performed. Error bars represent SEM of triplicate sample.

tact. We found that direct cell contact was required to inhibit the IL-4 response to α GalCer (Fig. 6a). As observed before (Fig. 5b), GM3-loaded APCs did not inhibit the IFN- γ response to α GalCer (Fig. 6b).

Discussion

The natural targets of most NKT cells described to date are microbial glycolipids,^{8–13} which is consistent with data suggesting that NKT cells can play an important role in innate immunity.¹⁶ Among mammalian-derived glycolipids, few have been shown to be true ligands of NKT cells. Phospholipid antigens can be recognized by mouse NKT hybridomas¹⁷ and glycosylphosphatidylinositol¹⁸ can bind to CD1d, although it is not clear that it is

recognized by NKT cells. Natural killer T cells that recognize sulfatide have also been described.¹⁹ Recently, isoglobotrihexosylceramide (Gal α 1-3, Gal β 1-4, Glc β 1,1 Cer; iGb3) has been shown to be a ligand of type I NKT cells²⁰ in human and mouse, although the robustness of the response to iGb3 and the tissue distribution and the level of expression of iGb3 remain to be clarified.^{21,22} Indeed, mouse dendritic cells do not express iGb3, even after stimulation with Toll-like receptor agonists.²² Recent reports have called into question the role of iGb3 in murine NKT-cell development.²¹

In the current report, we confirm that in mice immunized against GD3, NKT cells against GD3 can be detected although the frequency is relatively low (approximately 60 cells/ 2×10^5 splenocytes in these studies). This is slightly lower than the frequency reported for other antigen-specific NKT cells against phosphatidylinositol mannoside (0.26% of CD3⁺ cells⁸) and against d-glucuronosulceramide (PBS30) (0.18% of splenocytes).¹¹

The GD3 ganglioside is the first NKT-cell target that is also a relevant tumour antigen in humans. It is a glycolipid that is markedly overexpressed on virtually all melanomas as well as on other tumours of neuroectodermal origin but is expressed in few normal tissues and mostly at very low levels. Monoclonal antibodies against GD3 can induce tumour shrinkage in patients²³ and the observation that GD3 can also be recognized by NKT cells¹⁴ raises the possibility of both cellular and humoral responses to GD3.

In the current study, we have explored the fine specificity of mouse NKT cells that recognize GD3 and found that immunization with GD3 expands at least two populations of NKT cells that react with GD3. One population is CD4⁺ CD8⁻ and recognizes only GD3. Another population is CD4⁻ CD8⁻ and cross-reacts with GM3 but not with GM2, GD2, or LacCer. This difference in fine specificity suggests that the TCRs of these two NKT-cell populations are different, although we cannot rule out the possibility that CD4 plays a role in the specificity of the NKT cells. The TCR of the CD4⁻ CD8⁻ NKT cells requires at least one NANA molecule (because they recognize GM3 but not LacCer) although they can accommodate two NANAs (as in GD3). The TCR of the CD4⁺ CD8⁻ population are specific for GD3 and do not recognize gangliosides with only a single NANA. We recognize that the CD3⁺ NK1.1⁺ cells may have included a small proportion of conventional T cells expressing NK1.1. However, conventional T cells do not recognize glycolipids and our previous data showed that only α GalCer tetramer positive CD3⁺ NK1.1⁺ NKT cells were reactive to GD3.¹⁴

Neither population of GD3-reactive NKT cells recognized GD2, which differs from GD3 only by the addition of a terminal GalNAc molecule. This leads us to conclude that the TCRs of GD3-reactive NKT cells do not accom-

modate a terminal GalNAc. This may be a general pattern for TCRs of NKT cells because Zhou and colleagues demonstrated that iGb3, a glycolipid derived from lysosomes, is a primary ligand for both human and mouse NKT cells.²⁰ However, NKT cells did not react to iGb4, which is identical to iGb3 except for the addition of a terminal GalNAc.

Aside from GD3, none of the other gangliosides tested (or LacCer) induced an NKT-cell response that was detectable by IL-4 or IFN- γ production. The lack of an NKT-cell response against these other gangliosides does not appear to be the result of a failure to bind by CD1d (Fig. S2). The inability of LacCer to induce NKT-cell responses has been observed previously.²⁰

We discovered that GM3, when presented by APCs, suppresses the NKT-cell response to GD3. This suppression is CD1d-restricted and is a local effect that persists for at least 2 days. GM3 also inhibited the IL-4 response, but not the IFN- γ response, of NKT cells to α GalCer. This indicates that the suppressive effect may extend to NKT cells of various specificities and it also raises the possibility that the GM3-mediated suppressive effect is limited to Th2-like cytokines. We speculate that GM3 represents an altered ligand presented by CD1d that interacts with the TCR of the NKT cells and prevents Th2-like responses.

It does not appear that inhibition is the result of secretion of soluble factors or of a general paralysis of APC function. Indeed, GM3-loaded APCs had no effect on the ability of local APCs to present SIINFEKL peptide to class I-restricted T cells (Fig. S5). We found that direct cell contact between GM3-loaded APCs and NKT cells is required for this inhibitory effect. We are currently investigating the mechanism of this effect. Initial experiments have shown that loading GM3 onto CD1d⁺ myeloid-derived dendritic cells did not affect expression of DEC205, CD80, or CD86 (data not shown).

Gangliosides have long been observed to have a negative effect on T-cell proliferation.²⁴ Gangliosides can block T-cell proliferation to IL-2, mitogen, or lipopolysaccharide. This inhibitory effect is seen with many gangliosides and is not specific for one ganglioside.²⁵ Results of experiments have implicated several possible mechanisms of inhibition including interference with IL-2 binding,²⁶ inhibition of protein kinase C, inhibition of signal transduction,²⁷ in particular extracellular signal-regulated kinase (ERK) phosphorylation,^{28,29} and inhibition of dendritic cell function.³⁰⁻³² Many of these mechanisms may not be mutually exclusive.

The GM3-mediated inhibition of type I NKT-cell function we describe appears to be distinct from these previously-reported effects. First, the concentrations of ganglioside used in most of these other reports is quite high (10–50 μ g/ml), whereas we have used only GM3-pulsed APCs. Second, we have found that the inhibition is GM3-specific whereas the effects reported in these other

papers were not specific for a given ganglioside. AsialoGM2 has been shown to decrease by 50% the IL-2 secretion of a specific NKT-cell hybridoma cell line *in vitro*³³ but the specificity of this inhibition was not reported and it is unclear whether this effect is seen *in vivo*.

The role of NKT cells in anti-tumour immunity remains unclear. There are suggestions that NKT cells play a role in tumour immunosurveillance in that cancer patients seem to have decreased NKT-cell function.^{34–36} However, antigen-specific NKT cells have not been evaluated in cancer patients. It is interesting to speculate on the biological significance of GM3-mediated suppression of NKT cells. Since GM3 is ubiquitously expressed,³⁷ it is possible that GM3 on host APCs serves to tune the Th2 NKT-cell response, perhaps inhibiting reactivity to self-glycolipids. This merits further investigation.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. Glycolipid structure.

Figure S2. Gangliosides bind to CD1d *in vitro*.

Figure S3. Immunizing with GD3, GM3, GD2, GM2 or LacCer does not induce antigen-specific IFN- γ NKT-cell responses.

Figure S4. Splenocytes interacting with GM3-loaded APCs show decreased basal levels of IL-13 production.

Figure S5. GM3 does not inhibit the ability of APCs to present MHC-I and MHC-II restricted ovalbumin peptide.

Figure S6. TGF- β does not mediate inhibition of NKT-cell responses by GM3.

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Supplementary Figure 1. Glycolipid structures: LacCer (A), gangliosides GM3 (B), GD3 (C), GM2 (D) and GD2 (E). All the gangliosides contain a glucose-galactose core linked to ceramide. Either one or two NANA molecules are linked to the terminal galactose. GM2 and GD2 also have GalNAc linked to the terminal galactose. LacCer is not classified as a ganglioside since it does not contain NANA.

Supplementary Figure 2. Gangliosides bind to CD1d *in vitro*. The isoelectric focusing electrophoresis assay (IEF) to measure lipid loading onto mouse CD1d has been described [15]. 2 μ M of mouse CD1d were incubated at 37 °C for 1 hour with various concentrations of glycolipids. The loading of the glycolipid into CD1d was followed by IEF. Ganglioside-CD1d complexes are detected as the upper band as indicated.

Supplementary Figure 3. Immunizing with GD3, GM3, GD2, GM2 or LacCer does not induce antigen-specific IFN- γ NKT cell responses. Mice were injected with 10^5 syngeneic APCs loaded with GD3, GM3, GD2, GM2, LacCer or unloaded APCs. Splenocytes were collected on day 7 and tested for reactivity against syngeneic APCs loaded with GD3, GM3, GD2, GM2, LacCer or unloaded-APC (blank), as indicated. IFN- γ production was detected by ELISPOT assay. Each point represents the mean of triplicate wells from an individual mouse; horizontal lines indicate mean values.

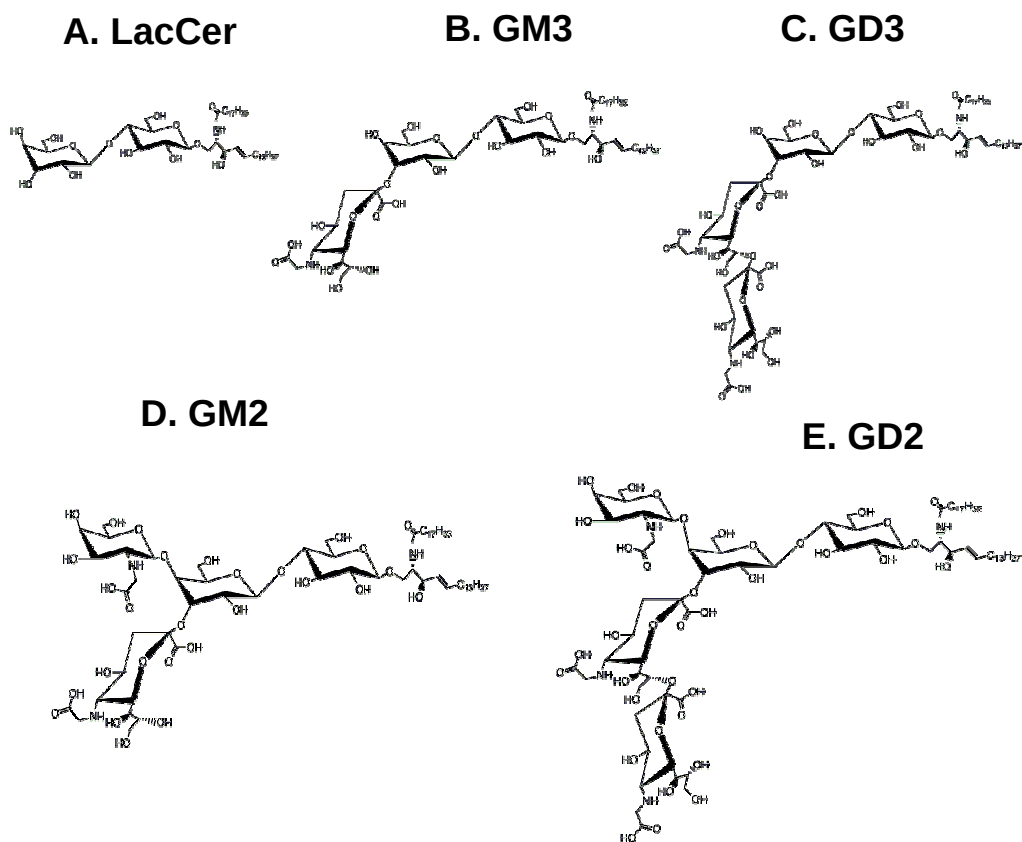
Supplementary Figure 4. Splenocytes interacting with GM3-loaded APCs show decreased basal levels of IL-13 production. Splenocytes incubated overnight with GM3-loaded APCs showed decreased IL-13 gene transcription (A). After 48 hours of

incubation, the level of IL-13 protein in the culture supernatants was measured by ELISA (B). Error bars represent SEM of triplicate samples of each condition.

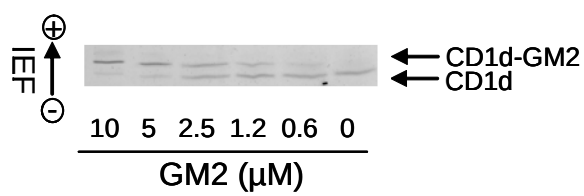
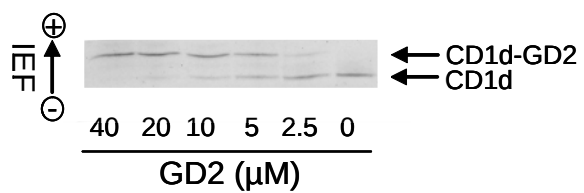
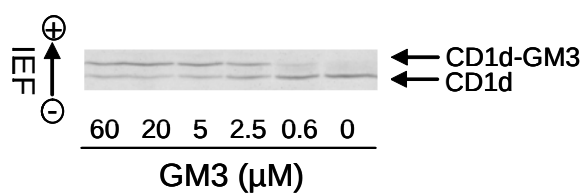
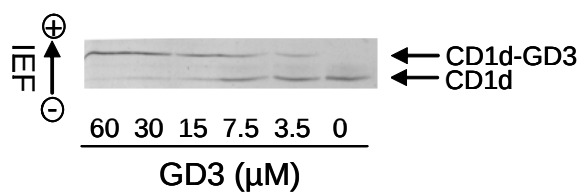
Supplementary Figure 5. GM3 does not inhibit the ability of APCs to present MHC-I and MHC-II restricted ovalbumin peptide. (A) Splenocytes were collected from mice immunized with SIINFEKL peptide-loaded APCs alone or mixed with APCs pulsed with GM3. Seven days later, splenocytes were collected and IFN- γ production was measured by ELISPOT assay. Each symbol represents the mean of triplicate values from a single mouse. (B) OT-II mice splenocytes were incubated with unloaded APCs (blank), OVA₃₂₃₋₃₃₉ peptide-loaded APCs, OVA₃₂₃₋₃₃₉ peptide-loaded APCs mixed with GM3-loaded APCs, or GM3-loaded APCs in vitro. IL-4 and IFN- γ production was measured by ELISPOT assay. Error bars represent SEM of triplicate samples of each condition.

Supplementary Figure 6. TGF- β does not mediate inhibition of NKT cell responses by GM3. (A) Gene expression level of TGF- β was measured by real-time PCR in unloaded APCs and GM3-loaded APCs after overnight incubation. (B) A mixture of GM3-loaded APCs and GD3-loaded APCs were administered by foot pad injection. Anti-TGF- β antibody or isotype control antibody were administered at day 0, 2, 4, 6 by intraperitoneally. Splenocytes were collected on day 7 and IL-4 production was tested by ELISPOT. GD3-loaded APCs injected mice were used as a positive control. Each symbol represents the mean of triplicate values from a separate mouse. Error bars represent SEM of triplicate of each condition.

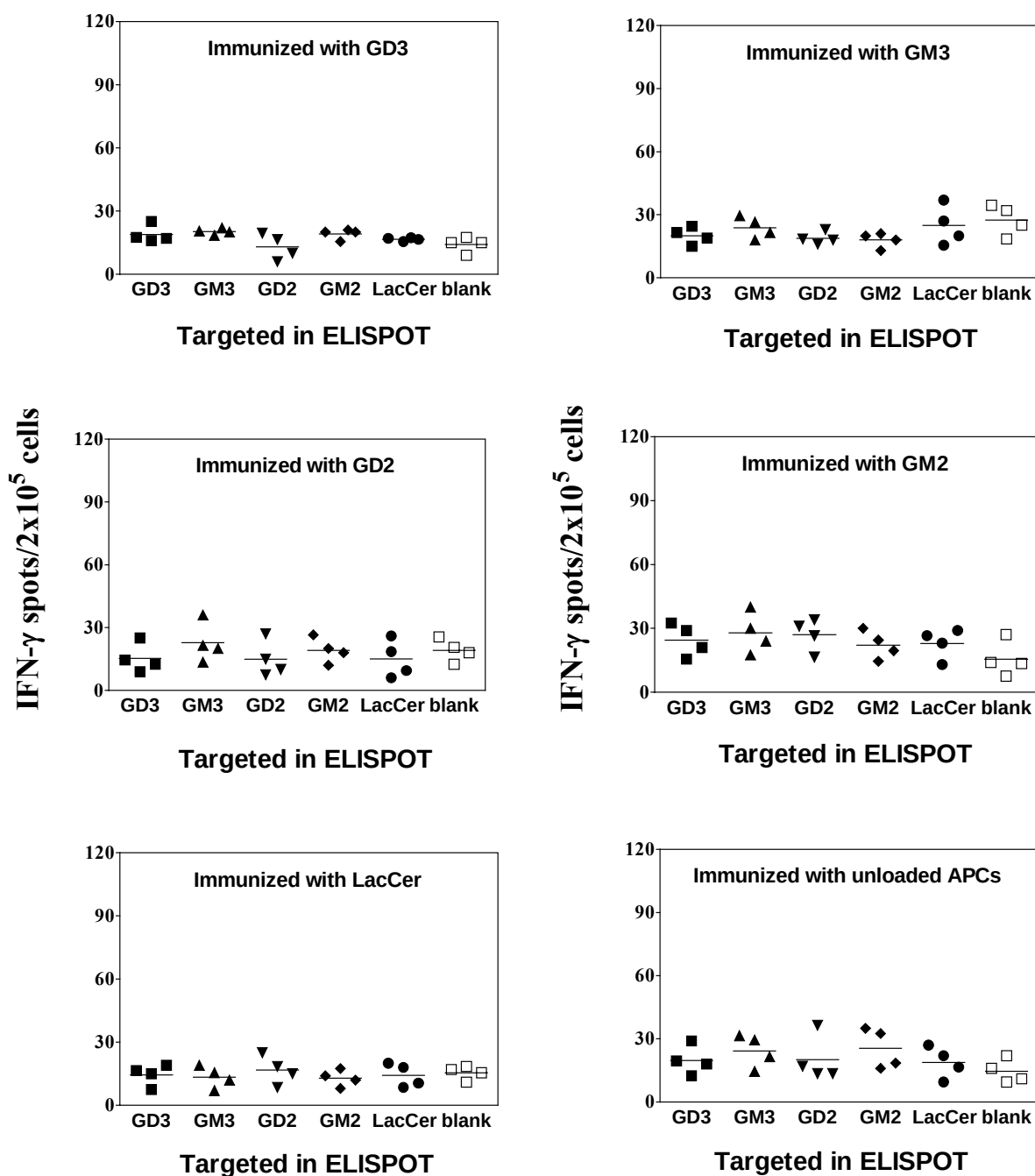
Supplementary Figure 1



Supplementary Figure 2

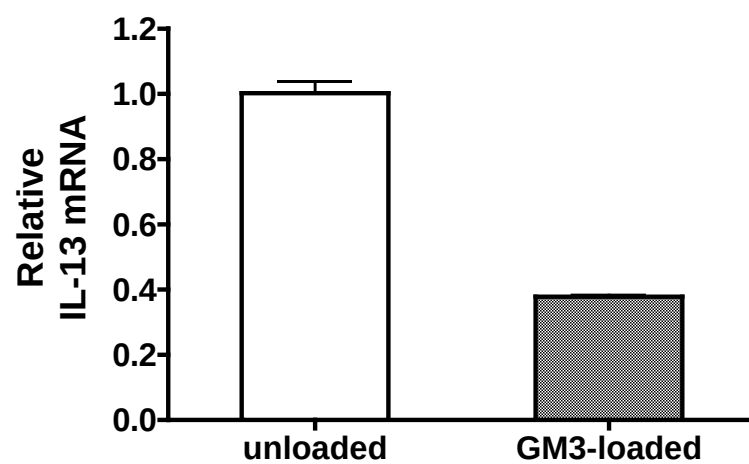


Supplementary Figure 3

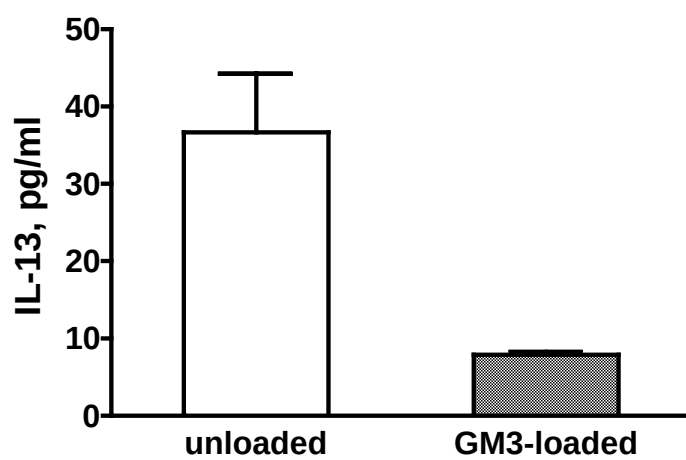


Supplementary Figure 4

A.

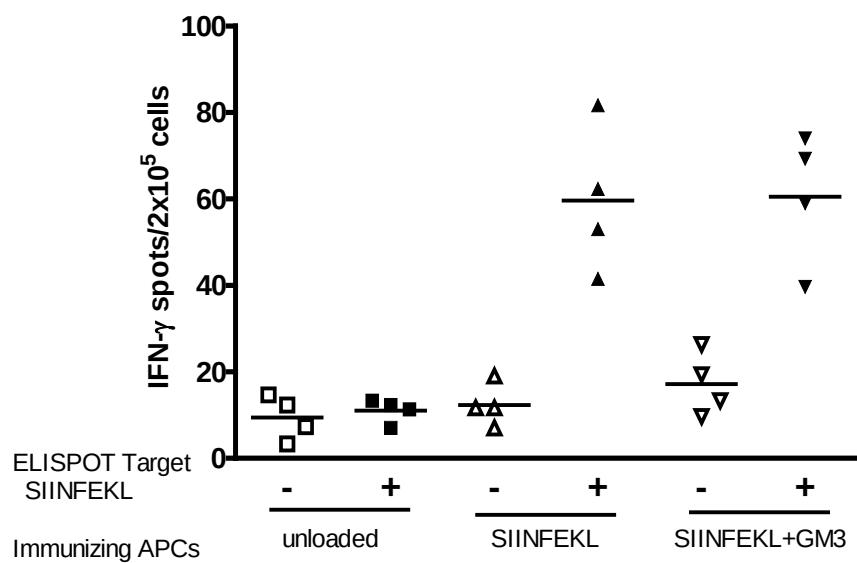


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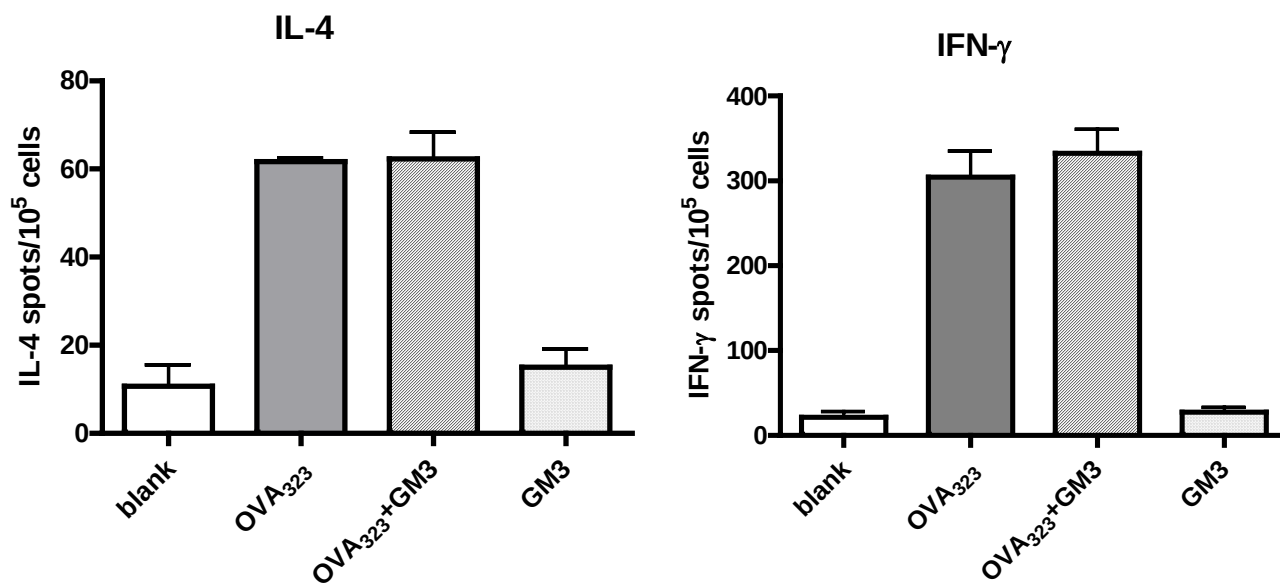


Supplementary Figure 5

A.

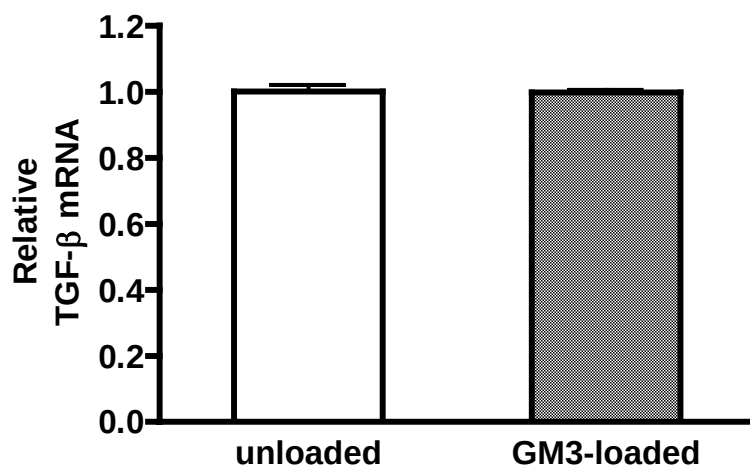


B.



Supplementary Figure 6

A.



B.

