Inhibition of glycolipid shedding rescues recognition of a CD1$^+$ T cell lymphoma by natural killer T (NKT) cells

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Neoplastic transformation of cells is accompanied by an aberration of cell surface glycolipid composition. These tumor-associated, altered glycosphingolipids are often shed into the tumor cell microenvironment and mediate immunosuppressive activity. The nature and form of glycolipids shed by a variety of tumor cell lines and the mechanism(s) of shedding have been well characterized. The murine T cell lymphoma line, L5178Y-R, is known to shed a tumor-associated glycolipid, gangliotriaosylceramide, into the culture medium. We analyzed the effect of glycolipids from L5178Y-R on antigen presentation by murine CD1d1 molecules. CD1d1 molecules present glycolipid antigens to a specialized class of T cells called natural killer T (NKT) cells that mainly express a T cell receptor α chain (Vα14Jα281) associated with Vβ chains of limited diversity. In the current report, we found that L5178Y-R cells express CD1 on their cell surface yet are unable to stimulate CD1d1-specific NKT cells. We hypothesized that the glycolipid(s) shed by L5178Y-R inhibited antigen presentation by CD1d1. Pretreatment of CD1d1$^+$ cells with conditioned medium from L5178Y-R inhibited CD1-specific stimulation of canonical (Vα14$^+$) but not noncanonical (Vα5$^+$) NKT cells. Exogenous addition of lipids extracted from L5178Y-R cells as well as purified gangliotriaosylceramide mimicked this effect. Inhibition of glycolipid shedding in L5178Y-R cells with D-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol resulted in the rescue of CD1d1 recognition by canonical (but not noncanonical) NKT cells. These results suggest that one means by which certain tumor cells can evade the host's innate antitumor immune response is by shedding glycolipids that inhibit CD1-mediated antigen presentation to NKT cells.

CD1 molecules are cell surface glycoproteins with structural similarity to MHC class I molecules. Two groups of CD1 genes based on amino acid sequence homology have been identified (1). Group 1 CD1 molecules consist of the human CD1a, b, and c, whereas CD1d molecules are the sole members of Group 2. CD1e is suggested to be an intermediate of both of these groups and it has been serologically defined. CD1 molecules can present a variety of both exogenous (e.g., mycobacterial lipid antigens) and endogenous lipid and glycolipid antigens to T cells (1–7). Furthermore, in collaboration with Joyce *et al.* (8), we have found that a major natural ligand of the mouse CD1d1 molecule is the normal cellular glycolipid, glycosylphosphatidylinositol. Antigen-specific restriction of a unique T lymphocyte subpopulation, termed natural killer T (NKT) cells, has been demonstrated for both human and mouse CD1d (9, 10). On activation, NKT cells promptly produce IL-4 and IFN-γ (among other cytokines) and can influence immune responses against autoantigens (11), tumors (12), and bacterial or parasitic infections (1, 13–17). NKT cells can also be activated in a CD1d-restricted manner by the synthetic glycolipid, α-galactosylceramide (1).

The antitumor activity of NKT cells has been demonstrated based on numerous *in vivo* studies with the CD1d ligand, α-galactosylceramide (reviewed in ref. 18). NKT cells can mediate the inhibition of tumor growth and metastases in experimental tumors by direct (IL-12-mediated) or indirect (activation of NK cells) mechanisms. Paradoxically, recent studies have implicated NKT cells in an inhibitory role during the host's antitumor immune response through a predominant T helper 2 response that includes the production of IL-13. Thus, NKT cells can play major immunoregulatory roles (both positive and negative) in the host's innate antitumor immune response (18).

One hallmark trait of transformed cell lines is altered glycolipid expression and shedding. Glycosphingolipids (GSLs) are membrane-bound glycoconjugates consisting of a lipophilic ceramide attached to a hydrophilic oligosaccharide chain. The absence of a negatively charged sialic acid on neutral GSLs distinguishes them from gangliosides. Tumor cell GSLs have been demonstrated to exert both positive and negative influences on host immunological effector cells (19). The immunosuppressive function of glycolipids *in vivo* is thought to be related to direct inhibitory effects by means of the modulation of T lymphocyte signal transduction and effector cell differentiation or development (19, 20). However, glycolipids may also influence T cell function by altering tumor cell antigen processing and presentation (21, 22). Despite extensive analysis of tumor cell glycolipid structure and shedding, little is known about the effects of these glycolipids on antigen presentation. Here, we have addressed the effect of shed glycolipids on CD1-specific antigen presentation to NKT cells. Through an analysis of a panel of CD1$^+$ murine tumor cell lines, we show that shed glycolipids from one tumor line, the murine L5178Y-R T cell lymphoma cell line, can inhibit endogenous CD1d-mediated antigen presentation. Furthermore, as no extensive analysis of NKT cells as antitumor effector cells against CD1$^+$ tumor cells has been reported, we have analyzed the recognition of the murine CD1$^+$ hematopoietic tumor cells by NKT cells and found that the tumor cells were not recognized by either canonical or noncanonical NKT cells. This defect in canonical NKT cell recognition could be overcome in one of these tumor cell lines by treatment of the tumor cells with an inhibitor of glucosylceramide synthase.

Methods

Cell Lines. The murine L5178Y-R (T cell lymphoma) and YAC-1 (T cell leukemia) cell lines were cultured in DMEM and RPMI medium 1640 media, respectively, supplemented with 2 mM L-glutamine (BioWhittaker) and 10% FBS (HyClone). The L5178Y-R cells were kindly provided by J. Yewdell and J. Bennink (Laboratory of Viral Diseases, National Institutes of Allergy and

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Abbreviations: CM, conditioned medium; GAD, glutamate decarboxylase; Gg3Cer, gangliotriaosylceramide; NKT, natural killer T; PPMP, D-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol; WT, wild type; PE, phycoerythrin.

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Infectious Diseases, National Institutes of Health), whereas the YAC-1 cells were a kind gift from R. M. Welsh (Univ. of Massachusetts Medical School, Worcester). The preB cell line 18.81 was also cultured in RPMI medium 1640 with the same supplements and also provided by R. M. Welsh. Murine L cell fibroblast lines DAP-3 (L-control) and DAP-3 transfected with *cd1d1* cDNA [L-CD1 (23), kindly provided by W. Paul (National Institutes of Health)] were cultured in DMEM with the same supplements as above.

T Cell Hybridomas. The Vα14$^+$ CD1d1-specific NKT cell hybridoma DN32.D3 (9) was cultured in Iscove's modified Dulbecco's medium supplemented with 5% FBS and 2 mM L-glutamine. The CD1d1-specific NKT cell hybridoma N37-1A12 (Vα5$^+$) and N38–2C12 (Vα14$^+$) have been described (3), and were maintained in RPMI medium 1640 supplemented with 10% FBS, 5.5×10^{-5} M mercaptoethanol, and 2 mM L-glutamine. The HLA-DR4-restricted, glutamate decarboxylase (GAD)-specific murine T cell hybridoma, 33.1, has been described (24).

Generation of L Cell Transfectants. Total RNA was extracted from L5178Y-R, YAC-1, and 18.81 cells with TriReagent (Molecular Research Center, Cincinnati). RNA was reverse-transcribed into cDNA and subsequently amplified with *cd1d1*-specific primers (25). Full-length *cd1d1* cDNA (1.1 kb) was subcloned into the *Eco*RV site of the pcDNA3.1-neo vector (Invitrogen). L-control cells were transfected with vector alone or vector with *cd1d1* cDNA inserts by standard calcium phosphate coprecipitation. Transfectants were grown in the presence of 500 μg/ml of G418 (Life Technologies, Gaithersburg, MD) for 2–4 weeks before analysis and used as bulk populations. Retroviral transduction of L-CD1d1WT (WT, wild type) cells with cDNA encoding the HLA-DR4 α and β chains was performed as described (26).

Treatment of Cells with Conditioned Medium (CM). After 5 days of culture, supernatants from L5178Y-R, YAC-1, and 18.81 cells were harvested by centrifugation at $250 \times g$ for 10 min. The clarified supernatants were used immediately for the treatments as follows. L-control and L-CD1 cells were incubated with fresh CM (6.0×10^6 cells per ml of CM) for 4 h at 37°C. The cells were subsequently washed three times with PBS, resuspended in Iscove's modified Dulbecco's medium, supplemented with 5% FBS and 2 mM L-glutamine and cocultured with or without the indicated NKT hybridomas for 20–24 h at 37°C. Fresh culture medium was used as a control. IL-2 release was measured by a standard sandwich ELISA with murine IL-2-specific mAb (PharMingen). In some experiments, L cells transfected with vector alone or the WT *cd1d1* cDNA in pcDNA3.1-neo (Invitrogen), generated in our laboratory as above, were used. The results were identical to those with L-control and L-CD1, respectively.

GAD Peptide Assay. L-CD1d1WT cells were transfected with the human HLA-DR4 α and β chains by retroviral transduction (26). Stimulation of the GAD-specific murine 33.1 hybridoma by these HLA-DR4$^+$ cells in the presence or absence of various concentrations of GAD peptide with or without L5178Y-R CM or gangliotriaosylceramide (Gg3Cer) for 20–24 h at 37°C was measured as described (24).

Inhibition of Glycolipid Shedding. To inhibit glycolipid shedding, tumor cells were cultured in the presence of 10 μM D-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP; Matreya, Pleasant Gap, PA) for 5 days as described (27). To evaluate the effect of purified glycolipids on CD1-specific antigen presentation, Gg3Cer (Matreya) was dissolved in ethanol by sonication and added to cells at a final concentration of 1–40 μM as indicated. Ethanol (0.05%) was used as the vehicle control.

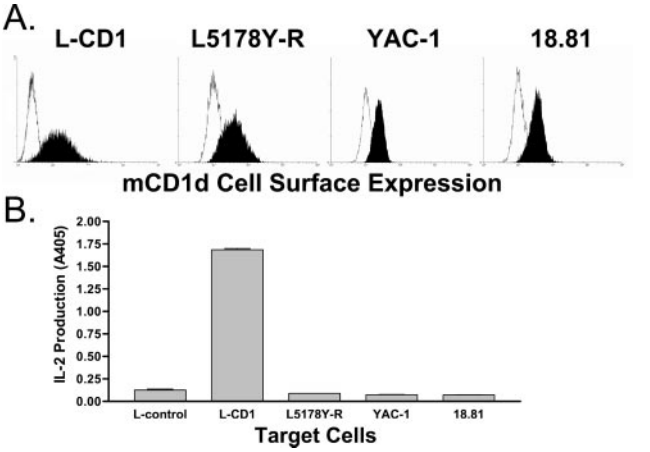

Fig. 1. Cell surface expression of CD1 by mouse hematopoietic tumor cell lines. (*A*) L-CD1, L5178Y-R, YAC-1, and 18.81 cells were stained with a PE-labeled anti-mouse CD1d mAb (filled histograms) or a PE-conjugated rat IgG2b as an isotype control (open histograms). The cells were analyzed by cytofluorography. (*B*) Murine CD1$^+$ hematopoietic tumor cells are not recognized by NKT cells. The Vα14$^+$ NKT cell hybridoma cell line, DN32.D3, was cocultured with L cells (negative control), L-CD1 cells (positive control), or murine CD1$^+$ hematopoietic tumor cells L5178Y-R, 18.81, and YAC-1. After a 24-hr incubation, culture supernatants were harvested and assessed for IL-2 production by ELISA. The bars represent the mean absorbance values at 405 nm from triplicate cultures ± SD.

Flow Cytometry. Cells were stained for cytofluorography with a phycoerythrin (PE)-labeled anti-mouse CD1d or PE-labeled rat IgG2b (isotype control; PharMingen) for 30 min on ice followed by washing 3 times in FACS buffer (Hanks' balanced salt solution + 0.1% BSA + 0.02% azide) as described (28). Cells cultured in the presence of PPMP were fixed in 0.05% paraformaldehyde in PBS before staining. Analysis was performed on a FACScan cytofluorograph (Becton-Dickinson).

Glycolipid Extraction and High-Performance Thin-Layer Chromatography (HPTLC). Cellular gangliosides were isolated from L5178Y-R cells that had been pelleted and extracted twice with chloroform–methanol (1:1 vol/vol). The total lipid extract was taken to dryness and saponified with methanolic KOH to remove phospholipids (29). Analysis of glycolipids was performed with 5×8 cm precoated Silica gel 60 HPTLC plates (Merck). The plates were developed in chloroform–methanol–water (65:25:4 vol/vol/vol). Glycolipids were visualized as purple bands with orcinol-HCl reagent spray.

Results

CD1 Cell Surface Expression by Murine Hematopoietic Tumors. As the cell surface expression of CD1 molecules is restricted mainly to hematopoietic tissues (1, 11), we analyzed three well known murine hematopoietic tumor cell lines for CD1 cell surface expression by cytofluorography. These tumor cells include representatives of T cell- (L5178Y-R, YAC-1) and pre-B cell-derived (18.81) tumors. All of these tumor cell lines expressed detectable levels of CD1 on the cell surface (Fig. 1*A*). Of these, L5178Y-R expressed the highest amount of CD1 among the T cell-derived tumor cells, whereas the 18.81 pre-B cells expressed comparable or higher levels of cell surface CD1 relative to the other tumor cell lines (Fig. 1*A* and data not shown).

CD1$^+$ Hematopoietic Tumor Cells Are Not Recognized by NKT Cells. We have shown that murine CD1 molecules are recognized by a unique subpopulation of T cells called NKT cells (9). To determine whether the CD1$^+$ hematopoietic tumor cells could be recognized by NKT cells, the tumor cells were cocultured with the canonical (i.e., Vα14Jα281 T cell receptor rearrangement) CD1-restricted

NKT cell hybridoma, DN32.D3 (9, 28). As controls, DN32.D3 cells were cocultured with mouse L cell fibroblasts transfected with (L-CD1) or without the murine *cd1d1* cDNA (L-control) (23). CD1-specific recognition was determined by measuring the IL-2 production in the coculture supernatants by ELISA. The CD1-specific NKT cell hybridoma, DN32.D3, failed to recognize any of the $CD1^+$ hematopoietic tumor cells (Fig. 1*B*). By contrast, L-CD1 cells could stimulate IL-2 production from the DN32.D3 NKT cell hybridoma. Therefore, even though the hematopoietic tumor cells expressed CD1 on the cell surface, this expression, by itself, was insufficient to confer sensitivity to recognition by NKT cells.

CD1d1 Molecules from the Hematopoietic Tumor Cells Are Recognized by NKT Cells. A potential explanation for the failure of the panel of murine $CD1^+$ hematopoietic tumor cells to trigger NKT cell activation could be spontaneous mutations in the *cd1d1* gene itself. To assess this possibility, *cd1d1* cDNA was generated from all three hematopoietic tumor cell lines with the resulting full-length cDNA being subcloned into the eukaryotic expression vector, pcDNA3.1. L cells transfected with these cDNAs were screened for CD1 cell surface expression by cytofluorography. L cells transfected with the *cd1d1* cDNA from L5178Y-R, YAC-1, or 18.81 cells (L-L5178Y, L-YAC-1, and L-18.81, respectively) expressed CD1 on their cell surface at detectable levels (Fig. 2*A*). To assess the functional expression of the tumor cell-derived CD1d1, NKT cell recognition was tested. The $CD1d1^+$-transfected cell lines were cocultured with the canonical ($V\alpha14^+$) and noncanonical ($V\alpha5^+$) DN32.D3 and N37-1A12 (3) NKT cell hybridomas, respectively, and recognition was analyzed by ELISA. All three CD1d1 transfectant cell lines could be recognized by both of the NKT cell hybridomas at levels at least as high as the L-CD1-positive control, whereas transfectants containing the vector alone failed to stimulate the NKT cells (Fig. 2*B*). Therefore, these results suggest that the *cd1d1* genes from the hematopoietic tumor cell lines are functionally normal.

L5178Y-R Tumor Cells Shed a Factor That Inhibits NKT Cell Recognition of CD1d1. It is well established that tumor cells can shed gangliosides and other glycolipids that inhibit T cell recognition and activation (30–37), including the generation of antitumor cytotoxic T cells (20). L5178Y tumor cells have been shown to shed glycolipids (31). Therefore, culture supernatants from these tumor cells containing shed macromolecules were tested for their effect on CD1d1 recognition by NKT cells. Fresh medium or CM from L5178Y-R cells was added to L-CD1 cells for 4 h, removed by washing, and these fibroblasts were cocultured with either the canonical NKT cell hybridoma, DN32.D3, or the noncanonical ($V\alpha5^+$) CD1-specific NKT cell hybridoma, N37-1A12 (3). L-CD1 cells were fixed in paraformaldehyde after treatment with culture medium and before incubation with NKT cells, thus preventing endocytosis of preexisting cell surface CD1d1 molecules or the expression of nascent CD1d1. L5178Y-R CM substantially blocked recognition of L-CD1 cells by DN32.D3 ($\sim$50% inhibition; $P < 0.0001$) (Fig. 3). In contrast, L5178Y-R CM had no effect on L-CD1 recognition by N37-1A12, suggesting that the CM caused a specific inhibition of CD1d1 recognition by a canonical NKT cell, as opposed to a down-regulation of cell surface CD1d1. In fact, L5178Y-R CM treatment had no effect on the cell surface expression of CD1d1 as analyzed by cytofluorography (data not shown). CM from YAC-1 and 18.81 tumor cells failed to affect NKT cell recognition of L-CD1 cells (data not shown). Therefore, these results suggest that the L5178Y-R tumor sheds a molecule(s) or factor(s) that can specifically inhibit CD1 recognition by canonical (i.e., $V\alpha14J\alpha281$–DN32.D3) but not noncanonical (i.e., N37-1A12) NKT cells.

Disruption of Tumor Cell Production of Glycolipids Restores NKT Cell Recognition of L5178Y-R Cells. To determine whether glycolipid shedding by L5178Y-R prevents NKT cell recognition (Fig. 3), the tumor cells were treated with vehicle (0.05% ethanol) or the

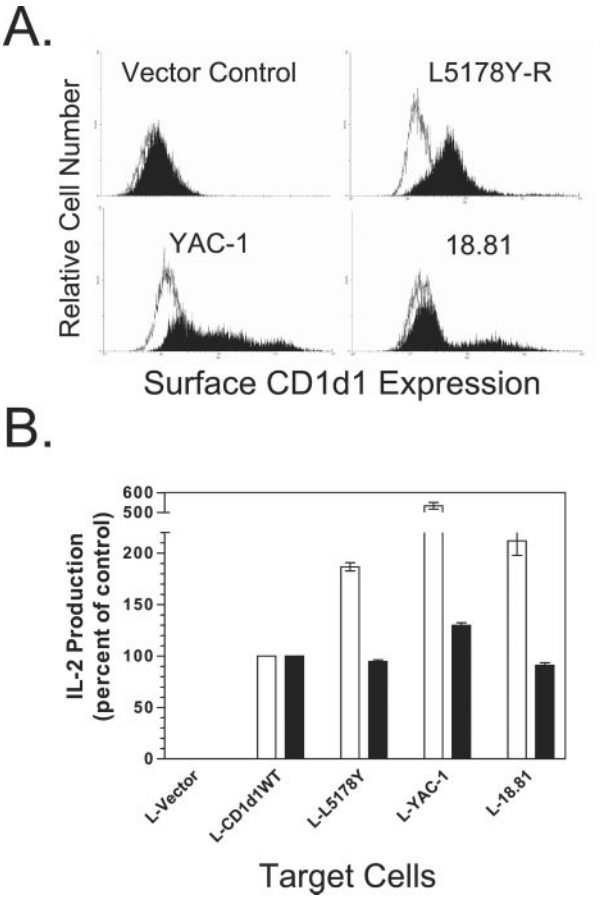

Fig. 2. Functional cell surface expression of CD1d1 on L cells transfected with *cd1d1* cDNA from murine $CD1^+$ hematopoietic tumor cells. (*A*) Murine L cell fibroblasts were transfected with the pcDNA3.1-neo vector alone or the vector containing the full-length *cd1d1* cDNA generated from L5178Y-R, YAC-1, or 18.81 cells. The cells were stained with a PE-labeled anti-mouse CD1 mAb (filled histograms). A PE-conjugated rat IgG2b (open histograms) served as an isotype control. Analysis was by cytofluorography. The data are representative of two independent experiments. (*B*) L cells transfected with vector only or vector containing the WT *cd1d1* cDNA (L-CD1d1WT) or that from L5178Y-R, YAC-1, or 18.81 cells were cocultured with the $V\alpha14^+$ (canonical; DN32.D3; white bars) or $V\alpha5^+$ (noncanonical; N37-1A12; black bars) NKT cell hybridomas for 24 h. Supernatants were harvested, and IL-2 production was measured by ELISA. The data shown are the mean of triplicate cultures ± SD.

glucosylceramide synthase inhibitor PPMP (27). PPMP and related compounds have been shown to abrogate tumor cell glycolipid biosynthesis within 3 days and glycolipid shedding within 5 days after treatment—without a significant effect on cell viability (35). Therefore, L5178Y-R cells were treated with vehicle or PPMP (10 μM) for 5 days before coculture with the CD1-specific NKT cell hybridomas, DN32.D3 or N37-1A12. It should be noted that the presence of PPMP during a 20–24-h coculture of L-CD1 with NKT cells did not affect IL-2 production by either hybridoma (data not shown). The canonical CD1d1-specific NKT cell hybridoma, DN32.D3, failed to recognize untreated L5178Y-R cells (Table 1). In contrast, a 5-day PPMP treatment of L5178Y-R cells permitted direct tumor cell recognition by DN32.D3. Interestingly, PPMP treatment did not influence recognition of L5178Y-R cells by the noncanonical NKT cell hybridoma, N37-1A12 (Table 1). Tumor cell treatment with 10 μM PPMP for 5 days substantially inhibited glycolipid biosynthesis including, notably, the production of Gg3Cer (Fig. 4) as reported (27). Therefore, these data suggest that the inhibition of glucosylceramide synthase by PPMP treatment

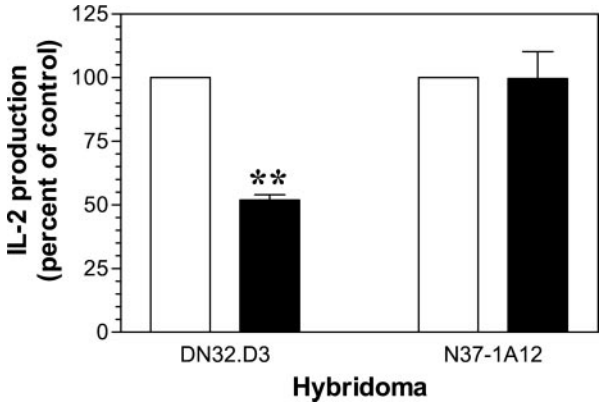

Fig. 3. CM from L5178Y-R cells inhibits recognition of CD1d1 by canonical (but not noncanonical) NKT cells. Murine L-cell fibroblasts transfected with the WT *cd1d1* cDNA (L-CD1d1WT) were cocultured with the canonical (Vα14$^+$) or noncanonical (Vα5$^+$) NKT cell hybridomas, DN32.D3 and N37-1A12, respectively. L cells transfected with vector only were similarly treated and served as negative controls (not shown). Following a 24-hr incubation, IL-2 levels in the supernatant were measured by ELISA. The bars represent percent IL-2 production in control (DMEM; white bars) or L5178Y-R CM (black bars) ± SD in triplicate, and the data are presented as percent of control (DMEM)-treated cells. **, $P < 0$ 0001.

restores NKT cell recognition of CD1d1 complexes on L5178Y-R cells.

Treatment of L5178Y-R cells with PPMP could potentially influence CD1d1 function via the up-regulation of this cell surface antigen. To address this question, mock- and PPMP-treated L5178Y-R cells used in the NKT cell assays were also stained for CD1 cell surface expression and analyzed by cytofluorography. Five or 10 μM PPMP treatment of L5178Y-R cells for 5 days had no effect on the cell surface levels of CD1 (data not shown). Therefore, these results suggest that the ability of PPMP to facilitate recognition of L5178Y-R cells by canonical NKT cells is not simply caused by an up-regulation of tumor cell CD1d1 cell surface expression.

Exogenous Gg3Cer Inhibits CD1d1 Recognition by NKT Cells. Analysis of the molecular structure of the inhibitory factor present in L5178Y-R CM was rendered difficult because of the complex nature of constituents present in FBS added to the medium. FBS contains high amounts of gangliosides (data not shown). Therefore, direct extraction of lipids from CM for analysis could lead to an erroneous interpretation. We were also unable to adapt L5178Y-R cells to serum-free medium. However, because the lipid profile of a total cellular extract reflects the glycolipids being shed by these cells, total cellular glycolipids were tested for their effect on CD1d1-mediated antigen presentation. Thus, to analyze the nature of the molecule(s) shed by the L5178Y-R tumor cells and that is responsible for the effect observed (Fig. 3), lipid extracts from vehicle or PPMP-treated L5178Y-R cells were tested. Lipids extracted from L5178Y-R cells inhibited recognition of CD1d1 molecules on L-CD1d1WT cells by the canonical NKT cell hybridoma (DN32.D3) similar to that observed with CM (Fig. 5). As expected, these L5178Y-R lipid extracts had no effect on CD1d1 recognition by the noncanonical N37-1A12 NKT cell hybridoma (data not shown). Interestingly, lipids extracted from PPMP-treated L5178Y-R cells failed to inhibit the recognition of CD1d1 by DN32.D3 NKT cells (Fig. 5). Thus, the data suggest that PPMP treatment of L5178Y-R cells blocked the synthesis and shedding of inhibitory molecules that would otherwise alter the recognition of CD1d1 by canonical (but not noncanonical) NKT cells.

As we showed previously that components shed by L5178Y-R cells were able to inhibit NKT cell recognition of L-CD1d1 cells and, because the inhibitory factor(s) was present in the lipid fraction of the cells, it was asked whether a predominant glycolipid shed by L5178Y-R cells was responsible for the observed effect on NKT cell recognition. It is well known that a major glycolipid species shed by L5178Y-R cells is Gg3Cer (31), and we found that there was an apparent reduction in Gg3Cer after PPMP treatment of L5178Y-R cells as assessed by TLC analysis (Fig. 4). Thus, L-CD1d1WT cells were treated with increasing concentrations of purified Gg3Cer for

Table 1. Effect of PPMP treatment of L5178Y-R cells on their recognition by NKT cells

NKT cell hybridoma	L5178Y-R cells treated with:				
	Vehicle	5 μM PPMP	*P* value	10 μM PPMP	*P* value
DN32.D3	224 ± 41	2229 ± 85	<0.0001	1924 ± 40	<0.0001
N37-1A12	<125	<125	—	<125	—

L5178Y-R cells were treated with vehicle (0.05% ethanol) or the indicated concentrations of PPMP for 5 days before washing and coculture with the NKT cell hybridomas, DN32.D3 (Vα14$^+$) or N37-1A12 (Vα5$^+$). The net mean IL-2 production (pg/ml) ± SD is indicated. The limit of detection for this ELISA assay was 125 pg/ml.

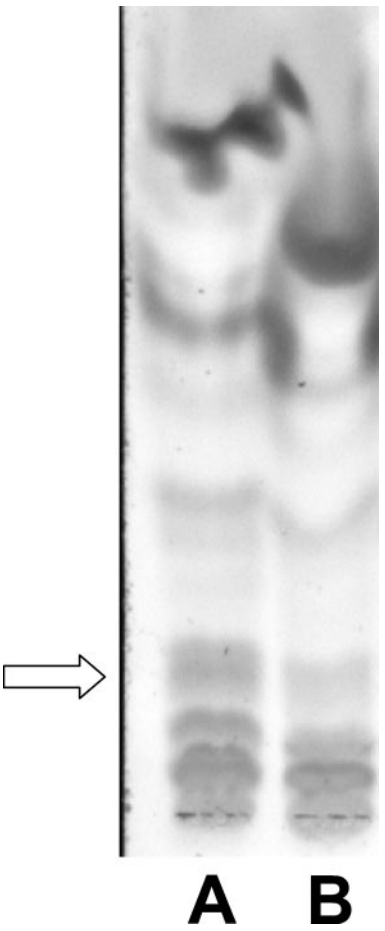

Fig. 4. High-performance thin-layer chromatography profile of total cellular lipids extracted from L5178Y-R cells. L5178Y-R cells were cultured for 5 days in vehicle (0.05% ethanol; lane A) or 10 μM PPMP (lane B). The pelleted cells were washed and extracted with C:M (2:1, vol/vol) by sonication. Contaminating phospholipids were removed by alkaline saponification (methanolic alkali). Cell equivalents were spotted for comparative analysis. The chromatogram was developed in C:M:W (65:25:4, vol/vol/vol), and glycolipids were detected by orcinol spray. The arrow indicates the standard relative mobility (R_f) of Gg3Cer. C:M:W, chloroform/methanol/water.

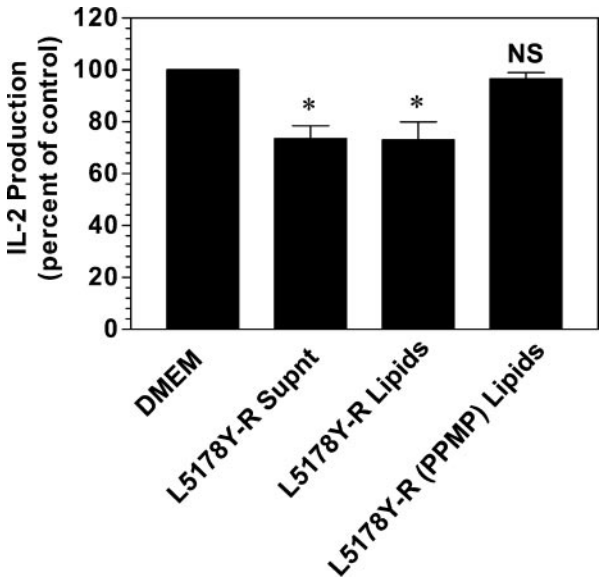

Fig. 5. Cellular lipids from L5178Y-R cells inhibit CD1 recognition by canonical NKT cells. L-CD1d1WT cells were treated with L5178Y-R CM or lipid extracts from vehicle or PPMP-treated L5178Y-R cells for 4 h, washed, and fixed in 0.05% paraformaldehyde before a 24-hr coculture with the DN32.D3 NKT cell hybridoma. IL-2 production in the supernatants was measured by ELISA and is represented as the percent IL-2 production relative to control (DMEM-treated) cells ± SD in triplicate wells. *, $P < 0.001$. NS, not significant.

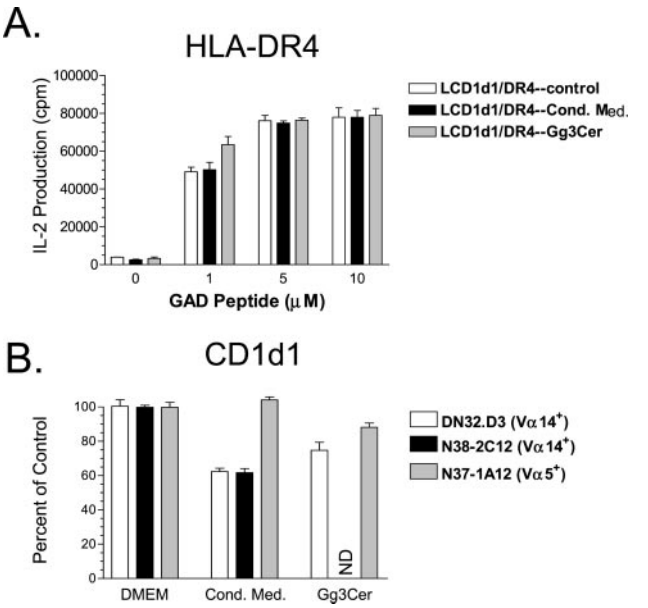

Fig. 6. Inhibitory effect of glycolipids on NKT cell recognition of L-CD1d1WT cells is CD1d1-mediated. L-CD1d1WT cells were transfected with HLA-DR4 cDNA and used as targets in a GAD peptide recognition assay with DR4-restricted, GAD-specific T cells (*A*) or for CD1d1 recognition by CD1d1-specific NKT cell hybridomas (*B*). The target cells were treated with or without L5178Y-R CM or 10 μM Gg3Cer as indicated. ND, not determined.

4 h, washed extensively, and cocultured with the canonical (DN32.D3) or noncanonical (N37-1A12) NKT cell hybridomas. Similar to the L5178Y-R CM (Figs. 3 and 5), Gg3Cer was able to inhibit NKT cell recognition of L-CD1d1WT cells by DN32.D3 (but not N37-1A12) NKT cells in a dose-dependent manner (Table 2). Gg3Cer treatment of L-CD1d1WT cells had no effect on the cell surface expression of CD1d1 (data not shown). Therefore, these results suggest that the Gg3Cer shed from L5178Y-R cells significantly contributes to the inhibition of NKT cell recognition of CD1 present on these tumor cells.

Inhibitory Effect of L5178Y-R CM and Gg3Cer Is Mediated by CD1d1. It is known that CD1d1 molecules traffic through the endocytic pathway like MHC class II molecules (1). To ensure that the inhibitory effects observed were mediated by the CD1d1 molecule itself rather than another factor unrelated to antigen presentation, we also analyzed the effects of L5178Y-R CM and Gg3Cer on MHC class II antigen presentation. Thus, L-CD1d1WT cells were transfected with the human HLA-DR4 α and β chains by retroviral transduction (26). HLA-DR4$^+$ L-CD1d1WT cells were then treated with or without various concentrations of the HLA-DR4-presented peptide derived from GAD in the presence or absence of L5178Y-R CM or Gg3Cer as done previously with the CD1-specific NKT cells. The GAD peptide-pulsed targets were then cocultured with the HLA-DR4-restricted GAD-specific 33.1 mouse T cell hybridoma, and IL-2 production was measured as described (24). Neither L5178Y-R CM nor Gg3Cer had any effect on GAD-specific stimulation of 33.1 (Fig. 6). As seen with the parental L-CD1d1WT cells, these inhibitors were able to reduce recognition of CD1d1 on the HLA-DR4$^+$ L-CD1d1WT cells by Vα14$^+$ (but not Vα14$^-$) NKT cell hybridomas. Therefore, these results strongly suggest that the inhibitory activity of L5178Y-R CM and Gg3Cer on L-CD1d1WT recognition by Vα14$^+$ NKT cells was mediated by CD1d1.

Discussion

The initial immune recognition of oncogenic transformation and subsequent destruction of transformed cells is a very critical process of the host's immune response to prevent tumor formation. However, tumor cells have evolved a myriad of ways to evade the complex antitumor immune mechanisms of the host (38). One such mechanism of tumor escape is the shedding of cell surface molecules by tumor cells into their microenvironment, leading to immunosuppression (19, 30, 38). Shedding of glycolipids has also been implicated as a mode of tumor cell evasion from detection and resultant dissemination to distant metastatic sites (39). Ladisch and his colleagues (20, 35, 37) have extensively studied the role of tumor gangliosides as soluble modulators of classical antitumor immune responses. In fact, recent chemotherapeutic and vaccine strategies include blocking glycolipid synthesis and using anti-glycolipid Abs, respectively (40, 41). In this context, the absence of NKT hybridoma

Table 2. Effect of Gg3Cer treatment of L-CD1 cells on their recognition by NKT cells

NKT cell hybridoma	Gg3Cer concentration, μM				
	Vehicle	1	10	20	40
DN32.D3 (Vα14$^+$)	1233 ± 77	1061 ± 34	973 ± 25	870 ± 24	766 ± 17
N37-1A12 (Vα5$^+$)	2128 ± 72	2090 ± 23	2068 ± 4	2068 ± 70	1846 ± 27

L-CD1d1WT cells were treated with vehicle (0.05% ethanol) or the indicated concentrations of Gg3Cer for 4 h before washing and coculture with the NKT cell hybridomas, DN32.D3 or N37-1A12. The net mean IL-2 production (pg/ml) in triplicate wells ± SD is indicated.

stimulation by $CD1^+$ tumor cell lines led us to question whether glycolipids shed from these tumor cell lines could alter antigen presentation by CD1 molecules. It has been reported that tumor gangliosides block antigen processing and presentation by classical MHC class I molecules (42). The inhibitory effect of CM from L5178Y-R cells on canonical NKT cell hybridoma stimulation by L-CD1 cells (Fig. 3) demonstrates that CD1d1 function also is disrupted by specific tumor cell-derived glycolipids. Gg3Cer was chosen as a candidate antigen for testing its inhibitory activity on CD1d1-mediated antigen presentation, because it is the predominant neutral glycolipid shed by L5178Y-R cells (31). Thus, as expected, purified Gg3Cer showed inhibitory activity after treatment of $CD1d1^+$ cells with this antigen (Table 2; Fig. 6).

Earlier studies analyzing tumor cell glycolipids generally compared the biological properties of cell lines or sublines with different cellular glycolipid profiles. Ladisch *et al.* (30) found that, among several murine AKR lymphoma cell sublines, cells with high ganglioside content were highly tumorigenic, cells with low ganglioside content were poorly tumorigenic, and the addition of gangliosides isolated from highly tumorigenic cells greatly enhanced tumor formation by ganglioside-deficient, poorly tumorigenic cells. Other studies have also demonstrated that a high ganglioside content is associated with high tumorigenicity (37, 40, 43) and metastatic potential (19, 40). Neutral glycosphingolipids have not been implicated as mediators of the biological effects in any of these studies. However, our results suggest a role for the neutral glycolipid, Gg3Cer, in the modulation of endogenous glycolipid antigen presentation by CD1d1 molecules.

The mechanism of the inhibitory action of these glycolipids on antigen presentation is as yet unknown. It has been shown that glycolipids could be shed as large membrane vesicles as well as micelles (36). Based on the critical micellular concentration of the shed glycolipid, the aqueous microenvironment could force the glycolipids to attain large aggregate or vesicular forms. These large vesicular glycolipids might become adsorbed to the hydrophobic ligand-binding groove of CD1 and thus inhibit antigen presentation. However, it would be expected that such a phenomenon could inhibit CD1 recognition by any NKT cell and, as we found, recognition of CD1d1 by the noncanonical $V\alpha5^+$ NKT cell hybridoma N37-1A12 was not altered by L5178Y-R CM (or purified Gg3Cer). Considering that the majority of shed glycolipids from hematopoietic tumor cells are in a monomeric form (36), we think it is likely that the shed Gg3Cer competes with the natural CD1d1-presented ligand recognized by canonical NKT cells (the identity of which is currently unknown).

Human dendritic cells down-modulate their capacity to take up soluble antigens in response to exogenously added or endogenously produced ceramides (44). Ceramide has also been shown to inhibit fluid-phase and receptor-mediated endocytosis in Chinese hamster fibroblasts (45). Kok *et al.* (46) could demonstrate involvement of early and late endosomes in glucosylceramide recycling. Based on these findings, the inhibition of CD1-specific antigen presentation by Gg3Cer could also be attributed to its effect on endocytosis. However, given the short period of $CD1^+$ cells' (4-h) exposure to CM or purified glycolipids, we think that it is unlikely that the shed glycolipid(s) inhibits endocytosis. It is most likely that the mechanism of inhibition is the result of the binding of the shed glycolipid(s) to the "accommodative" hydrophobic groove of CD1d1 (1), thereby leading to inhibition of (or competition with) the binding of the endogenous ligand. This hypothesis would account for the rescue in NKT cell recognition of CD1d1 observed with PPMP-treated L5178Y-R cells.

In conclusion, the present study presents strong evidence that the neutral glycolipid, Gg3Cer, enriched in the CM of murine L5178Y-R T lymphoma cells, can inhibit CD1d1-mediated antigen presentation to NKT cells. The inhibitory activity of the shed neutral glycolipids may explain their effect on antigen processing and presentation in general, and may be one means by which some tumor cells can evade the host's innate antitumor immune response. Further understanding and elucidating the mechanisms by which CD1d-mediated antigen presentation is modulated by glycolipids may lead to the discovery and use of new therapeutic agents targeted at glucosylceramide synthesis.

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