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Involvement of Secretory and Endosomal Compartments in Presentation of an Exogenous Self-Glycolipid to Type II NKT Cells[1]

Keshab Chandra Roy,* Igor Maricic,* Archana Khurana,† Trevor R. F. Smith,* Ramesh C. Halder,* and Vipin Kumar[2]*

Natural Killer T (NKT) cells recognize both self and foreign lipid Ags presented by CD1 molecules. Although presentation of the marine sponge-derived lipid αGalCer to type I NKT cells has been well studied, little is known about self-glycolipid presentation to either type I or type II NKT cells. Here we have investigated presentation of the self-glycolipid sulfatide to a type II NKT cell that specifically recognizes a single species of sulfatide, namely lyso-sulfatide but not other sulfatides containing additional acyl chains. In comparison to other sulfatides or αGalCer, lyso-sulfatide binds with lower affinity to CD1d. Although plate-bound CD1d is inefficient in presenting lyso-sulfatide at neutral pH, it is efficiently presented at acidic pH and in the presence of saposin C. The lysosomal trafficking of mCD1d is required for αGalCer presentation to type I NKT cells, it is not important for presentation of lyso-sulfatide to type II NKT cells. Consistently, APCs deficient in a lysosomal lipid-transfer protein effectively present lyso-sulfatide. Presentation of lyso-sulfatide is inhibited in the presence of primaquine, concanamycin A, monensin, cycloheximide, and an inhibitor of microsomal triglyceride transfer protein but remains unchanged following treatment with brefeldin A. Wortmannin-mediated inhibition of lipid presentation indicates an important role for the PI-3kinase in mCD1d trafficking. Our data collectively suggest that weak CD1d-binding self-glycolipid ligands such as lyso-sulfatide can be presented via the secretory and endosomal compartments. Thus this study provides important insights into the exogenous self-glycolipid presentation to CD1d-restricted T cells. ***The Journal of Immunology,*** **2008, 180: 2942–2950.**

The CD1 proteins are Ag-presenting molecules that have many characteristics similar to MHC molecules (1). Five different isotypes of CD1 proteins are expressed in association with β_2-microglobulin (β_2m)[3] and can be categorized into two groups: group I (CD1a, CD1b, CD1c and CD1e (being intermediate) in humans) and group II (CD1d in humans and mice) based on sequence homology (2). Unlike MHC molecules that present peptides, CD1 proteins bind and display a wide array of lipids, glycolipids, and lipopeptides (either foreign or self) to T lymphocytes expressing α- and β-chains of the TCR (3). CD1d presents lipids and glycolipids to at least two distinct populations of NKT cells. One of these is referred to as invariant (iNKT) or type I NKT cells, which use a single invariant α-chain (mouse Vα14-Jα18 and the homologous human Vα24-Jα18) in combination with a restricted number of β-chains (mouse Vβ8.2, Vβ7, and Vβ2, and human Vβ11) of TCR (4, 5). Type I NKT cells can recognize the foreign Ag αGalCer, a lipid found in the marine sponge *Agelas mauritianus* (6), in response to which they rapidly produce Th1 and Th2 cytokines (7–11). These cells also recognize bacterial lipids as well as a self-glycolipid, isoglobotrihexosyl ceramide iGB3 (4, 5). Other autoreactive CD1d-restricted T cells, which do not use the invariant TCR α-chain and use a wide variety of TCR β-chains, are referred to as type II NKT cells. A subset of the type II NKT cells is able to recognize the self-glycolipid sulfatide in both humans and in mice (5).

Like proteins, glycolipids also need processing to yield immunogenic complexes with CD1 molecules for successful presentation. APCs have evolved an Ag processing machinery and intracellular trafficking of Ag presenting molecules including CD1 to survey and bind with appropriate ligands for presentation to T cells. Recently adaptor proteins, several lysosomal enzymes, lipid transfer molecules, the biophysical environment inside the cell, and lipid carrier proteins have been shown to be involved in surveillance as well as in the processing and loading of lipid Ags onto CD1 molecules (12). It has been shown that a high affinity ligand to CD1d such as αGalCer does not require processing for presentation to iNKT cells (13). However its presentation to iNKT is inhibited in the absence of saposins, MTP, and adaptor protein AP-3 (14–16). Sulfatide is a promiscuous ligand which binds to human CD1a, CD1b, CD1c, and CD1d and does not require further processing because it forms a stable immunogenic complex with CD1 in vitro (17). We have previously reported that the murine non-invariant T cell hybridoma 19.3 recognizes sulfatide presented by CD1d (18). Bovine brain-derived sulfatide contains several species, including *cis*-tetracosenoyl-, tetracosenoyl-, palmitoyl-, and lyso-sulfatide.

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[3] Abbreviations used in this paper: β_2m, β_2-microglobulin; WT, wild type; TD, tail deleted; IEF, isoelectric focusing; CMA, concanamycin A; ER, endoplasmic reticulum; EE, early endosome; RE, recycling endosome; iNKT, invariant NKT.

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Recently we have published a three dimensional structure of *cis*-tetracosenoyl-sulfatide complexed with murine CD1d at 1.9 Å (19) showing binding of this natural self-glycolipid ligand.

Here we further define the Ag-fine specificity of the type II NKT cell hybridoma 19.3. We show that its TCR is extremely restrictive in that it recognizes lyso-sulfatide but not other glycolipids, including three other major species of sulfatide. Lyso-sulfatide binds with a weak affinity to mouse CD1d. Sulfatide bound mCD1d traffics into the endoplasmic reticulum (ER), early endosome (EE), and recycling endosome (RE) for its presentation at the cell surface. Also we report for the first time that trafficking of mouse CD1d and its Ag presentation ability is regulated by phosphatidyl inositol 3-kinase. Although the occurrence of cross-presentation of lipid Ags in vivo is inferred from earlier studies (20, 21), the present data further elucidate the intracellular pathways involved in the presentation of exogenous self-glycolipids bound by CD1d to type II NK T cells.

Materials and Methods

Mice and reagents

Female C57BL/6 and AP-3 mice were purchased from The Jackson Laboratory. They were maintained in specific pathogen-free conditions in vivarium at TPIMS. A20 cells transfected with the wild-type (WT) or tail-deleted (TD) mouse CD1d (mCD1d), Vα14$^+$ 1.2 NKT cell hybridoma, Hy-1.2 were kindly provided by Dr. M. Kronenberg (La Jolla Institute of Allergy and Immunology, La Jolla, CA) and were cultured in RPMI supplemented with 10% FBS and 2 mM L-glutamine. Sulfatide-reactive Vα14– hybridoma has earlier been described (18). Brefeldin A, primaquine, monensin, wortmannin, concanamycin A (CMA), glutaraldehyde, L-lysine, asialo-GM1 and Tween 20 were purchased from Sigma-Aldrich. Cycloheximide and lactacystin were purchased from A.G. Scientific. MTP inhibitor (iMTP, NUP-LAB687) was gifted by Drs. Gary Ksander and David Chao (Novartis Institute for Biomedical Research). Dr. Kronenberg kindly provided wild type, prosaposin, and cathepsin L knock out mice originally developed in the laboratories of Drs. Grabowski and A Rudenski, respectively. All Abs and conjugates used for FACS analysis were purchased from BD Biosciences. Recombinant mouse saposin C was provided by Dr. Masao Hiraiwa (Department of Neurosciences, University of California San Diego). Semisynthetic lyso-sulfatide, palmitoyl-sulfatide, *cis*-tetracosenoyl and tetracosenoyl sulfatide were purchased from Matreya Inc. Synthetic α GalCer was provided by Pharmaceutical Division of the Kirin Brewery Co. All lipids were dissolved in vehicle (0.5% polysorbate 20 (Tween 20) and 0.9% NaCl solution). Enhanced protein-binding 96-well ELISA plates were purchased from NUNC.

Flow cytometry

Staining for flow cytometry was performed as described previously (18). In brief, splenocytes or A20 cells transfected with WT- or TD-mCD1d were treated either in the presence or absence of inhibitors. Cells were washed twice with PBS and suspended in FACS buffer (PBS, 0.01% NaN_3, and 0.5% BSA). Resuspended cells were first blocked with Abs against FcR-γ (2.4G2) and then labeled with the indicated Abs. Finally cells were fixed with 1% paraformaldehyde. Flow cytometric analysis was performed on a FACSCalibur instrument using CellQuest software (BD Biosciences). PE-labeled mCD1d–α GalCer tetrameric complexes were made in a baculovirus expression system as described previously (22, 23). "Unloaded" mCD1d tetramers were prepared by preincubating biotinylated mCD1d protein with vehicle only. All experiments were repeated at least two times.

In vitro Ag presentation assay using APCs

Initial Ag presentation assays were performed to define the fine specificity of sulfatide-reactive hybridoma, Hy-19.3 in the presence different lipids. 5×10^4 of T cell hybridoma (Hy-19.3) were cocultured with irradiated splenocytes (4×10^5/well in triplicate) from 8 to 12 wk old C57BL/6 mice in the presence of graded concentrations of glycolipids (10^{-2} ng-1 μg for αGalCer and 0.05 μg-25 μg for other lipids). Supernatants from these 16-h cultures were used in sandwich ELISA to the measure IL-2 production.

In some lipid presentation assay, APCs including splenocytes collected from different WT and knock out mice or WT or TD mCD1d-transfected A20 cells (4×10^5/well) pulsed with lyso-sulfatide or αGalCer for 4h (irradiated but unfixed APC) or 2h (unirradiated, fixed APC). APCs 4×10^5/well were cocultured with 5×10^4/well T cell hybridoma, Hy-19.3 or Hy-1.2 for lyso-sulfatide or αGalCer, respectively in triplicate at 37°C in RPMI 1640 medium containing 10% FCS. Supernatants were harvested for IL-2 release assay as above. Data are expressed as mean ng/ml ± SD of triplicates. All experiments were repeated at least two times.

To study the lipid Ag pathway, WT mCD1d-transfected A20 cells ($2–4 \times 10^6$/ml) were incubated with a panel of Ag presentation pathway inhibitors at their optimum concentrations, including 300 mM primaquine, 100 nM CMA, 8–10 μg/ml brefeldin A, 50 mM monensin, 1.0 mM wortmannin for 2 h, 100 μg/ml cycloheximide for 5 h and 10 mM LAB-687 (iMTP) for 72 h before the addition of lipid Ags (lyso-sulfatide: 10 μg/ml and αGalCer: 200 ng/ml). Inhibitory reagents were titrated and class I-restricted OT-I T cells were used as controls for nonspecific cytotoxicity. Cells were further incubated for 2–4h at 37°C in continued presence of inhibitor, washed and fixed and used to stimulate T cells.

Fixation of APCs

WT mCD1d-transfected A20 cells treated either in presence or absence of inhibitor followed by washing twice and suspended in PBS (10×10^6/ml) containing 0.05% glutaraldehyde for 30 s at room temperature. Additional fixation was blocked by incubating with 0.2 M of L-lysine for 2 min. It was ascertained that the concentrations of the inhibitors and the fixation procedure used in the assay did not result in significant loss of cell membrane integrity of the APC as determined by trypan blue exclusion.

Ag presentation assay using plate-bound CD1d

Flat bottom 96-well plates were coated for 18 h with soluble CD1d recovered from baculovirus expression system (19, 22) at the concentration of 9.0, 3.0, 1.0 and 0.3 μg/well at 4°C in PBS (pH 7.4). Plates were washed three times with PBS and then incubated for another 4h at 37°C with indicated concentrations of lyso-sulfatide or αGalCer. After washing three times with PBS, 5×10^4/well T cell hybridomas (Hy-19.3 or Hy-1.2) were added in triplicate. Supernatants were collected after 17 h to measure IL-2 release.

To study the effect of pH on lyso-sulfatide loading and presentation, 1 μg of soluble mouse CD1d was coated on flat-bottom 96-well plates for 18 h at 4°C in 50 mM Na-acetate buffer (pH 5.0, 6.0 and 7.0). Plates were washed three times with same buffer and then incubated for another 4h at 37°C with lyso-sulfatide 1.25 μg/ml or αGalCer 500 ng/ml in 100 ml of buffer. Plates were washed thrice with PBS (pH 7.4) before addition of 5×10^4/well T cell hybridoma in triplicate. Supernatants were collected after 17 h to measure IL-2 release.

To investigate the role of saposin in lyso-sulfatide loading and presentation 1 μg of soluble mouse CD1d was coated on flat-bottom 96-well plates for 18 h at 4°C in 50 mM Na-acetate buffer (pH 5.0). Plates were washed three times with same buffer. 5 mg/ml lyso-sulfatide in 100 ml of 50 mM Na-acetate buffer (pH 5.0) in the presence of different concentrations of saposin C as indicated was added and plates were incubated for another 6 h at 37°C. Plates were washed three times with PBS (pH 7.4) before addition of 5×10^4/well T cell hybridoma, Hy-19.3 in triplicate. Supernatants were collected after 17 h to measure IL-2 release.

To compare the binding affinity of each four major species of sulfatide (*cis*-tetracosenoyl, tetracosenoyl, palmitoyl, and lyso) to CD1d the competition assay is designed as follows. One microgram of soluble mouse CD1d was coated on flat-bottom 96-well plate for 18 h at 4°C in PBS (PBS, pH 7.4). Plates were washed three times with PBS and then incubated for another 2 h at 37°C with a mixture of indicated concentrations of individual species of sulfatide with a fixed concentration of αGalCer (250 ng/ml) in 100 ml volume. Plates were washed thrice with PBS before addition of 5×10^4/well T cell hybridoma, Hy-1.2 in triplicate. Supernatants were collected after 17 h to measure IL-2 release.

Isoelectric focusing gel electrophoresis of sulfatide/CD1d complexes

The protocol is same as described earlier (19). In brief, recombinant mouse CD1d (9 μg/18 ml in PBS) was mixed with 4 μg (2.0 ml) synthetic *cis*-tetracosenoyl sulfatide, bovine brain-derived tetracosenoyl-, palmitoyl-, and lyso-sulfatide and incubated at 37°C for 6 h followed by washing with 500 ml of PBS and concentrated using 10 kDa m.w. cut-off centrifugal concentrators (Millipore). After adjusting the remaining volume, equal amounts of protein were subjected to isoelectric focusing (IEF) gel electrophoresis to monitor the loading efficiency of each species of sulfatide onto mCD1d.

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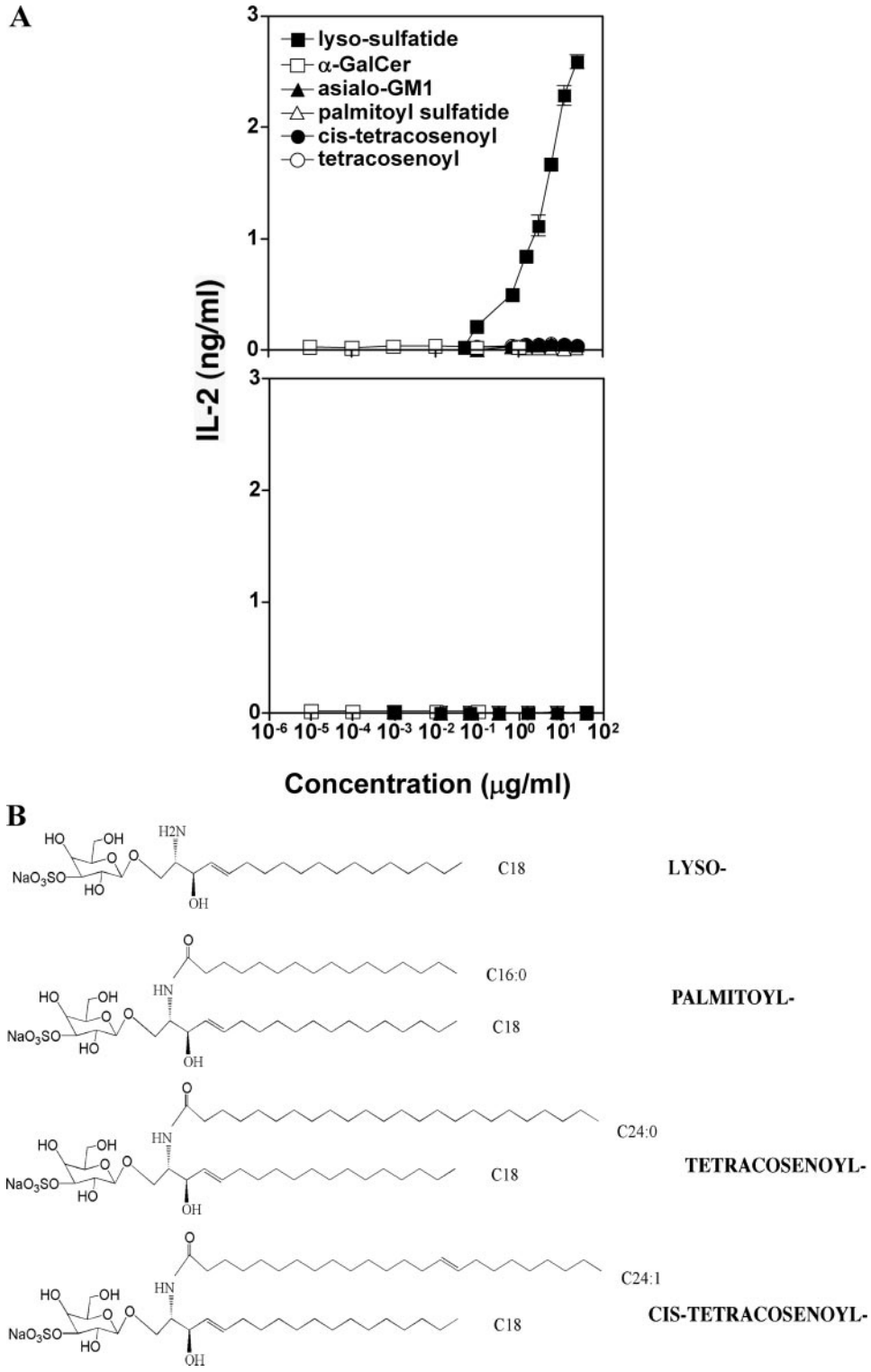

FIGURE 1. Recognition of lyso-sulfatide by a T cell hybridoma, Hy-19.3. *A*, Splenocytes from 8 to 12 wk old CD1d+/+ WT B6 (*top panel*) or CD1d−/− B6 (*lower panel*) mice were cocultured with Hy-19.3 (at a ratio of 8:1) in the presence of the indicated concentrations of lyso-sulfatide (■), *cis*-tetracosenoyl sulfatide (●), tetracosenoyl sulfatide (○), palmitoyl sulfatide (△), asialo-GM1 (▲), and αGalCer (□). Values are mean ± SD. The data shown here are the representative of at least three independent experiments. *B*, Chemical structure of different species of sulfatide used in the Ag presentation assay are shown.

Results

Fine specificity of a sulfatide-reactive type II NKT cell hybridoma

A panel of lipid Ags was used to study the fine Ag-specificity of the non-invariant Vα14$^-$ mouse type II NKT cell hybridoma Hy-19.3 which was previously shown to recognize bovine brain-derived sulfatides via CD1d (18). Our present data in Fig. 1*A* demonstrate that Hy-19.3 specifically recognizes lyso-sulfatide, and fails to be stimulated in the presence of other lipids including three other predominant species of bovine brain-derived sulfatides. Consistent with earlier data (18), presentation of lyso-sulfatide is also dependent upon CD1d, as APCs from CD1d+/+ (Fig. 1*A*, *top panel*) but not from CD1d−/− mice (Fig. 1*A*, *bottom panel*) are able to stimulate Hy-19.3. Unlike other species of sulfatide (*cis*-tetracosenoyl, tetracosenoyl, palmitoyl), lyso-sulfatide lacks an acyl chain (Fig. 1*B*). These data indicate that Ag recognition of a particular glycolipid by the TCR expressed on a type II NK T is highly specific.

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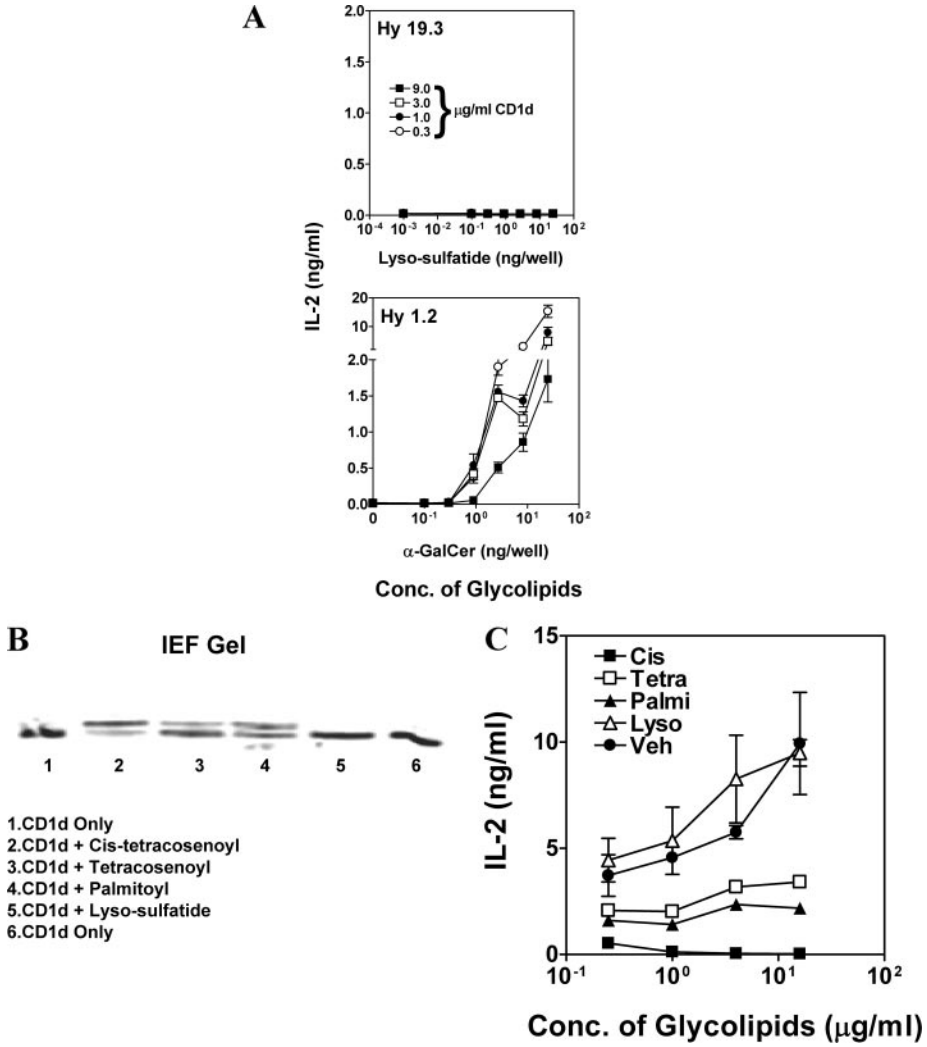

FIGURE 2. Lyso-sulfatide is a weak affinity ligand forming an immunogenic complex with soluble mCD1d in vitro (*A*) 96-well plates were coated with 9.0 (■), 3.0 (□), 1.0 (●), and 0.3 (○) μg/well of mCD1d and were allowed to bind with indicated concentrations of lyso-sulfatide (*upper panel*) and αGalCer (*bottom panel*) in PBS (pH 7.4) for stimulating Hy-19.3 or Hy-1.2 (14, *Materials and Methods*). Values are mean ± SD for IL-2 secretion. *B*, Lyso-sulfatide is poorly loaded onto mCD1d molecules as visualized by IEF gel. Nine micrograms of purified mCD1d protein was incubated with 4 μg of synthetic *cis*-tetracosenoyl sulfatide, bovine brain-derived tetracosenoyl sulfatide, palmitoyl sulfatide, and lyso-sulfatide and subjected to IEF gel electrophoresis (19). *C*, Lyso-sulfatide is unable to compete with αGalCer in vitro in ligand-binding competition assay. Fixed concentration of αGalCer (250 ng/ml) was mixed separately with *cis*-tetracosenoyl sulfatide (■), tetracosenoyl sulfatide (□), palmitoyl sulfatide (▲), lyso-sulfatide (△), and vehicle (●) and incubated for 2 h at 37°C with plate-bound mCD1d. After washing, IL-2 production by the αGalCer-reactive T cell, Hy-1.2 was determined in a typical 16–17 h assay (*Materials and Methods*). Values are mean ± SD. Data represents one of two individual experiments.

Lyso-sulfatide binds poorly with mouse CD1d

Studies were conducted to examine the binding ability of lyso-sulfatide to mCD1d. Lyso-sulfatide is not presented by immobilized CD1d to Hy-19.3, whereas αGalCer is successfully presented to Vα14$^+$ iNKT cells when incubated in PBS (pH 7.4, Fig. 2*A*). IEF gel studies show that compared with other species of sulfatide (*cis*-tetracosenoyl, tetracosanoyl, palmitoyl) there is no detectable loading of lyso-sulfatide onto CD1d as judged by the lack of shift of band in case of lyso-sulfatide (Fig. 2*B*). Similarly, in Ag competition experiments lyso-sulfatide is unable to compete against the high affinity ligand, αGalCer, although other sulfatides are able to displace αGalCer for binding with immobilized CD1d (Fig. 2*C*). These results show that lyso-sulfatide binds very weakly with mouse CD1d and is incapable of forming stable immunogenic complexes under these in vitro experimental conditions.

Presentation of lyso-sulfatide is restored in acidic pH and in the presence of saposin C

Next we examined whether acidic pH or the presence of lipid-transfer proteins, such as saposins, can enhance or restore the presentation of sulfatides under these in vitro conditions. Immobilized mCD1d can present lyso-sulfatide as well as αGalCer efficiently and stimulate Hy-19.3 and Hy-1.2 respectively when incubated in 50 mM Na-acetate buffer at pH 5 or 6. The efficiency of presentation of lyso-sulfatide by plate-bound CD1d is further increased significantly in the presence of recombinant mouse saposin C (Fig. 3*B*). This finding is consistent with an earlier study using another weak affinity self-glycolipid ligand, iGB3, for presentation to a type I NKT cell (14).

Enhanced presentation of lyso-sulfatide in the absence of CDld targeting into lysosomal compartments

Lysosomal targeting of mouse CD1d depends upon the tyrosine motif present in the cytoplasmic tail of CD1d (24, 25) as well as upon an adaptor protein, AP-3 (16). Data in Fig. 4 clearly demonstrate that presentation of lyso-sulfatide is significantly higher when APCs lack this Tyrosine motif as well as AP-3 (Fig. 4). On the other hand, as expected, presentation of Galβ(1 > 3)αGalCer is inhibited in the absence of the Tyrosine motif and AP-3. Consistent with previous findings we also found that surface expression of CD1d is increased when CD1d is not targeted into lysosomes for degradation (see Table I).

Presentation of lyso-sulfatide is not altered in the absence of functional prosaposin or cathepsin-L

Saposins, products of the precursor gene prosaposin, are present in the lysosomal compartment and have been shown to be required for the loading of lipids onto the CD1 groove (14). Although APCs lacking prosaposin show defects in presentation of αGalCer or Galβ(1 > 3)αGalCer to Hy-1.2, presentation of lyso-sulfatide to the HY-19.3 remains unchanged (Fig. 5*A*).

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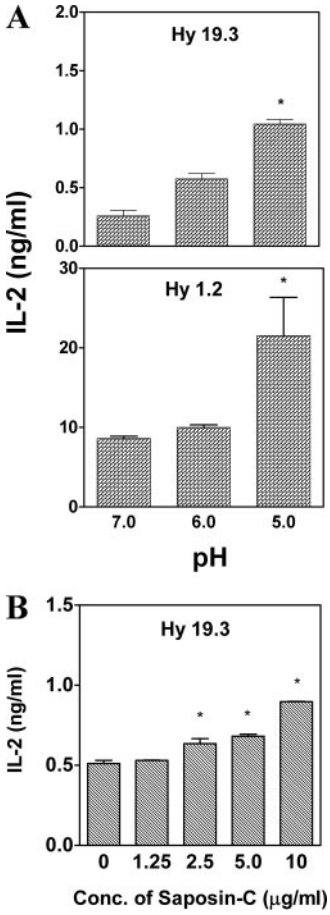

FIGURE 3. Lyso-sulfatide forms immunogenic complexes with mCD1d at acidic pH in vitro which are augmented in the presence of recombinant saposin C. Plate-bound CD1d was incubated with fixed concentrations of lyso-sulfatide either dissolved in acetate buffer at the indicated pH (*A*) or at pH 5.0 along with the indicated concentrations of recombinant saposin C. Values are mean ± SD. *, *p* values ~0.05 at pH 5. *B*, IL-2 release by the lyso-sulfatide-reactive T cell hybridoma, Hy-19.3 was assayed as described in the *Materials and Methods* section. Values are mean ± SD. *, *p* value ranged from 0.0001 to 0.028 at concentrations 2.5, 5, and 10 μg/ml. Data represent one of two independent experiments.

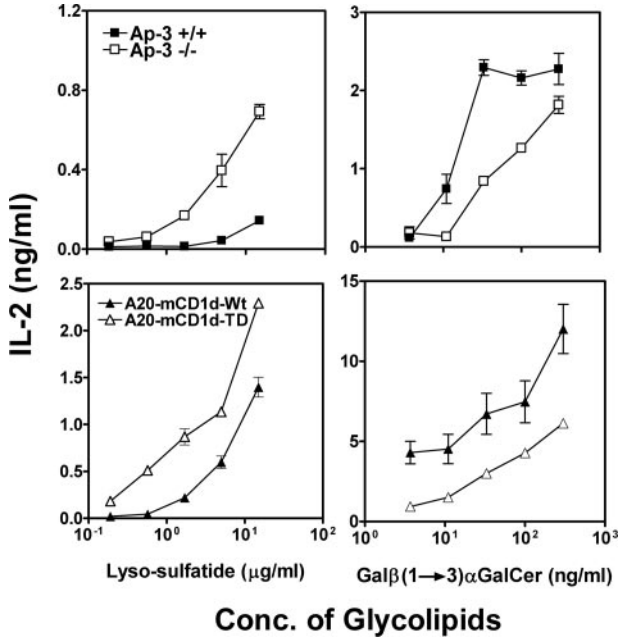

FIGURE 4. Mouse CD1d proteins not trafficking through lysosomal compartments are efficient in the presentation of lyso-sulfatide to type II NKT cells. Total spleen cells from *pearl* mice (AP-$3^{-/-}$) or WT (AP-$3^{+/+}$) C57BL/6 mice (*top panel*) and A20 cell line transfected with WT or TD mCD1d (*bottom panel*) were pulsed with either lyso-sulfatide (*left panel*) or Galβ(1→3)αGalCer (*right panel*). The type II NKT hybridoma Hy-19.3 and type I NKT hybridoma Hy-1.2 were cultured with the APCs pulsed with lyso-sulfatide and Galβ(1→3)αGalCer, respectively, and IL-2 release was determined. Values are mean ± SD. Data are representative of at least two experiments.

Surface expression of CD1d in prosaposin−/− mice in comparison to that in WT mice is not affected. Our data suggest that saposins present in the lysosomal compartment are not essential for lyso-sulfatide presentation in the context of CD1d molecules.

Cathepsin-L, a lysosomal protease, was proposed earlier to be required for the development of iNKT cells as well as presentation of endogenous ligand to them (26). In our assay system we observed that the presentation of exogenously supplemented lipid Ags including Galβ(1 > 3)αGalCer to Hy-1.2 and lyso-sulfatide to Hy-19.3 was not affected in cells deficient in the cathepsin-L gene product (Fig. 5*B*). Staining of liver mononuclear cells with CD1d/αGalCer tetramer shows reduced number of iNKT cells in these animals (unpublished data), consistent with the earlier report (26). Staining with CD1d/sulfatide tetramers+ cells indicated no significant difference in cathepsin-L+/+ (0.34%) or cathepsin-L−/− (0.38%) mice.

Requirements for presentation of exogenous lyso-sulfatide

To understand the intracellular processing/presentation pathway of lyso-sulfatide we used a panel of Ag presentation pathway inhibitors that have been used to study the processing and presentation of peptide Ags as well as lipid Ags by class I MHC and CD1 molecules, respectively. In each case concentration of the inhibitory agent was titrated and an optimum concentration was used at which there was no significant nonspecific effect on the viability of cells involved or on presentation of Ova peptide to class I MHC-restricted OVA-reactive OT-1 T cells (Fig. 6, *right panel*).

Brefeldin A disrupts anterograde protein transport from ER to Golgi (27) and induces retrograde transport of both resident and itinerant Golgi proteins to the ER in a fully reversible manner (28). The presentation of both lyso-sulfatide as well as αGalCer was not inhibited in the presence of brefeldin A (Fig. 6, *left* and *middle panels*). Our results indicate that presentation of lyso-sulfatide is not dependent on recycling of mouse CD1d. An interesting observation is that unlike MHC class I and B220 molecules, surface expression of CD1d is increased in the presence of BFA. It is noteworthy that BFA-induced increase in surface expression is a characteristic of mannose 6-phosphate/insulin-like growth factor II receptors (29, 30).

Monensin inhibits protein secretion by causing specific enlargement of Golgi-related compartments (31, 32), and being a Na^+/H^+-exchanging ionophore it increases endocytic vesicle pH above 6.0 (33). Our data show a significant inhibition of presentation of both lyso-sulfatide as well as αGalCer in the presence of monensin (Fig. 6, *left* and *middle panels*). This result implies that endosomal acidification plays an important role in exogenous lipid Ag presentation. As expected, a slight reduction of cell surface molecules including CD1d was observed after treatment with monensin (Table I).

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Table I. *Alterations in cell surface expression of CD1d under different experimental conditions*[a]

Treatment	CD1d	MHC Class I	B220	CD45
Brefeldin A	52 ± 5.70 ↑	28 ± 4.10 ↓	13.1 ± 0.96 ↓	ND
Monensin	19.0 ↓	21.4 ↓	12.4 ↓	7.6 ↓
AP-3 K/O	62 ± 1.97 ↑	ND	ND	ND
Primaquine	17.5 ± 3.02 ↓	–	6.0 ± 1.97 ↑	ND
Wortmannin	41.7 ↓	ND	ND	ND
Cycloheximide	48.6 ↓	ND	23.63 ↓	7.14 ↓
MTP-Inhibitor	44.5 ± 1.45 ↓	14.1 ± 4.49 ↓	–	–
Concanamycin A	–	ND	ND	ND

[a] Changes in expression of cell surface markers are expressed as percent values (%): mean ± SE (three experiments) or mean only (two experiments), by calculating the difference in MFI in each condition versus control by FACS analysis. ↓, Decrease; ↑, increase; –, unchanged; and ND, not done.

A generalized protein synthesis inhibitor cycloheximide (CHX) was found to significantly block lyso-sulfatide presentation as compared with αGalCer presentation (Fig. 6, *left* and *middle panels*). This suggests that that exogenously administered lyso-sulfatide can be presented via newly synthesized CD1d molecules.

An ER resident molecule microsomal triglyceride transfer protein (MTP) has been shown to function in transferring and/or loading of lipid ligands onto CD1d for presentation to iNKT cells (15). Presentation of lyso-sulfatide as well as αGalCer is inhibited when APCs are treated with the inhibitor, iMTP (LAB-687). Treatment with iMTP also causes down-regulation of surface expression of CD1d and MHC class I molecules of up to 50% and 25%, respectively (Table I). Our data (Fig. 6; *left* and *middle panels*) suggest that exogenous lipids may traffic through and bind to CD1d in the ER with the help of MTP. Also, our data corroborate earlier reports of iMTP-dependent specific down-regulation of surface expression of CD1d.

Wortmannin (WM) is a well-known inhibitor of phosphatidyl inositol 3-kinase (PI-3 kinase). Treatment of APCs with WM results in down-regulation of cell surface expression of CD1d molecules (Table I). This suggests that PI-3 K plays a significant role in trafficking of mouse CD1d. Furthermore, treatment of WM causes almost complete inhibition of lyso-sulfatide presentation (Fig. 6; *left panel*). These data clearly indicate a specific role of PI-3 K in trafficking of mouse CD1d and lipid Ag presentation.

Concanamycin A (CMA), an endosomal acidification inhibitor, prevents lyso-sulfatide presentation by wild type APCs (Fig. 6; *left panel*). Interestingly CMA also inhibits lyso-sulfatide presentation in the presence of APCs from AP-3KO mice (K. Roy and V. Kumar, unpublished data). However surface expression of CD1d was unchanged with this treatment. These data indicate that the pH of early as well as late endosomal compartments plays a significant role in presentation of weakly binding exogenous CD1d ligands.

The weak base primaquine (PQ) blocks trafficking of proteins through recycling endosomes and presentation of peptide Ags by MHC class II molecules as well as endogenous ligands to iNKT cells (34–36). We found that PQ down-regulates cell surface expression of mouse CD1d up to 20% whereas no effect on MHC class I or B220 molecules was observed (Table I). Moreover, treatment of PQ blocks presentation of both lyso-sulfatide and αGalCer to Hy-19.3 and Hy-1.2, respectively (Fig. 6; *left* and *middle panels*). These data collectively suggest that a fraction of CD1d molecules may traffic through recycling endosomes for the presentation of exogenous self-glycolipids via CD1d pathway.

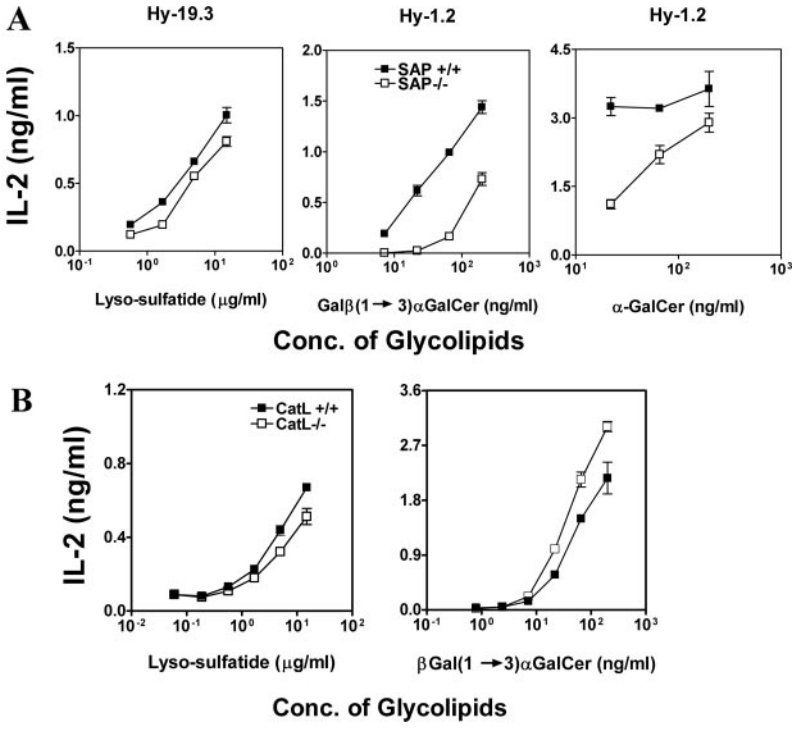

FIGURE 5. Lysosomal proteins saposins and cathepsin L are not important for lyso-sulfatide presentation by mCD1d. *A*, Total spleen cells from prosaposin knockout ($SAP^{-/-}$) or WT ($SAP^{+/+}$) mice were pulsed with lyso-sulfatide (*left*) Galβ(1→3)αGalCer (*middle*) or αGalCer (*right*). Hy-19.3 and Hy-1.2 were cultured with APC pulsed with lyso-sulfatide and Galβ(1→3)αGalCer as well as αGalCer, respectively. *B*, Total spleen cells from cathepsin L knockout ($CatL^{-/-}$) or WT ($CatL^{+/+}$) mice were pulsed with lyso-sulfatide (*left*) and Galβ(1→3)αGalCer (*right*). Hy-19.3 and Hy-1.2 were cultured with the APC pulsed with lyso-sulfatide and Galβ(1→3)αGalCer, respectively. IL-2 released after 17 h of coculture was determined by sandwich ELISA. Apart from splenocytes, thymocytes from the same mouse were also used as another source of APCs, and the response of each hybridoma toward respective Ag was similar (not depicted). Values are mean ± SD. These data are representative of one experiment conducted with APCs from two different sets of mice.

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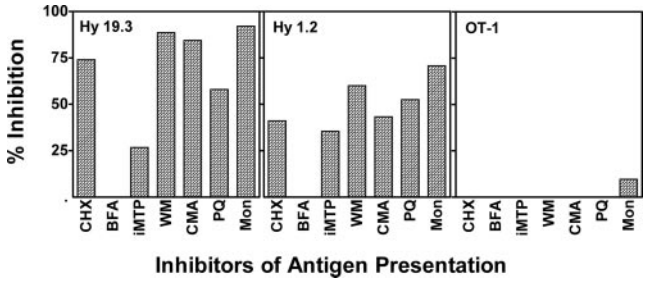

FIGURE 6. Exogenous presentation of lyso-sulfatide by mCD1d in the presence of inhibitors of Ag-presentation. The A20 cell line transfected with mCD1d was pretreated under different conditions (as described in *Materials and Methods*) and then pulsed with lyso-sulfatide (*left panel*) and αGalCer (*middle panel*) followed by fixation (except treatment of monensin) before the addition of Hy-19.3 and Hy-1.2, respectively. Released IL-2 was measured by sandwich ELISA. Down-regulation of Ag presentation in the presence of an optimum concentration of each inhibitor is shown as percentage of value corrected for the differential IL-2 production by the T cell hybridomas in response to each lipid in the presence of vehicle or inhibitor. Antigenic stimulation of class I MHC-restricted OT-1 T cells (*right panel*) by Ova peptide was used as a control for any nonspecific inhibitory effects. Data are the representative of at least two experiments.

Discussion

CD1 molecules are endowed with a unique hydrophobic groove that binds with a panel of Ags including self and foreign lipids, glycolipids, lipopeptides, phosphomycoketides, and small hydrophobic compounds in both secretory and endosomal compartments for presentation to T lymphocytes (3, 5, 37, 38). In the present study we have examined the involvement of Ag-processing/loading compartments in the exogenous presentation of a weak-binding self-lipid CD1d ligand, lyso-sulfatide to a highly specific type II NKT cell.

Here we have further defined the Ag fine-specificity of a sulfatide-reactive type II NK T cell hybridoma (Hy-19.3) (18), and found that it exclusively recognizes lyso-sulfatide. Our data clearly show that lyso-sulfatide is a weak CD1d-binding ligand as compared with other species of sulfatides. Although *cis*-tetracosenoyl-, tetracosenoyl-, palmitoyl-, and lyso-sulfatide belong to same sulfated-glycolipid group, lyso-sulfatide lacks an acyl chain. Thus single-chain lyso-sulfatide forms fewer hydrogen bonds and is likely to be accommodated in one of the hydrophobic pockets (A′ or F′) of mCD1d in a different orientation than other species of sulfatides with two chains occupying both pockets of mCD1d (19). This may lead to its specific recognition by a particular TCR whose structure is different from TCRs recognizing other sulfatide species.

It is clear that self-glycolipids that bind poorly to mCD1d, for example, IGB-3 and lyso-sulfatide individually are unable to form stable CD1d-tetramers and thereby do not stain type I or type II NKT cells, respectively (4, 19). Whereas CD1d-tetramers filled with the *cis*-tetracosenoyl sulfatide that bind very efficiently with mCD1d do form stable tetramers that are able to stain a distinct type II NK T cell population in liver mononuclear cells derived from both BL/6 and SJL/J mice (19). It is relevant to state that though lyso-sulfatide is a minor species, mCD1d-tetramers loaded with bovine brain-derived sulfatides stain the type II NK T HY19.3 but not type I NK T cells, HY-1.2 (18). It is likely that in this mixture of sulfatide species the higher CD1d binders provide stable binding energy for lyso-sulfatide and thus enabling bovine-brain derived sulfatides filled CD1d-tetramers to stain HY-19.3.

Our data suggest that lyso-sulfatide, being a weak CD1d-binding ligand, requires special conditions for efficient loading to CD1d, for example acidic pH (Fig. 3*A*) and presence of lipid transfer molecules (Fig. 3*B*), PI 3-kinase, or possibly other proteins. Consistent with the earlier reports (39, 40), it is likely that under these conditions the binding groove of mCD1d protein might be more exposed to solvent resulting in better binding of lyso-sulfatide. Though our in vitro binding studies to plate-bound CD1d (Fig. 3*B*) suggest that lipid-transfer proteins can enhance lyso-sulfatide binding, it is clear from the data using splenocytes from prosaposin−/− mice that saposins may not be required for the exogenous presentation of lyso-sulfatide in vivo. Our data in Fig. 5*A* show that lyso-sulfatide is presented efficiently whereas presentation of both αGalCer and Galβ(1 > 3)αGalCer (Fig. 5*A*) is inhibited in the absence of saposins. The inhibition of presentation of Galβ(1 > 3)αGalCer is due to perturbed trafficking of CD1d into the lysosomal compartment, which is prerequisite for the loading of these glycolipids onto the CD1d groove. Thus trafficking of mCD1d through lysosomal compartment is not essential for the exogenous presentation of lyso-sulfatide. However, it is important to mention here that saposins help in the degradation of sulfatides (41–43) by lysosomal hydrolase and arylsulfatase in the lysosomal compartment (44). In the absence of saposins, more sulfatides are available for recycling and presentation by CD1d. This is consistent with the enhanced lyso-sulfatide presentation in the absence of adaptor protein, AP-3 or in the presence of mCD1d lacking a tyrosine motif in the cytoplasmic tail (Fig. 4). In these two situations mCD1d is not targeted into the lysosomal compartment and recycles back to the plasma membrane from early endosomes (16, 24, 25) and results in increased cell surface expression of CD1d. Furthermore, inhibition of presentation of lyso-sulfatide following treatment with CMA of splenocytes from AP-3−/− mice, or the A20 cell line transfected with cytoplasmic tail mutant mCD1d (Keshab Roy, Vipin Kumar, unpublished observations) reinforces the idea that sulaftide/mCD1d traffics through endosomal compartment for presentation to type II NKT cells.

Although it has been shown earlier that Cathepsin L has a role in presentation of endogenous Ag (26), our data suggest that it has no role in exogenous presentation of lipid Ags such as, lyso-sulfatide and Galβ(1 > 3)αGalCer to type II and type I NK T cells, respectively.

Our data (see Fig. 6) indicate that both Concanamycin A as well as monensin, which block acidification of pH in the endosomal/secretory pathway are able to significantly inhibit the presentation of both lyso-sulfatide and αGalCer, although additional effects of these inhibitors resulting from perturbation of the endosomal pH (27–33) cannot be completely ruled out. Consistent with the role of secretory pathway in the presentation of glycolipids to type I and type II NKT cells, the protein synthesis blocking agent cyclohexamide (45) effectively blocks their presentation via CD1d. The microsomal triglyceride transfer protein or MTP facilitates loading of lipids onto mCD1d and helps in expression of mature CD1d on the cell surface (15). Furthermore, it has been postulated that the self-lipid glycophosphatidylinositol is loaded onto mouse CD1d in an analogous manner to the MHC class II-associated invariant chain in the ER and serves to stabilize the conformation of CD1d (46, 47). Our data suggest that exogenously added lyso-sulfatide and αGalCer could be loaded in the ER for the presentation to T cells (Fig. 6). Collectively this implies that exogenous presentation of recycling lipid Ags in CD1d pathway can occur following endocytosis through the ER where glycolipids are loaded with the help of lipid-transfer proteins such as MTP, similar to the pathway proposed for cholesterol recycling (48).

Down-regulation of surface expression of mCD1d as well as inhibition of presentation of both lyso-sulfatide and αGalCer following treatment with Primaquine suggest that a fraction of

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mCD1d molecules traffic through an early recycling endosomal compartment that is different from the unknown recycling compartment between the MIIC compartment (49) and plasma membrane. Based on sequence homology, mCD1d has been categorized in the group-II CD1 molecules, but its trafficking pattern correlates more with human CD1b, a group I CD1 protein (1, 3). Our data indicate that mCD1d may also traffic through early recycling endosomes, which is a characteristic feature of human CD1a. Thus mCD1d may be able to survey all self and foreign lipid Ags presented in different endo-lysosomal compartments, and being the only isotype of CD1 in the mouse can effectively replace the other isotypes present in humans.

Treatment with Wortmannin (50, 51) results in a significant down-regulation of the surface expression of CD1d. These data further suggests that phosphorylation of the tyrosine motif plays an important role in trafficking of CD1d. Indeed, the phospho-tyrosine motif, pYXXM present in insulin receptor substrate-1, platelet-derived growth factor receptor, and ErbB2, 3 receptors binds with the SH2 domain of the p85 subunit of PI 3-kinase (52–58). Additional studies in AP-3−/− mice are needed to further clarify specific interaction of PI 3-kinase on trafficking of CD1d protein. Blocking of Ag presentation in the presence of this inhibitor implies that PI 3-K-dependent trafficking of CD1d is important for the presentation of glycolipids to both type I and type II NKT cells.

Exogenous or endogenous glycolipids trafficking from lysosome to ER may be loaded in the secretory pathway (ER/Golgi) with the help of lipid-transfer proteins, such as MTP and presented on cell surface for recognition by NKT cells. The tyrosine motif (YXXZ) present in the cytoplasmic tail of CD1d enables the CD1d-β_2m-ligand complex to be internalized and enter into the early endosomal compartment. A fraction of CD1d returns back onto the plasma membrane through a primaquine-sensitive recycling endosomal compartment. Other molecules, such as AP-3 or PI3K interact to dock majority of CD1d proteins into the lysosomal compartment. Lysosomal resident enzymes (glycosidases, hexosaminidases etc.) give rise to immunogenic endogenous self-ligands. Lipid transfer proteins, and saposins help in editing or exchange of these lipid ligands in the hydrophobic groove of CD1d which eventually traverses to the plasma membrane for presentation to type I and type II NKT cells. The physiological relevance of presentation of self-glycolipids to either the type I or type II NK T cells is not well understood. However, our recent data demonstrated existence of a novel immune regulation pathway involving presentation of sulfatide to type II NK T cells and selective activation of plasmacytoid dendritic cells, ultimately resulting in anergy in type I NK T cell population (59).

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Disclosures

The authors have no financial conflict of interest.

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