

Activation state and intracellular trafficking contribute to the repertoire of endogenous glycosphingolipids presented by CD1d

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Myeloid antigen-presenting cells (APC) express CD1d molecules that present exogenous and endogenous lipid antigens that activate CD1d-restricted T cells, natural killer T (NKT) cells. NKT cell activation has been shown to mediate the potent downstream activation of other immune cells through cell–cell interactions and rapid, prolific cytokine production. Foreign antigens are not required for NKT cell activation. The endogenous lipids bound to CD1d are sufficient for activation of NKT cells in the setting of Toll-like receptor-induced cytokines. The most potent NKT cell antigens identified are glycosphingolipids (GSL). The GSL repertoire of endogenous ligands bound to CD1d molecules that are expressed in myeloid APC at steady state and in the setting of activation has not been delineated. This report identifies the range of GSL bound to soluble murine CD1d (mCD1d) molecules that sample the endoplasmic reticulum/secretory routes and cell surface-cleaved mCD1d that also samples the endocytic system. Specific GSL species are preferentially bound by mCD1d and do not solely reflect cellular GSL. GM1a and GD1a are prominent CD1d ligands for molecules following both the ER/secretory and lysosomal trafficking routes, whereas GM2 was eluted from soluble CD1d but not lysosomal trafficking CD1d. Further, after LPS activation, the quantities of soluble CD1d-bound GM3 and GM1a markedly increased. A unique α -galactose-terminating GSL was also found to be preferentially bound to mCD1d at steady state, and it increased with APC activation. Together, these studies identify the range of GSL presented by CD1d and how presentation varies based on CD1d intracellular trafficking and microbial activation.

antigen presentation | cluster of differentiation 1 | lipids

The cluster of differentiation (CD1) family of antigen-presenting molecules is a unique class of proteins that, in contrast to major histocompatibility (MHC) class I and II molecules, presents lipids, glycolipids, and lipopeptide antigens to T cells. CD1d-restricted T cells are often referred to as natural killer T (NKT) cells and have been implicated in host defense during infection (1–3), allergic responses (4), immune tolerance (5), and tumor immunosurveillance (6), and they are thought to play a role in autoimmune disease (7, 8). Their protean effects result in large part from their ability to rapidly recruit, stimulate, or influence the function of innate leukocytes, dendritic cells, and adaptive T and B cells (3).

One of the most potent NKT cell antigens, α -galactosylceramide (α -GalCer), has been an indispensable tool in the study of CD1d molecule and NKT cell biology, but it is not found in microbial pathogens. Related microbial and plant-derived α -glycosyl ceramides and glycerol-based lipids that are stimulatory are found in microbial organisms and pollen but are significantly less potent than α -GalCer (9–12). It has been well-documented that foreign antigens are not required for the activation of NKT cells. Self-reactivity or autoreactivity has been shown in murine and human NKT cells and was found to be necessary for NKT cell thymic selection (13, 14). Endogenous CD1d antigens have been shown to play an important role in peripheral NKT cell immunity. Several studies have shown

that, in the context of microbial infection, CD1d endogenous antigens are sufficient to mediate NKT activation. IL-12 produced by dendritic cells (DC) on stimulation was sufficient to activate NKT cells (1). This mechanism of NKT cell activation suggests that activation of DC by many Toll-like receptor (TLR) agonists can mediate NKT cell activation in a foreign-antigen independent manner (10, 15–18). In addition to the inflammatory cytokine production generated in response to TLR activation, APC undergo changes in their lipid biosynthetic pathways that alter their endogenous lipid repertoire and more potently activate NKT cells (17–19). These findings highlight the importance of endogenous antigen repertoire in NKT cell development and in the peripheral immune system under inflammatory conditions.

Similar to microbial lipid CD1d antigens, the majority of the endogenous antigens identified belong to two lipid classes, glycerol-based phospholipids and glycosphingolipids (GSL). Phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and lyso-PC have been shown to stimulate a small subset of NKT hybridomas in vitro (20–22). A number of mammalian GSL have been identified as NKT cell antigens. Several gangliosides, including GM1 and GD3, have been shown to be presented by CD1 (7, 23, 24). More recently, sulfatide has been shown to be presented by all CD1 isoforms (25, 26). Sulfatide has been shown to be produced in activated THP-1 cells, suggesting a possible increased role in the setting of inflammation (19). The GSL isoglobotrihexosylceramide (iGb3) can stimulate both murine and human NKT cells (27), but the physiologic role of iGb3 remains unclear (28, 29).

A variety of lipids have been eluted from human CD1d molecules including phospholipids, sphingomyelin, and GSL, suggesting that CD1 molecules can accommodate diverse lipids (22, 30, 31). The majority of studies to date that examined the repertoire of lipids bound by CD1d have used transfected B cell lines and identified PI, PC, lysophospholipids, GM3, and sphingomyelin (20, 22, 30, 31). These studies have been informative and have drawn attention to these lipid classes. However, the spectrum of CD1d ligands in myeloid lineage APC has not been examined. The range of GSL and phospholipids expressed by a specific cell type may have a significant impact on the lipid ligands bound by CD1 molecules. For example, the GSL repertoire in B cells has been shown to undergo serial changes with B cell maturation (32, 33). These changes in GSL

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Table 2. Relative abundance of selected GSL in RAW cells and GSL bound to mCD1d-Fc expressed at steady state and in the setting of activation (LPS)

GSL species	Untreated RAW cells		LPS-treated cells	
	Cells	mCD1d-Fc	Cells	mCD1d-Fc
GA2	174 (2.4%)	1,199 (18.1%)	257 (9.3%)	1,535 (12.9%)
GM3	100 (1.4%)	140 (2.1%)	94 (3.4%)	1,265 (10.7%)
GM2	359 (5.0%)	672 (10.2%)	190 (6.8%)	1,022 (8.6%)
GA1	69 (1.0%)	0 (0%)	39 (1.4%)	223 (1.9%)
GM1a	2,596 (36.3%)	781 (11.9%)	1,007 (36.3%)	2,404 (20.3%)
α Gal GSL	ND	86 (1.3%)	ND	556 (4.7%)
GM1a (NeuGc)	320 (4.5%)	45 (0.7%)	75 (2.7%)	596 (2.5%)
GD1a	3,158 (44.2%)	1,181 (17.9%)	646 (23.3%)	2,581 (21.7%)

GSL species abundance was quantified by calculating peak areas (mV \times seconds) in the NP-HPLC chromatograms of 2AA labeled glycans released from untreated or LPS-treated RAW cells or eluted from mCD1d-Fc expressed in these cells. Relative abundance for each GSL species is also shown as a percentage of total GSL (in brackets). ND, not detected.

(10.2%), in order of relative abundance. Additional GSL glycans eluted included GM3, GA1, GM1a (NeuGc), and LacCer. A significant amount of GA2 was eluted in all sample sets, although the relative abundance was variable (Fig. 2B). These peaks were not observed in the ceramide glycanase-digested eluates of the MHC I-Fc control (Fig. 2B).

Comparison of mCD1d-Fc Eluted Lipids with RAW GSL Profile. GD1a and GM1a, the most predominant RAW cell GSL, were eluted from soluble mCD1d-Fc but were not the most predominant GSL species eluted (Fig. 2). Soluble mCD1d preferentially bound GD1a, GM1a, GM2, and GA2 (Fig. 2B; Table 2). These findings suggest that the GSL bound to soluble mCD1d do not solely reflect the GSL content of the RAW cells. Instead, it reveals preferential binding of specific GSL to mCD1d. In addition, finer mapping of smaller peaks was also performed. GSL glycan analysis with this methodology is capable of identifying GSL glycans that are present in as low as 1% of the total sample (29). Given the importance of α -galactose (Gal)-expressing GSL as CD1d antigens, exoglycosidase digests were performed to identify the α -Gal-terminating GSL glycans bound to mCD1d (Fig. 3). These analyses also allow for differentiation between Gb3 and iGb3. We were able to confirm the presence of the Gb3 peak and the absence of iGb3 in the lipid eluates from soluble murine CD1d-Fc. The retention times for Gb3 and iGb3 were clearly distinct and confirmed with known standards (29). An additional α -galactose-bearing GSL glycan at 4.22 glucose units (GU) that will be referred to as α -Gal-GSL was also identified. This GSL glycan was not observed in the total RAW cell GSL glycan

analysis and suggests preferential enrichment of this GSL by mCD1d (Table 2; Fig. 3A). However, the GU and retention times of the α -Gal-GSL peak and exoglycosidase digest peaks did not correspond directly to any GSL in our glycan database, and thus, its identity could not be determined.

GSL Profile of Cell Surface-Cleavable mCD1d. The ability of GSL to be loaded and exchanged on mCD1d is likely influenced by many factors including cellular localization, GSL availability, and accessory proteins, such as microsomal triglyceride transfer protein (MTP), saposins, and GM2 activator. Because soluble mCD1d-Fc is directly secreted from the cell, it would only have exposure to the GSL available in the ER and along the secretory routes. To analyze intact mCD1d that traffics through the lysosome and recycling pathways, a cytoplasmic tail (CT)-containing, cell surface-cleavable construct was developed. This cell surface-cleavable construct had the added advantage of not necessitating detergent cell lysis to release mCD1d.

The cell surface-cleavable mCD1d Tobacco Etch Virus (mCD1d-TEV) CT construct was designed as a single chain construct with β 2-microglobulin. The TEV cleavage sequence was inserted into the connecting peptide region of mCD1d so that the wild-type mCD1d transmembrane and cytoplasmic regions remained intact. Importantly, this construct was shown to traffic properly by confocal microscopy as demonstrated by marked colocalization with lysosomal-associated membrane protein-1 (LAMP-1), which was also observed for wild-type, full-length CD1d (Fig. S2). The proper intracellular trafficking and folding of mCD1d-TEV-CT

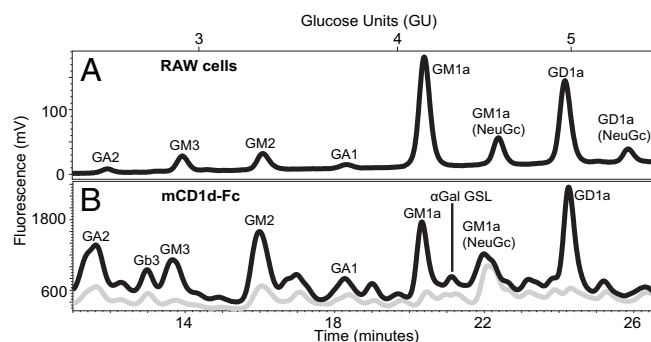


Fig. 2. Identification of GSL eluted from soluble mCD1d-Fc expressed in RAW cells and comparison with total cellular RAW cell GSL. Lipids were extracted from (A) RAW cells or eluted from (B) mCD1d-Fc (black) or MHC class I-Fc (gray) expressed in these cells. GSL glycan headgroups were characterized by labeling with 2AA and NP-HPLC analysis. Results are representative of six sample sets.

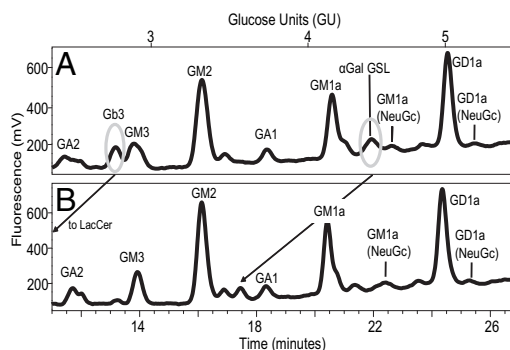


Fig. 3. Identification and confirmation of α -galactose-expressing GSL eluted from mCD1d-Fc. mCD1d-Fc eluted GSL glycans (A) before and (B) after digest with coffee bean α -galactosidase (cbag) were analyzed by NP-HPLC. Encircled peaks represent α -Gal-terminating glycan species that are lost after digest with cbag. Arrows indicate the shift in retention time of various species after exoglycosidase digest. Results are representative of three sample sets.

This work highlights that TLR activation of myeloid APC has a marked effect on the GSL repertoire of cells, and it revealed that the GSL eluted from all forms of CD1d did not mirror endogenous GSL or the marked changes to the cellular GSL pool after LPS activation. Instead, there were specific changes to the GSL bound to mCD1d on LPS activation. For example, although the most dramatic change to the RAW cell GSL pool was an almost 50% decrease in GD1a, the relative abundance of GD1a eluted from soluble mCD1d expressed by RAW transfectants stimulated with LPS was essentially unchanged (Table 2). In contrast, the relative expression of GM1a in the cellular GSL pool was unchanged with LPS stimulation, but it increased in the eluates of mCD1d-Fc expressed in LPS-stimulated RAW transfectants. These findings highlight the dichotomy between cellular GSL expression and the GSL bound to mCD1d. Changes to total cellular GSL content, subcellular GSL localization, lipid transfer, and GSL synthetic enzyme activity may all be contributors to alterations to the repertoire of endogenous GSL bound to CD1d in activated APC.

Although the focus of this report is mCD1d, modification of the endogenous GSL repertoire by activation and changes observed in GSL loading and editing for molecules that traffic through the lysosome are likely important for other CD1 molecules. These findings may be relevant in the case of human CD1b where the lysosomal microenvironment has been shown to be important in facilitating the loading of lipids (37) and possibly, with specific endogenous GSL. In addition, CD1e has been shown to play important roles in CD1b antigen loading and to rapidly redistribute from the Golgi network to the lysosome in myeloid DC matured by LPS (38); additionally, CD1e could play a role in selecting the endogenous GSL that are bound to CD1b in both the steady state and in the setting of myeloid DC activation.

This report does not address the phospholipids, sphingolipids, or lipopeptides that are bound to mCD1d. In addition, our methods do not identify sulfatide or GSL with glycan head groups smaller than lactose. The GSL that were identified in this study may only partially overlap with the GSL bound to mCD1d in other APC, which may have a different or more limited GSL repertoire as discussed above. It is interesting to speculate that the differences observed in the autoreactivity of NKT cells to specialized murine tissues, such as the spleen and thymus, may reflect differences in the endogenous pool of GSL or other lipid classes (39).

These analyses describe the repertoire of GSL bound to mCD1d and show that the endogenous GSL bound can be altered by intracellular trafficking, cellular GSL content, and changes to the activation state of APC. The GSL identified define the main GSL antigen complexes available for TCR recognition. This may be compared with the repertoire of peptides bound by MHC molecules, which include hundreds of distinct peptides containing common anchor residues specific for each MHC allele (40). In contrast, the repertoire of bound GSL reveals that a limited number of specific GSL dominate and that the pattern results from CD1d preferentially selecting only certain species. Further, because polymorphisms of CD1d molecules are very limited, this repertoire of antigens is likely to be very similar among individual animals or humans. Together, changes in GSL along with inflammatory cytokines work together to elicit NKT cell activation. These studies describe this CD1d GSL repertoire and provide a window into its changes under physiological conditions that alter T cell activation.

Materials and Methods

Soluble mCD1d-Fc and Cell Surface-Cleavable mCD1d Constructs. The generation of the soluble, single-chain β 2m-mCD1d-mouse IgG2a constant fragment mCD1d-Fc (21) has been previously described. In the case of the MHC class I Fc control, the extracellular domain of mCD1d was replaced by the extracellular domain of MHC class I. The extracellular domain of MHC class I was cloned using full-length HLA-B27 cDNA in pSR α -neo, generously provided by Masahiko Sugita (Kyoto University, Kyoto, Japan), as a template. A single-chain, β 2m-mCD1d cell surface-cleavable construct was generated by inserting the seven amino acid protease cleavage sequence for TEV (Glu-Asn-Leu-Tyr-Phe-Gln-Gly) into the connecting peptide sequence of mCD1d with minimal disruption of the wild-type

mCD1d sequence and without changing the number of amino acid residues in the connecting peptide segment (.ILYWDRQAPVG. was changed to .ILYWDENLVYFQG.). This generated a single-chain β 2m-mCD1d TEV (mCD1d-TEV-CT) construct with β 2m covalently linked to the extracellular domain of mCD1d followed by the connecting peptide region containing the TEV cleavage site and the complete wild-type mCD1d transmembrane region and CT. All PCR primer sequences used to generate the constructs are available on request.

Cell Lines and Transfectants. The murine myeloid lineage monocyte/macrophage RAW 264.7 and HeLa cell lines were obtained from the ATCC. The mCD1d-Fc, MHC class I Fc, wild-type mCD1d full-length and mCD1d-TEV constructs were transfected into RAW cells by electroporation, and stable clones were isolated by limiting dilution under selection with G418 sulfate (Invitrogen). The Fc constructs were screened by ELISA as described below. The mCD1d-TEV constructs were screened by cell-surface staining and flow cytometry. For the confocal microscopy experiments, full-length, wild-type mCD1d and mCD1d-TEV constructs were transiently transfected into HeLa cells with FuGENE 6 (Roche). When the soluble Fc transfectants were propagated for the production of soluble Fc, they were switched to complete media in which the standard FCS was replaced with 10% Ultra Low IgG FCS (Invitrogen). For the LPS-stimulation experiments, identical numbers of RAW cells and RAW transfectants were incubated in media or media containing LPS at 25 μ g/mL (*Escherichia coli*; Sigma) for 48 h.

Affinity Purification and Lipid Extraction. The soluble fusion proteins were purified from equal volumes of culture supernatants by affinity chromatography using recombinant rmp protein A Sepharose Fast Flow agarose (GE Healthcare). The cell surface-cleaved mCD1d-TEV protein was purified by affinity chromatography using anti-mCD1d monoclonal antibody mAb (19G11; provided by Albert Bendelac, Chicago, IL) or control anti-MHC class I (W6/32) mAb (ATCC) coupled to agarose by the Aminolink Plus immobilization kit (Thermo Scientific). The soluble Fc bound to protein A Sepharose and the cleaved mCD1d TEV proteins bound to anti-mCD1d or control MHC class I mAb-conjugated beads were washed with at least 20 volumes of PBS and drained before their direct extraction with chloroform/methanol (1:1 vol/vol) for 3 h at room temperature. The beads were pelleted by centrifugation, and the supernatants were aspirated and stored. The beads were then incubated in chloroform/methanol (1:2 vol/vol). The final extraction was performed with chloroform/methanol/water (4.8:3.5:1 vol/vol/vol). The extractions were pooled and stored at -80°C pending analysis. All of the solvents, including the water, were HPLC grade. Analysis of total cellular GSL was performed on the lipids extracted from RAW or LPS-treated RAW cell pellets using the same solvent protocol described above.

GSL Glycan Isolation, Fluorescent Labeling, and HPLC Analysis. The GSL glycan analyses were carried out as previously described (41). Briefly, lipids were purified from nonhydrophobic contaminants by passing the eluted mCD1d or MHC class I organic extracts over a SepPak C18 column solid-phase extraction column. The glycan headgroups of the GSL were cleaved from the ceramide backbone using ceramide glycanase (*Macrobdella decora* ceramide glycanase; Europa Bio-products) or purified from *Hirudo medicinalis* (D. Neville and T. Butters). The released glycans were labeled with the fluorescent marker anthranilic acid (2AA) and were analyzed by normal phase-HPLC (NP-HPLC) as described in ref. 41. Retention times of different species were converted to GU values, which were determined with reference to an external standard, a ladder of glucose oligomers obtained from a partial hydrolysate of dextran resolved with each sample set. The number of glucose residues in each dextran peak was plotted against the retention times of each of the peaks to obtain a standard curve using a fifth-order polynomial line fit. Preliminary identification of GSL glycans was done by comparison of GU values from published database of known standards (41). This method was found to be both sensitive and specific with standard deviations from the GU values listed in Table 1 in the range of 0.006–0.021 (41). Glycan identities were confirmed after digest with various exoglycosidases. A digest blank control was analyzed with each sample. Each lipid sample was split into two equal aliquots. One aliquot was treated with ceramide glycanase and fluorescently labeled before HPLC analysis. The other aliquot, designated digest blank, was not treated with ceramide glycanase before fluorescent labeling and HPLC analysis. This digest blank contained only non-GSL-derived sugars. The chromatogram peak areas of various species were normalized by subtracting the digest-blank peak area to distinguish GSL and non-GSL-derived sugars.

Exoglycosidase Digests. Additional characterization of the fluorescently labeled glycans was performed by exoglycosidase digestion. The fluorescently labeled glycans were dried down and resuspended in enzyme digest buffer (sodium acetate 100 mM at pH 6) and one or more of the following: *Throbacter ureafaciens* sialidase (ABS; Roche), bovine testes β -galactosidase (BTG; Prozyme), Jack bean β -N-acetyl-

hexosaminidase (JBH; Prozyme), or coffee-bean α -galactosidase (Boehringer). The samples were incubated overnight at 37 °C. Proteins were removed by centrifugation through a 10-KDa molecular weight cut-off filter before HPLC analysis.

Cleavage of Cell-Surface mCD1d-TEV. Full-length, wild-type mCD1d and cell surface-cleavable mCD1d-TEV-CT RAW transfectants were detached by brief incubation with 0.5% trypsin-EDTA, washed with PBS, and incubated in TEV buffer (50 mM Tris at pH 8.0, 0.5 mM EDTA, and 0.01% Triton X-100) for 2 h at 4 °C. AcTEV protease (Invitrogen), an enhanced form of TEV protease, was added as per manufacturer's instructions in several of the assays. After incubation, the

cells were pelleted, and the supernatants frozen at –20 °C. Equal amounts of the supernatants were passed over anti-mCD1d or MHC class I control mAb conjugated agarose beads as described above.

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