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Identification of Self-lipids Presented by CD1c and CD1d Proteins*[S]

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The CD1 family consists of five proteins that are related to the peptide-presenting MHC class I family. T cells can recognize the presentation of both foreign and self-derived lipids on four CD1 family members. The identities of the self-lipids capable of stimulating autoreactive T cell responses remain elusive or controversial. Here, we employed mass spectrometry to analyze the lipid content of highly purified CD1c and CD1d protein samples. We report the identification of 11 novel self-lipids presented by CD1c and nine by CD1d. Rigorous controls provide strong evidence that the identified lipids were specifically loaded into the lipid-binding site of the CD1 molecules. The diverse but distinct population of lipids identified from each CD1 family member implies each present a different subset of self-lipids, and the enrichment of particular motifs indicates that the lipids that are presented by CD1 family members could be predicted. Finally, our results imply the CD1 system surveys the endoplasmic reticulum, Golgi apparatus, and/or secretory compartments, in addition to its well characterized surveillance of the endocytic and lysosomal compartments.

The innate and adaptive immune systems protect against infectious challenges and cellular aberrations that can encumber the ability of their host to survive and reproduce. The antigen presenting cells of the immune system survey their surrounding environment and present two of the major constituents of life to the adaptive immune system: proteins and lipids. The presentation of such a diversity of potential immunogens involves three distinct systems: the MHC class I family presents proteins from the cytosol, the MHC class II family presents proteins from the endosomal and lysosomal compartments, and the CD1 family presents lipids. All three systems are capable of presenting immunogens from both endogenous and exogenous sources to the adaptive immune system (1–3).

The CD1 family is related to the MHC class I family in sequence and structure. The first two domains, which are known as α1 and α2, form the lipid-binding site. This site contains two to four hydrophobic pockets that are flanked on both sides by α-helices. T cells can recognize both the head group of the presented lipid and the α-helices of the CD1 protein. The α1 and α2 domains are situated above a third domain, α3, which non-covalently associates with a β_2 microglobulin (β_2M)[2] light chain. The α3 domain is then followed by a transmembrane domain and a cytoplasmic tail (4–7). Four members of the CD1 family are known to present lipids to the immune system: CD1a, CD1b, CD1c, and CD1d. Each has unique structural characteristics and a distinct trafficking pathway, which, when combined with the specificity of accessory proteins involved in lipid processing and presentation, defines the lipid-presenting capabilities of each CD1 family member (8–15).

The CD1 proteins fold in the endoplasmic reticulum (ER) where they are first loaded with lipids. Next, they traffic to the Golgi apparatus and then through the secretory pathway to the plasma membrane. Once at the cell surface, CD1 proteins are regularly internalized into the endosomal and/or lysosomal compartments where each family member follows a unique trafficking pathway. In humans, the majority of CD1a traffics to the recycling endosomes, whereas most CD1c and CD1d traffic to the early and late endosomes. CD1b primarily traffics to the lysosomal system (16–20). The CD1 proteins can be reloaded with lipids from these compartments. Some lipids require the action of accessory proteins for their efficient loading, whereas others do not (11, 14, 15, 21); accessory proteins can be involved in the selection, processing, loading, and/or presentation of a lipid (9, 10, 12, 13, 22). From here, the CD1 proteins traffic back to the cell membrane where they present their contents to the immune system. T cells can recognize the presentation of foreign and self-lipids (16–20), and immune cells bearing the ILT4 receptor can recognize the presentation of self-lipids by CD1d (23).

Self-lipids that are naturally presented by members of the CD1 family are capable of stimulating the activation of autoreactive T cells, with reports indicating the presentation of self-lipids by a single CD1 family member can be recognized by between 1/10–1/300 T cells in peripheral blood (24–27). Several publications have investigated the identity of these self-lipids through the detection of lipids that are present in CD1 protein samples. However, the findings have been contradictory and/or controversial. For instance, the initial findings suggested 90% or more of the lipids loaded into CD1d were glyco-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–3.

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[2] The abbreviations used are: β_2M, β_2 microglobulin; ER, endoplasmic reticulum; PI, phosphatidylinositol; BN-PAGE, blue native PAGE; HRV, human rhinovirus; GalCer, galactosylceramide; PC, phosphatidylcholine.

sylphosphatidylinositol (28). However, two later studies suggested that phosphatidylinositols (PIs) rather than glycosylphosphatidylinositol were the primary self-lipids loaded into CD1b and CD1d in the ER (29, 30). In contrast, isoelectric focusing of human CD1b and CD1d samples suggested these proteins were loaded with multiple lipid species that were carrying different charges (31, 32). Furthermore, three additional publications suggested plurality in the self-lipids that were loaded into CD1b and CD1d samples. These inconsistencies may have arisen from the protein purification strategies employed, the presence of impurities in the protein samples, and/or the absence of controls that strongly verify the lipids identified from the protein samples were specifically loaded in the lipid-binding domains of the CD1 proteins (28–31, 33, 34).

We report an approach that identified lipids that were loaded into the lipid-binding domain of CD1 family members. The approach was devoid of detergents and protein purification steps that can alter the repertoire of lipids loaded into the proteins. In addition, the protein samples were purified through three consecutive chromatography steps to reduce nonspecific protein and lipid contaminations, and then the purity of the samples was characterized through reducing and non-reducing SDS-PAGE, blue native (BN) PAGE, and MS. Using CD1c and CD1d protein samples with undetectable levels of impurities, we investigated the self-lipids that were loaded into these samples, which had trafficked through the ER, Golgi apparatus, and secretory compartments prior to their purification. We incorporated several novel controls that strongly implied the detected lipids were specifically associated with the lipid-binding site of the CD1 proteins. In total, 18 lipids were identified that represent six different classes of lipid. We detected a highly reproducible population of lipids from CD1c samples that was distinct from those detected from CD1d. These findings suggest that each member of the CD1 family has unique preferences in the self-lipids they present. Furthermore, it indicates the CD1 family members are surveying the ER, Golgi apparatus, and secretory compartments through which these protein samples had trafficked prior to their purification. The surveillance of these compartments, in addition to their widely appreciated surveillance of the endosomal and lysosomal systems, adds to the diversity of lipids the CD1 family can present to the immune system.

EXPERIMENTAL PROCEDURES

Protein Production, Purification, and Analyses—The protein constructs have been described previously (36). Briefly, CD1c and CD1d were engineered to be covalently linked to β_2M through a linker containing six repeats of glycine-serine $(GS)_6$.The transmembrane domain and cytoplasmic tail of CD1c-β_2M and CD1d-β_2M were replaced with a human rhinovirus (HRV) 3C protease site followed by an Fc tag. The Fc tag facilitated protein purification through Protein A affinity chromatography, whereas the HRV 3C protease site allowed its proteolytic elution from the column.

The constructs were stably transfected into HEK293T cells using the lentivirus system (38). The proteins were purified from the supernatant of the HEK293T cells. First, cellular debris and aggregates were removed from the supernatant by centrifugation at 3500 rpm for 20 min at 4 °C and then filtration through a 0.22-μm filter. The supernatant was passed through a protein A affinity chromatography column twice, and then the column was washed with 5 column volumes of PBS. Next, the proteins were proteolytically eluted from the column using HRV 3C protease. The protease contained a GST fusion tag, which facilitated its removal from the eluate using glutathione-Sepharose beads. After the eluate was incubated with the glutathione-Sepharose beads for 3 h at 4 °C on a roller shaker, the beads and their associated GST-tagged HRV 3C protease were removed from the eluate through 0.22-μm filtration.

After this first round of protein purification, the CD1c-β_2M and CD1d-β_2M protein samples, now with their Fc tags removed, were further purified through size-exclusion chromatography. The fractioned elutes that contained homogenous protein, as determined by non-reducing SDS-PAGE, were further purified through ion-exchange chromatography to reduce lipid contaminants. The proteins were eluted in fractions from the ion-exchange column using a continuous gradient. Fractions containing homogenous protein, as determined by non-reducing SDS-PAGE, were pooled into a single aliquot.

In addition to these Fc-tagged protein samples, His_6-tagged protein samples were also employed. The His_6 tag replaced the transmembrane domain and cytoplasmic tail (instead of the HRV 3C protease site and Fc tag on the aforementioned protein samples). Like the Fc-tagged proteins, these had β_2M covalently linked to CD1 through a $(GS)_6$ linker. These His_6-tagged protein constructs were also stably transduced into HEK293T cells. The supernatant was collected after 3–5 days, centrifuged, and filtered to remove cellular debris and aggregates. The supernatant was then loaded onto a nickel-nitrilotriacetic acid-agarose column. The proteins were eluted from the nickel-ion affinity chromatography columns in fractions using a linearly increasing imidazole gradient. All of the fractions containing homogenous protein, as determined by non-reducing SDS-PAGE, were pooled into a single aliquot and then further purified through size-exclusion chromatography and ion-exchange chromatography as described above. Using this approach, a CD1d-β_2M-His_6 sample was produced. Comparison of this sample with CD1d-β_2M would ensure the different purification techniques did not alter the population of lipids that were loaded into the protein. In addition, a hybrid CD1d sample was produced that had its lipid-binding site, specifically its α1 and α2 domains, replaced with the equivalent domains of HFE. Like the CD1 family, HFE is a member of the MHC class Ib family; however, it is known not to load or present lipids (2). This sample (HFE/CD1d-β_2M-His_6) was used as one of the negative controls.

The purification of all protein samples through affinity chromatography, size-exclusion chromatography, and ion-exchange chromatography produced highly purified soluble monomeric proteins and, in addition, likely reduced the amount of nonspecific lipid contamination. The final protein purity was assessed through reducing and non-reducing SDS-PAGE, BN-PAGE, and MS.

αGalCer-pulsed CD1d-β_2M Control—To demonstrate the lipids that were identified from CD1d-β_2M samples were neither artifacts nor contaminants, a CD1d-β_2M sample was

pulsed with αGalCer. Displacement of the previously identified lipids with αGalCer would indicate they had been loaded in the lipid-binding site of the CD1d-β_2M proteins.

This sample was generated using the method described by Li *et al.* (36). Briefly, a CD1d-β_2M sample was pulsed with 40 molar excess of αGalCer, whereas another CD1d-β_2M sample was pulsed with the vehicle control. The samples were incubated overnight at 37 °C in an orbital shaker and subsequently purified through size-exclusion chromatography and ion-exchange chromatography to remove excess lipids. The protein samples had been washed by ~35 column volumes prior to their elution from the ion-exchange column. These added purification steps purified soluble monomeric protein from aggregates and impurities. The final protein purity was again assessed on SDS-PAGE, BN-PAGE, and MS.

Extraction and Analyses of Lipids—Lipids were extracted from the CD1c-β_2M, CD1d-β_2M, αGalCer-pulsed CD1d-β_2M, vehicle-pulsed CD1d-β_2M, CD1d-β_2M-His_6, and HFE/CD1d-β_2M-His_6 samples using the method of Bligh and Dyer (39). Briefly, each of the protein samples was transferred into an amber glass tube with a Teflon-lined cap, and PBS was added to a final volume of 1.6 ml. Then, 2 ml of chloroform and 4 ml of methanol were added to make the single-phase Bligh-Dyer mixture, which consists of chloroform/methanol/PBS (1:2:0.8, by volume). This solution was vigorously mixed with a vortex and subjected to sonic irradiation in a bath apparatus for 5 min. This single-phase extraction mixture was then centrifuged at 500 × *g* for 10 min in a clinical centrifuge to pellet the protein precipitate. The supernatant was then transferred to a fresh tube where 2 ml of chloroform and 2 ml of PBS were added to generate the two-phase Bligh-Dyer mixture, which consists of chloroform/methanol/PBS (2:2:1.8, by volume). After vigorous mixing, each tube was centrifuged as stated previously to resolve the phases. The upper phase was removed and discarded. The lower phase was dried under a stream of nitrogen, and the dried lipids were stored at −20 °C until analysis.

LC/MS of lipids was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps, and a SCL-10A system controller) coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer. LC was operated at a flow rate of 200 μl/min with a linear gradient as follows: 100% of mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 4 min. Mobile phase A consisted of methanol/acetonitrile/aqueous 1 mM ammonium acetate (60/20/20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 μm, 2.1 × 50 mm) was obtained from Agilent (Palo Alto, CA). The post-column splitter diverted ~10% of the LC flow to the ESI source of the mass spectrometer.

RESULTS

Detection of Lipids Associated with Highly Purified CD1c and CD1d Protein Samples—CD1c-β_2M, CD1d-β_2M, αGalCer-pulsed CD1d-β_2M, vehicle control-pulsed CD1d-β_2M, CD1d-β_2M-His_6, and HFE/CD1d-β_2M-His_6 samples were purified from the supernatants of HEK293T cells. The Fc-tagged proteins were triple-purified through protein A affinity chromatography, size-exclusion chromatography, and ion-exchange chromatography, whereas the His_6-tagged proteins were triple-purified through nickel-ion affinity chromatography, size-exclusion chromatography, and ion-exchange chromatography. SDS-PAGE, BN-PAGE, and MS assessed the purity of the final protein samples, which suggested they were of high purity and contained undetectable levels of impurities. Fig. 1 shows the constructs used in this study and representative examples of the purity that was obtained during analyses.

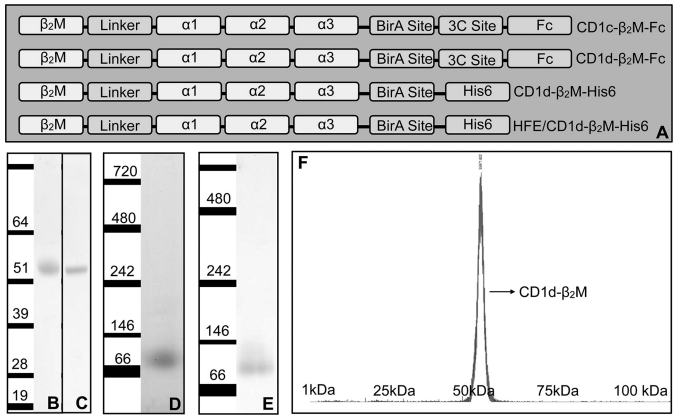


FIGURE 1. **The protein samples used for the analyses of self-lipids presented by CD1c and CD1d.** *A*, the constructs that were stably transduced into HEK293T cells. Fig. 2, *B–F*, shows representative analyses of the protein purity that was obtained. *B* and *D*, the analyses of CD1c-β_2M purity on a non-reducing SDS-PAGE and a BN-PAGE gel, respectively. *C* and *E*, the analyses of CD1d-β_2M purity on a non-reducing SDS-PAGE and a BN-PAGE gel, respectively. *F*, the analysis of CD1d-β_2M by MALDI-TOF MS.

The lipids that were detected from CD1c-β_2M samples were diverse and distinct from the lipids detected from CD1d-β_2M samples. These results were highly reproducible upon triplication (supplemental Fig. 1). The lipids detected from the CD1c-β_2M and CD1d-β_2M-His_6 samples were compared with those from HFE/CD1d-β_2M-His_6, which is a hybrid CD1d protein that had its lipid-binding domains (the α1 and α2 domains) exchanged for those of HFE (a related protein that is known not to present lipids). The lipids that were detected from the CD1c-β_2M and CD1d-β_2M-His_6 samples were not detectable from the HFE/CD1d-β_2M-His_6 sample (Fig. 2, *A–C*). Instead, only low levels of lipids were detectable from the HFE/CD1d-β_2M-His_6 sample, which was consistent with the background levels detected in the blank, suggesting very low levels of nonspecific lipid contamination. To provide further evidence, the lipids detected from the CD1c-β_2M and CD1d-β_2M samples had been specifically loaded into the lipid-binding site, a novel control was employed. A CD1d-β_2M sample was pulsed with αGalCer. This sample was compared with a CD1d-β_2M sample that was pulsed with the vehicle control. Both samples were purified through size-exclusion chromatography and ion-exchange chromatography before lipids were extracted from the samples and analyzed by MS; the results are displayed in Fig. 2, *C–E*. In the vehicle-pulsed CD1d-β_2M sample, a diverse population of lipids was again detectable. However, in the αGalCer-pulsed CD1d-β_2M sample, only αGalCer was detectable. Thus, αGalCer had displaced the previously detected lipids, which indicates they had been specifically loaded into the lipid-binding domain of the CD1d-β_2M sample.

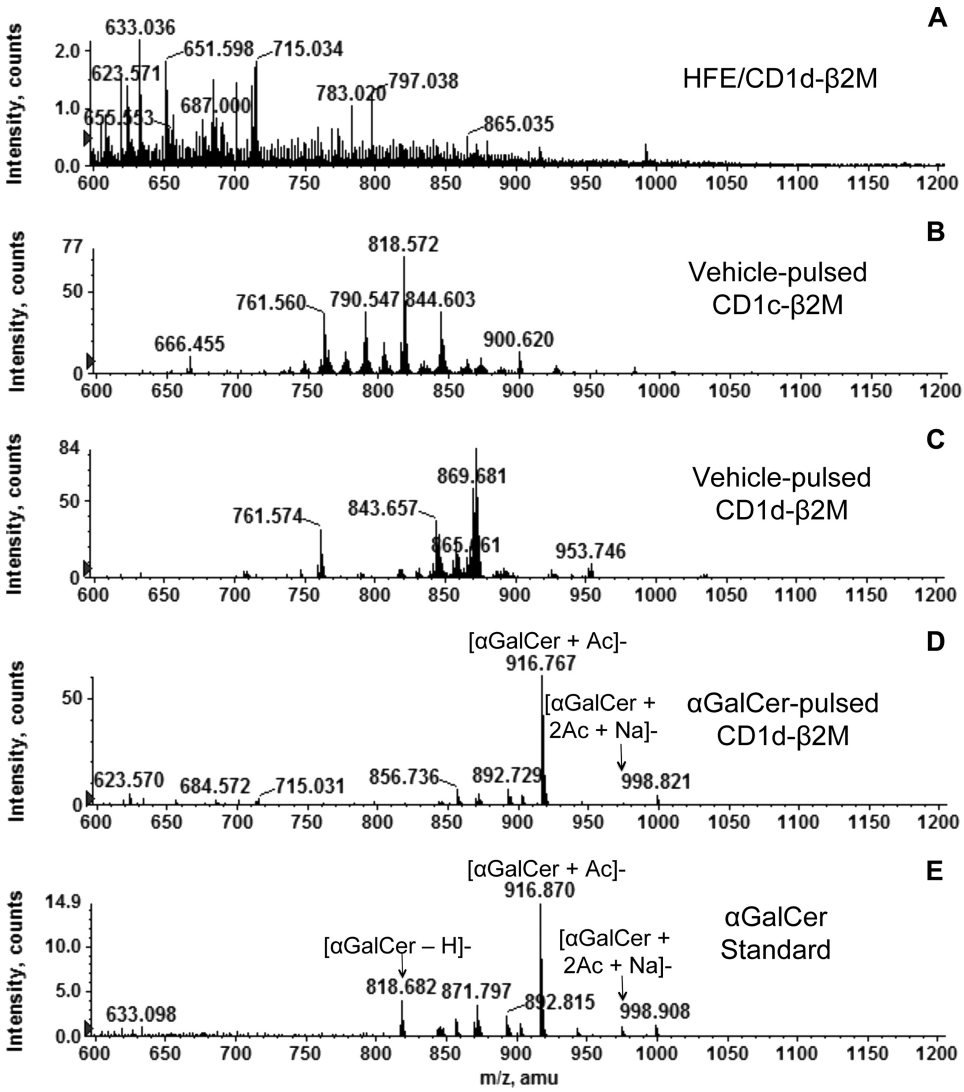


FIGURE 2. **Lipids detected from the CD1c-β_2M and CD1d-β_2M samples were specifically associated with the lipid-binding domain of the proteins.** The lipids were extracted from the samples using the method of Bligh & Dyer and subjected to reverse phase LC/MS analysis using a QSTAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) operating in the ESI negative ion mode. *A–C*, the lipids that were detected between 9.5 to 13.0 min from HFE/CD1d-β_2M, CD1cβ_2M, and CD1dβ_2M samples, respectively. *D* and *E*, the lipids that were detected from the αGalCer-pulsed CD1dβ_2M samples and the αGalCer standard, respectively.

Identification of Detected Lipids—The lipids that were detected from the CD1c-β_2M and CD1d-β_2M samples were identified through the exact mass of the parental ion and the daughter ions that resulted from a collision-induced dissociation. Fig. 3 shows two representative examples of identification. In total, 18 lipids were identified from CD1c-β_2M and CD1d-β_2M samples; these are described in Table 1, and their structures are shown in Fig. 4. Two of the lipids detected from CD1d-β_2M could not be identified because the exact mass of their parental and daughter ions could be identified as the closely related anomers and isomers (supplemental Fig. 3). These lipids are described as unidentified globo/isoglobo-trihexosylceramide (d18:1/24:1(15Z)) and globo/isoglobo-tetrahexosylceramide (d18:1/24:1(15Z)) for labeling purposes only; their identification has not been confirmed.

The lipids that were identified from CD1c had distinct properties compared with those identified from CD1d (see Table 2). For instance, phosphatidylcholines and other glycerophospholipids were the predominant lipid species detected from CD1c-β_2M samples, whereas sphingomyelins and glycosphingolipids were the primary lipid species detected from CD1d-β_2M samples. Although no polyunsaturated lipids were detected from CD1d-β_2M, four of the 11 lipids identified from CD1c-β_2M contained at least one acyl chain with a polyunsaturation. The lipids identified from CD1d-β_2M were, on average, four carbons longer than those identified from CD1c-β_2M. These findings imply that each member of the CD1 family has unique specificities in the self-lipids they present.

Several publications identified PIs from CD1d samples (28–30); however, we were unable to detect PIs from our CD1d-β_2M samples. PIs were readily detectable from lipids extracted from HEK293T cells and the supernatant from which the protein had been purified, indicating our approach was capable of detecting this lipid. Therefore, this discrepancy may have resulted from the use of different cell lines for the protein production or the different purification strategies employed.

Determining Location of Lipid Loading—To confirm the lipids were detectable from the cells that produced the protein, lipids were extracted from HEK293T cells and analyzed. All of the lipids identified from the CD1c-β_2M and CD1d-β_2M samples were identified from HEK293T cells. To investigate whether the identified lipids could have exchanged into the

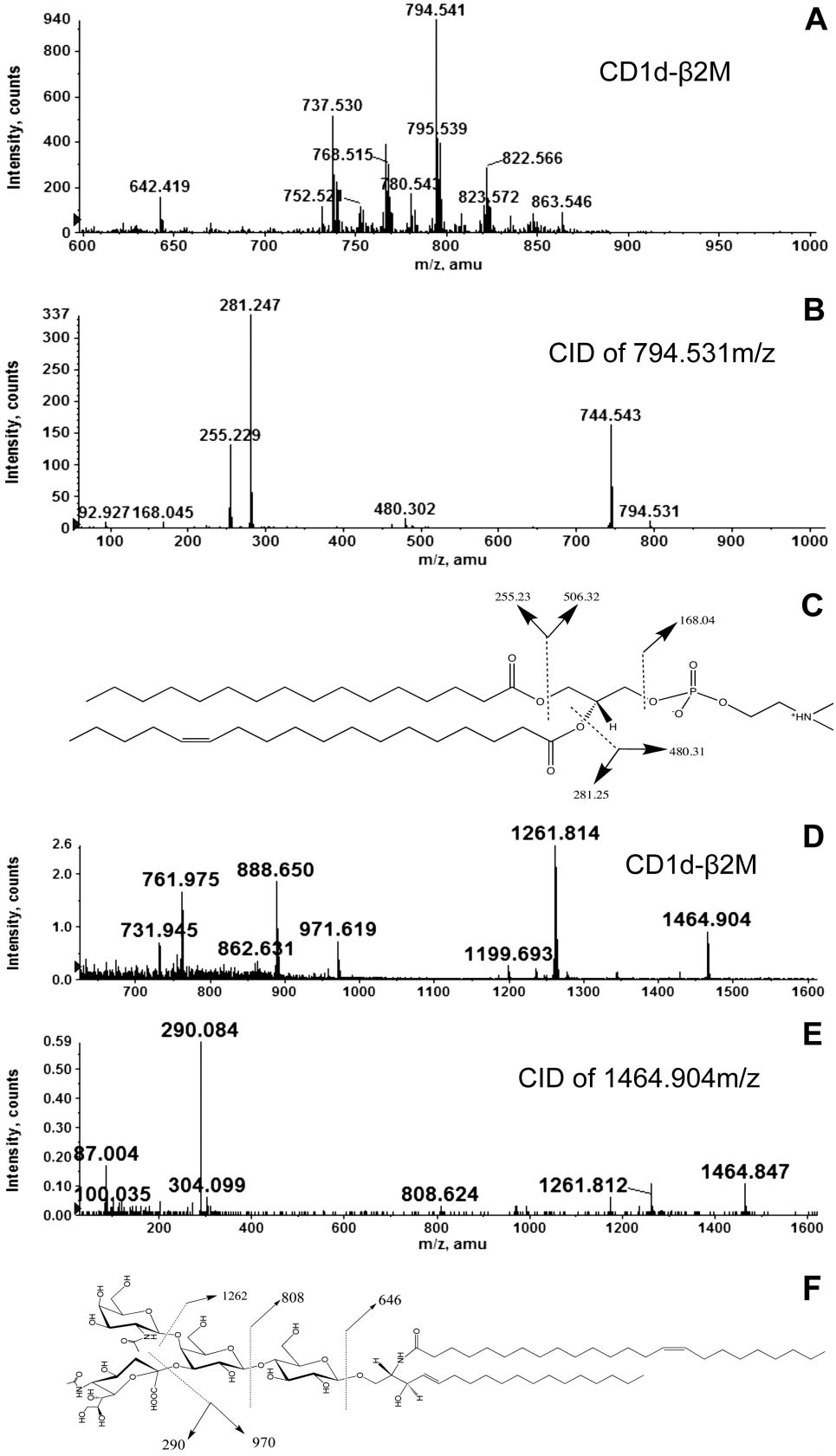


TABLE 1
The lipids identified from CD1cβ_2M and CD1dβ_2M samples

Category	Class	Common name	Systematic name	Exact mass	CD1c	CD1d
Sphingolipid	Sphingomyelin	SM (d18:1/16:0)	*N*-(hexadecanoyl)-sphing-4-enine-1-phosphocholine	703.58	Yes	Yes
Sphingolipid	Sphingomyelin	SM (d18:1/22:0)	*N*-(docosanoyl)-sphing-4-enine-1-phosphocholine	787.67	No	Yes
Sphingolipid	Sphingomyelin	SM (d18:1/24:1(15Z))	*N*-(15Z-tetracosenoyl)-sphing-4-enine-1-phosphocholine	813.68	No	Yes
Sphingolipid	Sulfatide	C24:1 sulfatide	(3′-sulfo)Galβ-Cer(d18:1/24:1(15Z))	889.63	No	Yes
Sphingolipid	Ganglioside	GM3 (d18:1/24:1(15Z))	NeuAcα1–3Galβ1–4Glcβ-Cer(d18:1/24:1(15Z))	1262.82	No	Yes
Sphingolipid	Ganglioside	GM2 (d18:1/24:1(15Z))	GalNAcβ1–4(NeuAcα1–3)Galβ1–4Glcβ-Cer(d18:1/24:1(15Z))	1465.90	No	Yes
Sphingolipid	Neutral glycosphingolipids	Unidentified globo/isogloboside-trihexosylceramide (d18:1/24:1(15Z))	N.D.[a]	1133.78	No	Yes
Sphingolipid	Neutral glycosphingolipids	Unidentified globo/isogloboside-tetrahexosylceramide (d18:1/24:1(15Z))	N.D.[a]	1336.85	No	Yes
Glycerophospholipid	Diacylglycerophosphocholines	PC (16:0/16:1)	N.D.[a]	731.55	Yes	No
Glycerophospholipid	Diacylglycerophosphocholines	PC (16:0/18:1)	N.D.[a]	759.58	Yes	Yes
Glycerophospholipid	Diacylglycerophosphocholines	PC (18:1/18:1)	N.D.[a]	785.59	Yes	No
Glycerophospholipid	Diacylglycerophosphocholines	PC (18:1/18:2)	N.D.[a]	783.58	Yes	No
Glycerophospholipid	Diacylglycerophosphocholines	PC (18:1/20:1)	N.D.[a]	813.62	Yes	No
Glycerophospholipid	Diacylglycerophosphocholines	PC (18:1/22:0)	N.D.[a]	843.67	Yes	No
Glycerophospholipid	Diacylglycerophosphoinositol	PI (18:0/18:1)	N.D.[a]	863.56	Yes	No
Glycerophospholipid	Diacylglycerophosphoinositol	PI (18:0/20:2)	N.D.[a]	889.57	Yes	No
Glycerophospholipid	Diacylglycerophosphoinositol	PI (18:0/20:3)	N.D.[a]	887.56	Yes	No
Glycerophospholipid	Diacylglycerophosphoinositol	PI (18:0/20:4)	N.D.[a]	885.54	Yes	No

[a] N.D., not determined.

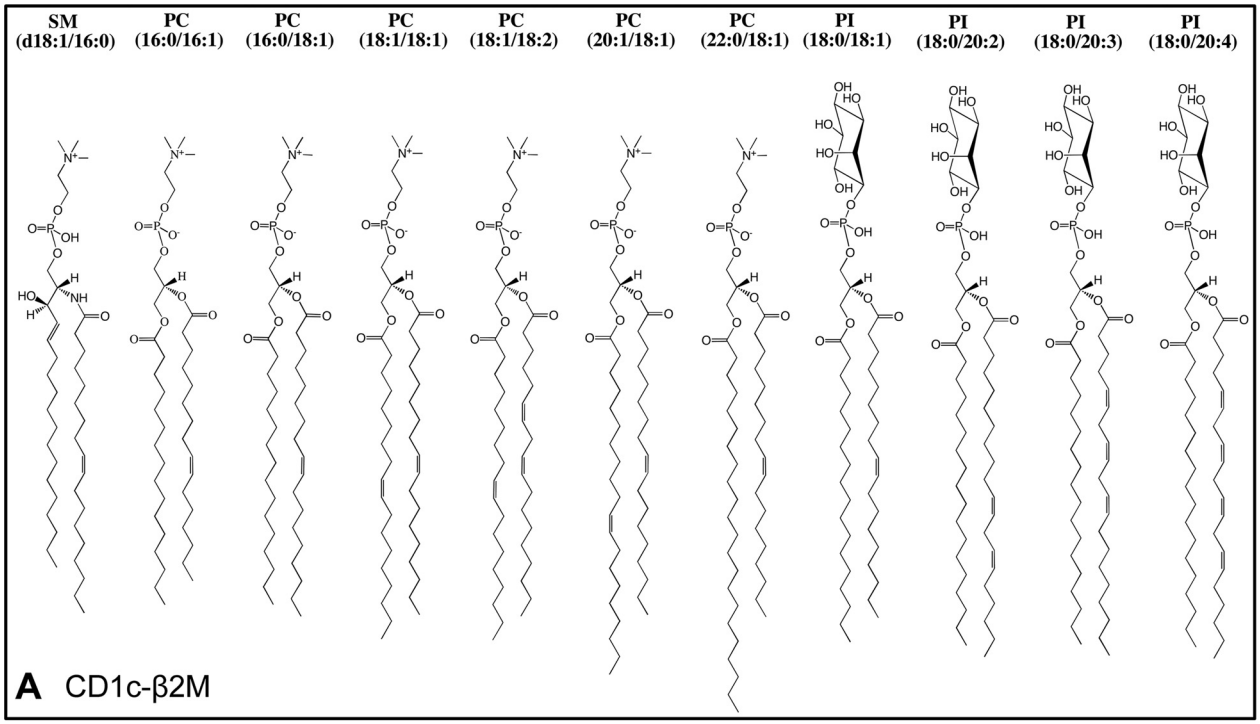
protein after they had been secreted into the supernatant, lipids were extracted from the HEK293T cell supernatant and analyzed. These findings are described in supplemental Table 1. SM d18:1/22:0), SM (d18:1/24:1(15Z), C24:1 ST, GM2 (d18:1/24:1(15Z), GM3 (d18:1/24:1(15Z), and the two unidentified globo/isogloboside lipid species were not detected in the supernatant; these lipids were most likely loaded in intracellular compartments. The remaining lipids were detected in both the HEK293T cells and supernatant, indicating they could have been loaded within the cell or once they had been secreted into the supernatant.

DISCUSSION

Autoreactive T cells are capable of recognizing the presentation of self-lipids by CD1 family members, but the identities of these lipid immunogens remain elusive (24–27). We investigated the identity of self-lipids that were loaded into CD1c and CD1d proteins produced by mammalian cells. Lipids were readily detectable from CD1c-β_2M and CD1d-β_2M samples, whereas only background levels were detectable from the hybrid HFE/CD1d-β_2M negative control sample. This indicates the lipids that were detected from the CD1c-β_2M and CD1d-β_2M samples had been specifically loaded into the lipid-binding site of the proteins. Furthermore, the distinct population of lipids that were identified from the CD1c-β_2M and CD1d-β_2M samples, which were highly reproducible, corroborates this assertion and indicates each CD1 family member has unique preferences in the self-lipids they present. Finally, αGalCer could displace the lipids that had been identified from CD1d-β_2M samples, implying those lipids had been loaded into the lipid-binding site of CD1d prior to their displacement by αGalCer. Together, these findings strongly insinuate the lipids that were identified from the CD1c-β_2M and CD1d-β_2M samples were specifically loaded into the lipid-binding site of the proteins.

Although others have reported the identification of foreign lipids that can be immunogenic when presented by CD1c (40–46), we are the first to report the direct identification of self-lipids that are naturally loaded into CD1c-β_2M molecules; 11 novel self-lipids were identified. Based on the recently solved crystal structure of CD1c loaded with mannosyl phosphomycoketide (47), which is a lipid derived from mycobacteria that is recognized by CD1c-restricted T cells (40, 43, 44), we can predict how CD1c accommodates the identified self-lipids. CD1c has a large hydrophobic immunogen-binding cavity (1780 Å), which is larger than both CD1a and CD1d (1280 and 1650 Å, respectively) but smaller than CD1b (2200 Å) (4, 5, 7, 47–52). The single hydrophobic moiety of mannosyl phosphomycoketide, a methylated hydrocarbon, was loaded into the A′ pocket of CD1c, which is a wide tunnel that circles clockwise around the A′ pole. Just after the portal to the A′ pocket is its

FIGURE 3. **Identification of lipids detected from CD1 samples.** Lipids were extracted from CD1d-β_2M using the method of Bligh and Dyer and subjected to reverse phase LC/MS analysis, using a QSTAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA), operating in the ESI negative ion mode. *A*, the lipids detected from CD1d-β_2M samples. *B*, the lipid responsible for the 794.534 peak was subjected to a collision-induced dissociation (*CID*). The exact mass (*m/z*, atomic mass unit (*amu*)) of the daughter ions and the mother ion facilitated its identification from the LipidMaps database as phosphatidylcholine (16:0/18:1); its structure is shown in *C*. The lipids that were detected from CD1dβ_2M samples between 7.0 and 9.0 min are shown in *D*. The collision-induced dissociation of the mother ion at 1464.904 exact mass resulted in the daughter ions (*E*) that facilitated the identification of the mother ion as GM2 (d18:1/24:1(15Z)); its structure is shown in *F*.


SM (d18:1/16:0)
PC (16:0/16:1)
PC (16:0/18:1)
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PC (22:0/18:1)
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A CD1c-β2M

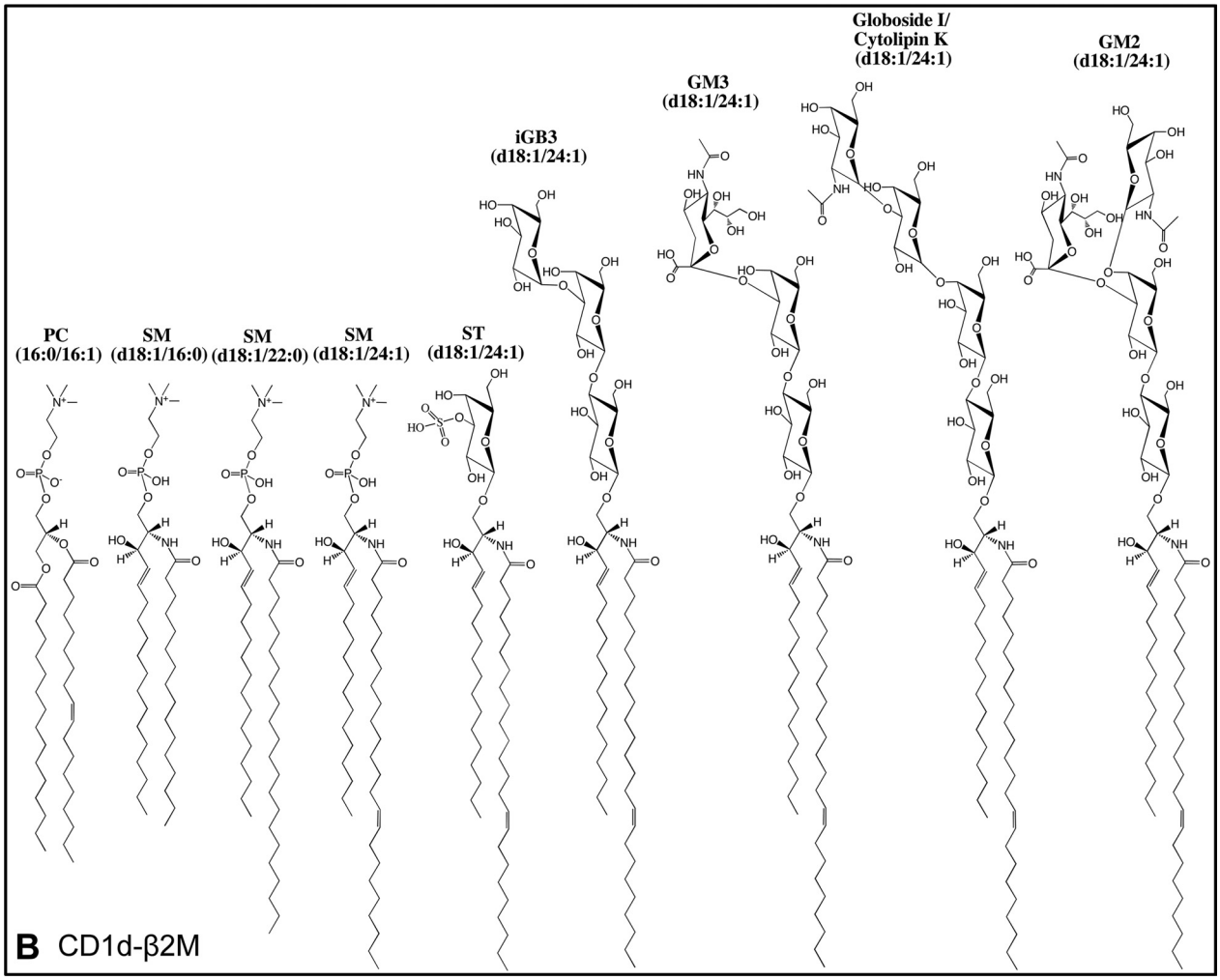
PC (16:0/16:1)
SM (d18:1/16:0)
SM (d18:1/22:0)
SM (d18:1/24:1)
ST (d18:1/24:1)
iGB3 (d18:1/24:1)
GM3 (d18:1/24:1)
Globoside I/ Cytolipin K (d18:1/24:1)
GM2 (d18:1/24:1)
B CD1d-β2M

TABLE 2
Comparison of lipid properties

	CD1c-β_2M	CD1d-β_2M
Average length of hydrophobic moieties (carbons)	36.40	40.40
Average difference in acyl chain length (carbons)	1.45	4.88
Polyunsaturated hydrophobic moiety	36.36%	0.00%
Hydrophobic moiety is a ceramide	9.09%	88.89%
Ceramide is d18:1/24:1(15Z)	0.00%	75.00%
Hydrophobic moiety is a diacylglycerol	90.90%	11.11%
Glycolipid	36.36%	55.55%
Glycosphingolipid	0%	55.55%
Sphingolipid	9.09%	33.33%
Phospholipid	100.00%	44.44%
Glycerophospholipid	90.90%	11.11%

narrowest point, which may restrict the presentation of bulkier lipids. The terminus of the A′ pocket connects with the unique E′ portal, which allows large lipid tails to exit into the solvent (47). We predict the clockwise orientation of the pocket favors the loading of the polyunsaturated (up to four) and longer acyl chains (up to 22 carbons) of the glycerophospholipids we identified from the CD1c-β_2M samples. The hydrophobic residues surrounding the portal to the A′ pocket interacted with the headgroup of mannosyl phosphomycoketide and aligned it in an orientation that exposes it to T cell receptor recognition (47). We predict similar interactions with the phosphocholine and phosphoinositol headgroups of lipids we identified from CD1c. The portal of the A′ pocket is adjoined with the F′ pocket; there was no wall separating these two cavities. Unlike other CD1 family members, the F′ pocket is not concealed but rather forms an open channel exposed to the solvent. Although this channel was only loaded with a hydrocarbon spacer in the published structure (47), it is likely the F′ channel accommodates the larger and bulkier amino acid residues of the lipopeptides presented by CD1c (46). We predict the open confirmation of the F′ pocket favors the loading of shorter alkyl chains of the self-lipids we identified from CD1c-β_2M, because this orientation is likely to have the least number of hydrophobic residues exposed to the solvent.

Nine diverse lipid species were identified from CD1d-β_2M samples, including gangliosides, globo/isoglobosides, sulfatide, sphingomyelins, and phosphatidylcholines. All of the self-lipids that we identified from the CD1d-β_2M samples were consistent with the constraints of CD1d that have been identified in the published structures (5, 50, 52, 64, 65). This contrasts with the findings of Cox *et al.* (53) who identified lipids that were too long and contained too many hydrophobic moieties to load into a single monomeric CD1d molecule. The diversity of lipids identified from CD1d-β_2M samples contrasts with the earlier findings of Joyce *et al.* (28), De Silva *et al.* (29), and Park *et al.* (30), which each identified a single or predominant lipid species. The identification of gangliosides GM2 and GM3 from CD1d-β_2M samples corroborates the later findings of Muindi *et al.* (34) in murine CD1d and Cox *et al.* (53) in human CD1d, but unlike these studies, we did not detect additional gangliosides. In support of the findings of Yuan *et al.* (33), we identified a large number of sphingomyelin and phosphatidylcholine species from CD1d-β_2M samples; however, we did not identify any that were polyunsaturated. Uniquely, we detected two members of the globo/isogloboside family, although their precise identification could not be determined using the MS techniques employed. Interestingly, one of the possible identifications of the smaller globo/isogloboside is isoglobotrihexasylceramide, which remains a highly controversial putative self-lipid capable of stimulating natural killer T cell (54–63). Further research will be required to elucidate the identity of the two detected globo-isoglobosides. A recently reported technique could distinguish between the possible anomers and isomers (54, 55), but a large CD1d-β_2M sample would be required to thoroughly scrutinize these two lipids, which constitutes only a portion of the lipids loaded into the CD1d-β_2M proteins.

The inconsistencies with previous publications may have resulted from differences in protein production, purification strategies, and/or the presence of impurities in the protein samples (28–31, 33, 34). Unlike previous studies, the protein production and purification strategy employed in this study was devoid of detergents, which could alter the population of lipids that are loaded into the protein samples. In addition, the robust purification strategy that we employed reduced the detection of nonspecific lipids to background/undetectable levels. Finally, the incorporation of strong positive and negative controls further strengthened confidence that the detected lipids were specifically associated with the lipid-binding domain of the CD1 proteins.

The repertoire of lipids identified from the CD1c-β_2M and CD1d-β_2M samples were distinct, with the exception of two lipids that were presented by both. This implies the following: (i) each CD1 family member has a unique preference in the self-lipids they present; (ii) CD1 proteins are not loaded with a single or predominant lipid species that perform only a chaperone function while the protein is in transit through the ER, Golgi apparatus, and secretory compartments, similar to the invariant chain for MHC class II molecules; (iii) the lipid-presenting members of the CD1 family are sampling the lipids in the ER, Golgi apparatus, and secretory compartments, and upon reaching the cell surface, presenting these lipids to the immune system. The surveillance of lipids from these compartments is in addition to their well accepted sampling of the endosomal and lysosomal compartments. This implies the CD1 family surveys the compartments where both the majority of lipids are synthesized (ER, Golgi apparatus, and secretory compartments) and catabolized (endosomal and lysosomal systems). This comprehensive coverage of lipid metabolism is equivalent

FIGURE 4. **The diverse but distinct repertoire of lipids identified from CD1c-β_2M and CD1d-β_2M samples.** *A*, the structures of lipids identified from CD1cβ_2M: one sphingomyelin (*SM*), six phosphatidylcholines (PCs), and four PIs were identified from CD1cβ_2M samples. The location of the unsaturations contained within the acyl chains of the PCs and PIs were not determined, so their locations within the figure are for illustrative purposes only. *B*, the structures of the lipids identified from CD1dβ_2M: one PC, three sphingomyelins, sulfatide (*ST*), and four glycolipids were identified from CD1dβ_2M. The location of the unsaturation on PC (16:0/18:1) was not determined, so its location in the structure is for illustration purposes only.

to that provided by both the MHC class I and MHC class II systems for the surveillance of proteins (33, 34).

The possibility that lipids may have exchanged into the CD1c-β_2M and CD1d-β_2M proteins after they had been secreted was explored. Several of the lipids that were identified from CD1c-β_2M and CD1d-β_2M samples were detected in the HEK293T cells and culture medium, and therefore, these lipids may have exchanged into the proteins after they were secreted. Nevertheless, their identification from CD1c-β_2M and CD1d-β_2M samples indicates these lipids are probably presented to the immune system, irrespective of the location they were loaded. The lipids that were only detected from the protein samples and the HEK293T cell samples had loaded into the CD1c-β_2M and CD1d-β_2M proteins while they were trafficking through intracellular compartments and are presumably presented to the immune system upon reaching the cell surface.

A few caveats accompany the results of this study. A proportion of the lipids loaded into CD1 family members in the ER, Golgi apparatus, and secretory compartments are likely to be replaced when they are reinternalized through the endosomal and/or lysosomal compartments. Therefore, the lipids identified in this study may not represent the full diversity of lipids that can be presented. Furthermore, the removal of the intracellular domain may have altered the population of lipids that were loaded in our CD1c-β_2M and CD1d-β_2M protein samples. Our controls could not eliminate the possibility that several of the lipids we identified may have originated from the supernatant and were loaded into the CD1 proteins once they reached the cell surface. However, this seems unlikely given the substantial excess that was necessary when we performed deliberate lipid exchange experiments. The repertoire of lipids presented by CD1 family members could vary depending on the cell type, their microenvironment, and/or their anatomical location within tissues and organs. The CD1c-β_2M and CD1d-β_2M samples used in this study were produced by HEK293T cells, which may not present the natural diversity of lipids. Finally, the diversity of lipids identified in this study raises the possibility that changes in lipid metabolism resulting from stress during infection, cancer, and/or autoimmunity could be reflected in the population of self-lipids presented to the immune system by members of the CD1 family.

The protein production and purification strategy employed in this study, combined with the strong controls, provide a robust approach for detecting and identifying lipids that are specifically loaded into the lipid-binding domain of CD1 family members. Because the efficiency of lipid ionization can vary, the addition of lipid standards in future experiments would allow the quantitation of individual lipid species and the determination of lipid protein-binding stoichiometry. The approach reported here could be adapted to identify both self- and foreign lipids that are presented by CD1 family members during disease.

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