Structural determination of lipid antigens captured at the CD1d–T-cell receptor interface

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Glycolipid antigens recognized by $\alpha\beta$ T-cell receptors (TCRs) drive the activation of invariant natural killer T (iNKT) cells, a specialized subset of innate T lymphocytes. Glycolipids with α -linked anomeric carbohydrates have been identified as potent microbial lipid antigens for iNKT cells, and their unusual α -anomeric linkage has been thought to define a “foreign” lipid antigen motif. However, mammals use endogenous lipids to select iNKT cells, and there is compelling evidence for iNKT cell responses in various types of sterile inflammation. The nature of endogenous or environmental lipid antigens encountered by iNKT cells is not well defined. Here, we sought to identify lipid antigens in cow's milk, a prominent part of the human diet. We developed a method to directly capture lipid antigens within CD1d–lipid–TCR complexes, while excluding CD1d bound to nonantigenic lipids, followed by direct biochemical analysis of the lipid antigens trapped at the TCR–CD1d interface. The specific antigens captured by this “TCR trap” method were identified as α -linked monohexosylceramides by mass spectrometry fragmentation patterns that distinguished α - from β -anomeric monohexosylceramides. These data provide direct biochemical evidence for α -linked lipid antigens from a common dietary source.

NKT | iNKT | lipid antigen | CD1d | glycolipid

Adaptive mammalian T lymphocytes are well known for their use of combinatorial diversity to generate a broad repertoire of antigen receptors, a process that evolved to recognize newly encountered peptide epitopes from pathogens. However, certain subsets of human T cells, such as invariant natural killer T (iNKT) cells, mucosal-associated invariant T cells, and germline-encoded mycolyl-reactive T cells reliably generate T-cell receptors (TCRs) that are conserved among genetically diverse humans, and in some cases, across species (1). Recent data indicate that T cells expressing such invariant TCRs comprise a significant fraction of all human T cells, estimated in the range of 10–20% (2). Furthermore, these conserved T-cell subsets are not restricted by MHC-encoded antigen-presenting molecules, but instead can recognize different chemical classes of antigens presented by nonpolymorphic antigen-presenting molecules, including CD1, MR1, or HLA-E (3). One of these conserved T-cell populations, iNKT cells, recognizes specific glycolipids presented by CD1d molecules. Following activation, iNKT cells respond rapidly with vigorous cytokine production that can profoundly shape immune responses (4).

Although a glycolipid antigen for iNKT cells was identified from a marine sponge more than 20 y ago as part of a chemical library screen (5), the origins and chemical identities of physiologically relevant antigens that drive selection and activation of iNKT cells remain controversial. Natural antigenic glycolipids have been detected from various foreign sources, including nonpathogenic α -proteobacteria (6–8), spirochetes (9), and other microbes, such as *Streptococcus pneumoniae* (10) and *Bacteroides fragilis* (11, 12). These known microbial lipid antigens for iNKT cells share a key

biochemical feature, namely an α -anomeric linkage of one hexose sugar to the lipid backbone. Mammals abundantly produce β -linked hexosylceramides, glucosylceramide and galactosylceramide, but were not previously thought to produce α -linked hexosylceramides. Therefore, a major principle of antigen recognition by iNKT cells has been that self-glycolipids are β -linked and not strongly recognized, whereas pathogens produce lipids that are recognized as foreign based on their α -anomeric linkage (5). However, pathogen-derived lipids presumably do not explain the TCR- and CD1d-dependent activation of iNKT cells during positive selection in the thymus, or in settings such as viral infection, cancer, or sterile inflammation (4). Endogenous lipids have long been proposed to activate iNKT cells in these settings.

It is possible that the longstanding view that mammalian monohexosylceramides are always β -linked is incorrect. Kain et al. showed that antibodies raised against α -linked glycosphingolipids could stain mammalian tissues (13). We recently fractionated mammalian milk-derived lipids, finding that antigenic substances were enriched in the hexosylceramide-containing fraction, and that antigenicity was not abrogated by β -specific glycosidases or chromatographic depletion of β -glucosylceramides (14, 15). Both studies point to the possibility that α -linked lipids are produced in mammalian tissues. However, if they exist, they are likely scarce compounds and they have not been

Significance

Invariant natural killer T (iNKT) cells are activated quickly and play a key role in the control of many microbial infections via their ability to rapidly secrete cytokines and chemokines that enhance many immune responses. Microbial glycolipid antigens that activate iNKT cells have been identified as α -anomerically linked glycolipids. However, the nature of the endogenous lipid antigens that are important contributors to the biology of iNKT cells has been unclear. In this study, antigenic lipids from cow's milk were isolated using a T-cell receptor trap method, and their stereochemical structures were determined to be hexosylceramides with α -linked hexose headgroups, thus identifying dietary lipid antigens for iNKT cells.

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Fig. 2. TCR trap assay. (A) Synthetic α -glucosylceramide was loaded in recombinant CD1d molecules, mixed with recombinant iNKT cell TCRs (2C12), and run over a size-exclusion chromatography column. (B) Lipids were eluted from the early fraction excluded from the column and analyzed by linear ion trap MS. A dominant ion corresponding to the α -glucosylceramide standard was recovered and few other ions were detected. (C) The activity-enriched milk monohexosylceramide fraction was loaded in CD1d and treated as in A. (D) The early fraction, CD1d alone, or TCR alone, was resolved on an SDS/PAGE gel and total proteins stained, yielding bands corresponding to TCR, CD1d, and β 2M. Data are representative of two independent experiments performed with separate starting material. mAU, milliabsorbance unit.

of the early eluting fraction made with milk lipids (Fig. 2C) was substantially lower than that of the early fraction made with CD1d carrying synthetic α -glucosylceramide (Fig. 2A), suggesting that TCRs bound to a small fraction of the total pool of CD1d–lipid complexes present. However, because this peak was observed at the same elution time as the synthetic α -glucosylceramide standard-loaded CD1d, it suggested that the milk lipid-derived ternary complex carried a high-affinity antigen.

Nano electrospray analysis of all eluted milk lipids in the late fraction containing CD1d and TCR monomers found numerous ions, and the mass intervals among the most abundant ions were diverse, suggesting the presence of structurally diverse lipids from many classes (Fig. 3A, Upper). In contrast, the early fraction, which contained CD1d–TCR complexes, showed fewer eluted ions (Fig. 3A, Lower). The lower peak height from the size-exclusion tracing and the less-complex MS profiles suggested that the iNKT cell TCR selected small subset of CD1d–milk lipid complexes from among all CD1d–lipid complexes present in the starting material. In fact, nearly all nano ESI-MS signals derived from the early-eluting CD1d–TCR complexes could be accounted for by a single alkane series comprised of lipids of m/z 710.7, apparent chain-length analogs (m/z 724.7, 738.7, 752.6, 794.7, 808.8, 822.8, 836.8), and the isotopes of these lipids.

TCR Ligands Identified Using Nano ESI-MS. Next we identified the lipid ligands trapped-in or excluded-from CD1d–TCR complexes using shotgun nano electrospray with multistage ion-trap MS. This method provides high sensitivity, broad detection, and structural information from collision-induced dissociation (CID), but offers relatively low mass accuracy. Considering the lipids eluted from the late fraction containing CD1d and TCR monomers, CID-MS of selected ions at m/z 738.4, 753.5, and 808.5 identified phosphatidylethanolamine, sphingomyelin, and phosphatidylcholine (Fig. 3B–D). In addition, fragments from the ion at m/z 722.6 corresponded to a hexosylceramide whose two fatty-acid chains had a combined lipid tail length of C34 with one unsaturation (Fig. 3E). This “type 1” monohexosylceramide collision pattern was characterized by a dehydration [loss of 18 u (atomic mass unit), m/z 704.5],

loss of a hexosyl unit (162 u, m/z 560.5), and loss of both fragments (loss of 180 u, m/z 542.4). In contrast, ions from early fraction-containing CD1d–TCR complexes did not show detectable signals for phosphatidylethanolamine, sphingomyelin, or phosphatidylcholine. Instead, complexes in the early fraction released matched expected masses of a “type 2” hexosylceramide, which unlike type 1 patterns, lacked a dehydration ion but did show a loss of 162 u. In fact, the abundant ions in the early fraction were all accounted for by single alkane series corresponding to an monohexosylceramide, with an overall lipid length ranging between C33 (m/z 710.7) and C42 (m/z 836.8). Separate analysis of six members of this alkane series demonstrated highly reproducible type 2 collision pattern (Fig. 3F).

The detailed profiles of these released lipids suggested several general conclusions. First, monomeric CD1d proteins were bound to phospholipids, sphingomyelin, type 1 hexosylceramide, as well as other lipids that were not specifically identified (Fig. 3A). Second, TCR–CD1d complex formation largely or completely excluded CD1d bound to phosphatidylcholine (m/z 808.6 with the m/z 749.3 product), sphingomyelin (m/z 753.6 with m/z 694.4 product), phosphatidylethanolamine (m/z 738.5 with m/z 695.3 product), and the type 1 hexosylceramide with a monounsaturated lipid tail (m/z 722.6 with m/z 542.4 fragment). Third, lipids eluted from the TCR–CD1d complexes contained type 2 monohexosylceramides ranging from C33–C42 in chain length (Fig. 3A). Furthermore, the characteristic patterns of loss in CID analyses by type 1 (18, 162, and 180 u) and type 2 (162 u) hexosylceramides implicated a differing conformation of these ceramide-based lipids. This interpretation is consistent with the TCR discriminating structural classes of lipids including glycosylceramides, and possibly α - vs. β -linked hexosylceramides.

The molar concentrations of hexosylceramides eluted from the early and late complexes were determined using HPLC-MS, in comparison with standards, and this material was tested for the ability to elicit cytokine production from an iNKT cell hybridoma, DN32. The late fraction did not have detectable activity, whereas the molar activity observed in the early fraction was similar to that of a synthetic antigen for iNKT cells known as α -galactosylceramide, KRN7000 (Fig. 3G). Thus, our approach was successful as a TCR-based affinity trap for selecting antigenic glycolipids that ligate TCR to CD1d out of a complex mixture of natural lipids. The equipotency of type 2 hexosylceramides and α -galactosylceramide was consistent with the type 2 hexosylceramides being an α -linked lipid. Although only trace amounts of the type 2 monohexosylceramide could be recovered from ternary complexes of CD1d–lipid–TCR, 1D-proton NMR spectroscopy carried out on a 900-MHz instrument identified a doublet with a chemical shift of 4.85 ppm, consistent with the anomeric proton resonance of α -monohexosylceramide (Fig. S1) (15).

Identification of Ligands at High Mass Resolution. To further biochemically characterize the lipids that promoted the formation of TCR–lipid–CD1d complexes, we separately analyzed eluents from milk lipid-treated protein monomers present in the late fraction, untreated CD1d, and untreated TCR (Fig. 4). Here, we took advantage of the higher mass accuracy of a quadrupole time-of-flight (Q-ToF) mass detector coupled with chromatographic separation in normal-phase HPLC-MS to confirm the identity of the key ions. Using this method, the major ions previously identified by nano ESI-MS were confirmed and assigned based on high mass accuracy and HPLC retention time, compared with standards as phosphatidylcholine (38.1 min, m/z 786.601), phosphatidylethanolamine (20.5 min, m/z 716.523), type 1 hexosylceramide (4.1 min, m/z 700.578), and type 2 hexosylceramide (4.1 min, m/z 702.590, 716.604, and 786.682) (Fig. 4). No substantial false-positive signals were derived from lipids released from the TCR, indicating that the lipids detected were carried by CD1d. As seen with the nanospray method (Fig. 3), CD1d alone and mixed CD1d and TCR monomers bound phosphatidylcholine, phosphatidylethanolamine, and type 1 monohexosylceramides (Fig. 4 and Fig. S2), but these were excluded from CD1d–TCR complexes. Conversely, type 2 hexosylceramides eluted with low or undetectable signals from milk lipid-treated monomers, but were highly enriched in

Fig. 3. Linear ion-trap MS analysis of CD1-lipid-TCR complexes. (A) Lipids extracted from the TCR trap assay fractions indicated in Fig. 2C were analyzed by linear ion-trap MS. Prominent ions from the late fraction were identified as (B) phosphatidylcholine, (C) sphingomyelin, (D) phosphatidylethanolamine, and (E) type 1 monohexosylceramide. (F) All major ions in the early fraction were an alkane series, and collision of six of the most abundant ions generated type 2 monohexosylceramide patterns. (G) Lipids were extracted from the early fraction, quantified, and cocultured with the DN32 iNKT cell hybridoma and CD1d-transfected RAW-264.7 cells. Experiments were performed on lipids extracted two independent TCR trap assays.

CD1d-TCR complexes. Thus, CD1d bound many lipids, but the iNKT TCRs selectively captured CD1d bound to type 2 hexosylceramides (Fig. S3).

Structural Identification of Type 1 and 2 Hexosylceramides. Despite their strikingly different patterns of binding within—or being excluded from—CD1d-TCR complexes, normal-phase HPLC-MS showed that type 1 and 2 hexosylceramides had equivalent retention time and the same underlying molecular composition, except for variations in lipid tail length and unsaturation. CID-MS experiments using the ion-trap architecture, however, showed different fragmentation patterns for type 1 and type 2 hexosylceramides (Fig. 3). We next asked whether anomeric variants could be distinguished by CID-MS. First, we produced synthetic standards differing only in the stereochemistry of their hexose attachment.

Both glucosyl and galactosyl variants were synthesized, with dihydrosphingosine (d18:0) as their lipid backbones based on the molecular composition of type 2 hexosylceramides identified in the milk-derived early fraction. These synthetic standards had a similar retention time by normal-phase HPLC to the early-eluting milk lipids (Fig. S4). The synthetic standards were tested for their ability to activate the DN32 iNKT cell hybridoma after loading in CD1d-expressing RAW264.7 cells. For both glucosyl and galactosyl synthetic standards, activity was observed for the α -linked variants, but not for the β -linked variants (Fig. 5A).

Positive-mode ion-trap CID-MS of sodiated adducts of these synthetic hexosylceramides was carried out under conditions that matched those used for milk-derived compounds (Fig. 3), and over a range of collision energy settings (Fig. 5B). Collision of the α -glucosylceramide synthetic standard demonstrated almost

Fig. 4. HPLC-MS analysis with Q-ToF MS detection. Lipids extracted from TCR trap assay fractions were analyzed by Q-ToF HPLC-MS experiments to provide high mass accuracy confirmation of the structures deduced in Fig. 3, to measure lipid binding to TCR and to measure retention time in comparison with standards. HPLC tracings are shown for selected targeted ions based on predicted masses for the structures identified in Fig. 3. Experiments were performed on lipids extracted two independent TCR trap assays.

exclusively a primary loss of 162 u, matching the pattern for type 2 milk-derived hexosylceramides. In contrast, β -glucosyl standards matched the fragmentation pattern seen for type 1 hexosylceramides from milk, showing a loss of 18 u at a similar frequency as loss of 162 u. Although both patterns involved the loss of 162 u as a hexosyl unit, analysis over a range of collision energy demonstrated that β -glucosylceramide also lost 18 u and 180 u, whereas α -glucosylceramide showed neither of these fragmentations at a substantial frequency. Parallel analyses of α - and β -galactosylceramide standards showed similar characteristic α - and β -collision patterns (Fig. S5). Matched synthetic α - and β -glucosylceramide and galactosylceramide standards based on the type 1 hexosylceramide lipid backbone structure showed similar characteristic α - and β -collision patterns, demonstrating that the anomeric linkage discrimination by this technique was not limited to dihydrosphingosine-based glycosphingolipids (Fig. S6). Ion-trap CID-MS of α -galactosylceramide with a phytosphingosine base also fragmented with the pattern matching type 2 hexosylceramides. We concluded from these experiments that linear ion-trap MS could sensitively distinguish α - from β -hexosylceramides under the conditions measured for a range of lipids with differing tail lengths and unsaturations.

We compared the collision patterns of the major hexosylceramide ions from cow's milk lipids. Unfractionated cow's milk glucosylceramides demonstrated a fragmentation pattern characteristic of β -hexosylceramide standards (Fig. 5C), consistent with the main lipid in the starting material being β -glucosylceramide. The activity-enriched cow's milk glucosylceramide showed a β -hexosylceramide collision pattern, but with an increased frequency of a type 2 pattern characterized by the loss of the sugar headgroup (162 u). The antigenic lipids captured in the TCR trap assay showed the fragmentation pattern typical of α -hexosylceramides. We concluded from these experiments that the TCR trap captured type 2 α -linked hexosylceramides from cow's milk. To address the possibility that these type 2 hexosylceramides were derived from microbes present in cow's milk, we purified the monohexosylceramide fraction from calf thymus, a mammalian lipid source previously shown activate iNKT cells (15). Several ions with the m/z seen in cow's milk were also present in calf thymus, including ions corresponding to type 2 hexosylceramides m/z 724.7 and 808.8 (Fig. S7). Linear ion-trap CID-MS of untreated calf thymus hexosylceramides showed a characteristic β -hexosylceramide pattern. After activity enrichment of thymus hexosylceramides by treatment with β -glucosidase and TLC fractionation, as in Fig. 1, we observed a collision pattern consistent with the presence of α -hexosylceramides, based on a substantial increase in the abundance of the ion representing a primary loss of the sugar headgroup.

Discussion

Uncovering the specific antigens recognized by iNKT cells is central to understanding their roles in health and disease. Although lipid antigens for iNKT cells have been identified in the microbial world (6–10), experimental evidence has demonstrated important roles for endogenous and environmental lipid antigens. Several candidate endogenous lipid antigens have been proposed (13, 14, 20–22), although technical limitations in the ability to detect these antigens has hampered our ability to determine their contextual roles. Here, we provide direct biochemical evidence for α -hexosylceramide derived from a mammalian source, cow's milk.

There is increasing evidence that iNKT cells encounter lipid antigens at mucosal surfaces. CD1-dependent immunomodulatory lipids have been identified in gut microbes (11, 12), pollen (23), house dust (24), and in our work, cow's milk. We have previously demonstrated that cow's milk contains substantially more lipid antigen activity than human or mouse milk. Although native triglycerides are removed from infant formula during processing, bovine polar lipids, including lipid antigens for iNKT cells, are retained in the final product (15). Studies in germ-free mice suggest that the iNKT cell compartment can be shaped by CD1d-lipid signals in the enteric tract (11, 25), and thus early-life exposure to cow's milk-based infant formula might similarly shape human mucosal iNKT cells in early life. In adult mice, orally delivered antigen activates iNKT cells in the gut (26), suggesting that exposure to dietary lipid antigens in adult life may also have consequences. Interestingly, it is unclear whether cows have iNKT cells (27), as their CD1d does not present the long-chain α -galactosylceramide commonly used as a tool in mice and humans (28). Bovine CD1d can present short-chain α -galactosylceramides (28, 29), and it is possible that an increased presence of α -hexosylceramides in cow's milk and tissues could lead to thymic deletion of iNKT cells, as has been observed in mice in the presence of exogenous α -galactosylceramide (30).

Although the bulk solids in milk derive overwhelmingly from mammary glands, we cannot formally rule out that colonizing microbes synthesized, or played a role in the synthesis of the α -hexosylceramides present in cow's milk. Several features of our data argue against possible contamination of milk by microbial products. We have previously observed iNKT cell-stimulating activity in the monohexosylceramide fractions of the milks, sera, and internal organs of multiple animal species (14, 15). The N -acyl chain use of the α -hexosylceramides that we identified in this report mirrors that of the bulk β -glucosylceramides in cow's milk, suggesting that these lipids are of mammalian origin. We also identified type 2 hexosylceramides from calf thymus with the same m/z as those in cow's milk. These thymic hexosylceramides, like those found in cow's milk, shifted from a β -hexosylceramide pattern toward a characteristic α -hexosylceramide collision pattern.

Fig. 5. Linear ion-trap MS distinguishes α - from β -hexosylceramides. (A) Lipid standards were synthesized based on the structures of the type 2 monohexosylceramides and tested in coculture with the DN32 iNKT cell hybridoma and CD1d-transfected RAW-264.7 cells. (B) MS-MS of sodiated adducts demonstrated characteristic fragmentation patterns for α - and β -glucosylceramide synthetic standards when studied over a range of collision energy settings. (C) Linear ion-trap MS-MS was performed on the cow's milk unenriched, activity-enriched, and TCR trap early fraction lipids. Three experiments were performed for A and B. Experiments in C were performed on lipids extracted two independent TCR trap assays.

after activity enrichment. Currently, the level of α -hexosylceramide identified from cow's milk, a relatively abundant and potent natural source of lipid antigen, is near the limit of detection for the TCR trap assay system. However, we are continuing to improve the system so that lipids can be captured and identified from smaller amounts of source material.

The stereochemistry of organic molecules can have significant biological consequences. Determination of stereochemistry has largely been accomplished by NMR. However, for rare compounds, such as endogenous iNKT cell lipid antigens, purification of sufficient material for complete, multidimensional NMR analysis is currently not possible. As shown in this report and others (31, 32), CID-MS can also be used to determine stereochemical features. We tested α - and β -linked synthetic standards with both sphingosine and dihydrosphingosine bases, and found consistent characteristic α - and β -fragmentation patterns, showing that this detection method was not limited to certain lipid backbones. Interestingly, these collision patterns were only observed with selected positive-mode adducts, including sodium and rubidium. We hypothesize that for the β -hexosylceramide structure, positive adducts with a sufficiently large valence, such as sodium and rubidium, can coordinate elements on both sides of the glycoside bond, stabilizing the glycoside bond during collision, thus reducing the propensity for cleavage of the sugar headgroup. For α -hexosylceramide, this headgroup stabilization may be sterically hindered, thus allowing for fragmentation across the glycoside bond at a lower energy.

Identification of CD1d-presented lipid antigens has largely relied on indirect evidence, such as extrapolation from cells deficient in lipid synthesis genes (22, 33), or fractionation of

crude lipid sources with activity tracking within these fractions (14, 20). These approaches have been useful, but do not directly demonstrate recognition by the TCR, and rely on correlations between two or more assays. Here, we used a direct method for antigen discovery, the TCR trap assay. This approach uses ternary complex formation both as the preparative method to recover lipids of interest and as the assay demonstrating the lipid's role in ligating CD1d and TCR. The TCR trap assay has also been used successfully to identify CD1a ligands (17), and could be used to identify ligands for other CD1 isoforms, or even MR1. This approach will allow tracking of rare lipids from virtually any source, including from complex mixtures, or limited starting materials, which represent common challenges when starting with mammalian tissues and cells.

Materials and Methods

Initial lipid activity enrichment was performed as previously described (15). MS analyses were performed on a Thermo-Fisher LXQ ion-trap mass spectrometer or an Agilent 6520 Accurate-Mass Q-ToF with HPLC. For details of experimental conditions and analysis, see *SI Materials and Methods*.

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