Cardiolipin Binds to CD1d and Stimulates CD1d-Restricted γδ T Cells in the Normal Murine Repertoire

Mélanie Dieudé, Harald Striegl, Aaron J. Tyznik, Jing Wang, Samuel M. Behar, Ciriaco A. Piccirillo, Jerrold S. Levine, Dirk M. Zajonc and Joyce Rauch

*J Immunol* 2011; 186:4771-4781; Prepublished online 9 March 2011;
doi: 10.4049/jimmunol.1000921
http://www.jimmunol.org/content/186/8/4771

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/03/07/jimmunol.1000921.DC1

**References**
This article cites 57 articles, 26 of which you can access for free at:
http://www.jimmunol.org/content/186/8/4771.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cardiolipin Binds to CD1d and Stimulates CD1d-Restricted γδ T Cells in the Normal Murine Repertoire

Mélanie Dieudé, Harald Striegl, Aaron J. Tyznik, Jing Wang, Samuel M. Behar, Ciriacio A. Piccirillo, Jerrold S. Levine, Dirk M. Zajonc, and Joyce Rauch

Cardiolipin (CL), a major phospholipid in bacterial cell walls, is sequestered from the immune system in mammalian mitochondria and is, therefore, a potential danger signal. Based on growing evidence that phospholipids constitute natural ligands for CD1 and that CD1d-restricted T cells recognize phospholipids, we hypothesized that CD1d binds and presents CL and that T cells in the normal immune repertoire respond to CL in a CD1d-restricted manner. We determined the murine CD1d-CL crystal structure at 2.3 Å resolution and established through additional lipid loading experiments that CL, a tetra-acylated phospholipid, binds to murine CD1d with two alkyl chains buried inside the CD1d binding groove and the remaining two exposed into the solvent. We furthermore demonstrate the functional stimulatory activity of CL, showing that splenic and hepatic γδ T cells from healthy mice proliferate in vitro in response to mammalian or bacterial CL in a dose-dependent and CD1d-restricted manner, rapidly secreting the cytokines IFN-γ and RANTES. Finally, we show that hepatic γδ T cells are activated in vivo by CD1d-bearing dendritic cells that have been pulsed with CL, but not phosphatidylcholine. Together, these findings demonstrate that CD1d is able to bind and present CL to a subset of CL-responsive γδ T cells that exist in the spleen and liver of healthy mice and suggest that these cells could play a role in host responses to bacterial lipids and, potentially, self-CL. We propose that CL-responsive γδ T cells play a role in immune surveillance during infection and tissue injury.

The Journal of Immunology, 2011, 186: 4771–4781.
stimulate a murine γδ T cell hybridoma (16, 17), but neither CD1 restriction nor generalizability to ex vivo T cells was assessed. Synthetic CL was not stimulatory to human NKT cell clones, but it efficiently blocked CD1d presentation of an antigenic glycolipid (15). Taken together, these findings suggest that phospholipids can serve as natural ligands for CD1d and induce strong CD1d-restricted T cell responses.

CL is a unique phospholipid that can be of both mammalian and bacterial origin. In prokaryotic cells, particularly Gram-negative bacteria, CL is a major phospholipid component of the cell membrane. In contrast, in eukaryotic cells, CL is absent from the cell membrane, but accounts for as much as 20% of mitochondrial lipid (because of its bacterial symbiotic origin). Thus, in healthy mammalian cells, CL is normally sequestered from the immune system.

The presence of CL in prokaryotic cell membranes, along with its absence in healthy eukaryotic cell membranes, suggests that prokaryotic CL may serve as a danger signal to the mammalian immune system. It is not clear, however, whether eukaryotic mitochondrial CL is normally presented to the immune system. It is not clear, however, whether eukaryotic mitochondrial CL is normally presented to the immune system in the context of CD1d, similar to MHC class I (MHC I) presentation of peptides from mitochondrial self-proteins (18). We hypothesize that by binding to CD1d, CL constitutes an important signal that induces a CD1d-restricted innate-like immune response to pathogens. In this study, we demonstrate that CL is a natural ligand for CD1d and that the normal immune repertoire contains CD1d-restricted T cells that are responsive to this phospholipid Ag.

Materials and Methods
Phospholipid preparation
Phospholipids and disulfatide (3,6-di-O-sulfo-β-D-C12-galactosylceramide) were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. For lipid binding and crystallography studies, mCD1d was obtained from the Hetero-compound Information Centre Uppsala (compound CDL) and truncated at all three N-linked glycosylation sites of CD1d. Refinement process was judged by monitoring the free R factor (Rfree) for cross-validation (23). The model was rebuilt using the program Coot (24). Water molecules were assigned in COOT (25) for >3σ peaks in an Fo − Fc map and retained if they satisfied hydrogen-bonding criteria and returned Fc − Fcr density > 3σ after refinement in REFMAC. Starting coordinates for the TM-CL ligand were obtained from the Protein Data Bank (PDB) coordinates for the mCD1d–TM-CL structure are available at the PDB under accession number 3MA7. The software programs Pymol (DeLano Scientific, Palo Alto, CA) was used to prepare Fig. 2. The PDB2PQR server (30) and the program APBS (31) were used to calculate the electrostatic surface potentials in Fig. 2.

In vitro loading of lipid Ags
Aliquots of 10 μl purified mCD1d at a concentration of 20 μM were loaded overnight at room temperature in the presence of four to six times molar excess of each ligand, PC and disulfatide were dissolved in DMSO (1 to 2 mg/ml), whereas all CL species were dissolved in ethanol (4 mg/ml). Final concentration of DMSO and ethanol in the loading experiment did not exceed 3–5%. Loading was performed in the presence of 100 mM Tris-HCl (pH 7). After lipid loading, samples were centrifuged (14,000 × g, 10 min) and 4 μl supernatant was used for isoelectric focusing (IEF) analysis. CD1d–lipid complexes were separated using precast gels (PhastGel 5-8 IEF) and the PhastSystem (GE Healthcare Biosciences, Piscataway, NJ). Staining of mCD1d was performed with Coomassie blue, and successful lipid loading is identified by a gel shift of the mCD1d band in relation to the control lanes.

Marine T cell preparation
All animal experiments were conducted according to the relevant Canadian and international guidelines and were approved by the McGill University Animal Care Committee. Specific pathogen-free C57BL/6 mice were obtained from Harlan Sprague Dawley, and TCRβ-deficient mice (The Jackson Laboratory, Bar Harbor, ME) were bred in-house. CD1d−/− mice backcrossed for 12 generations (N12) in the C57BL/6d background, which were kindly provided by Drs. Peter van den Elzen and Rusiang Tan (University of British Columbia, Vancouver, Canada), were originally derived by Drs. Steven Balk and Mark Exley (Harvard Medical School) (32). Anesthetized mice were perfused through the heart with PBS (pH 7.3), and the perfused spleen and liver were harvested postmortem. T cells from spleen or liver were purified by negative selection using a murine T cell enrichment kit (EasySep; StemCell Technologies, Vancouver, BC, Canada). Total splenic or hepatic T cells (98% CD3+ cells) were isolated by negative selection using a murine T cell enrichment kit (EasySep; StemCell Technologies, Vancouver, BC, Canada). The isolated T cell population contained both γδ and γδ T cell subsets. In certain experiments (where specified), isolated T cell subsets were labeled with CFSE using the CellTrace CFSE Cell Proliferation Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. In experiments using purified γδ T cells, the cells were isolated using a two-step TCRβ–γδ T cell isolation kit (Miltenyi Biotec, Auburn, CA). Briefly, a first-step enrichment of T cells by negative selection (removal of CD11b- and CD45R-positive cells) was followed by positive selection of TCR γδ-positive cells.
**T cell stimulation with phospholipids**

Purified total T cells (50 μl; 2.5 × 10^6/ml) or γδ T cells (50 μl; 1 × 10^7/ml) were stimulated with different final concentrations of phospholipids (30–60 μl of a 1 mg/ml suspension), including native bovine heart CL (also referred to as bovine CL, native CL, or CL), hydrogenated CL (hCL), or bovine heart PC, diluted in complete RPMI 1640 medium, in presence of APCs (splenocytes treated with mitomycin C; 150 μl; 8 × 10^6/ml). The cells were cultured in a 96-well plate for 24 or 48 h at 37°C (5% CO2). Blocking experiments were performed using anti–I–A (Y3P; generously provided by Dr. Philippa Marrack, Howard Hughes Medical Institute, National Jewish Medical and Research Center, Denver, CO), anti-CD1d (19G11) (33), or anti-H2Dd (34-2-12; BD Biosciences, Mississauga, ON, Canada). Cell proliferation was measured using a BrdU proliferation kit (Roche Diagnostics, Indianapolis, IN) or CFSE-labeled cells. T cells were characterized by flow cytometry using mAbs to murine CD3, IFN-γ, or β2 TCR, and CD3 TCR (BD Biosciences). In certain studies (where specified), cells were stained with mAbs to murine CD25, CD69, Vγ2, Vγ3, Vγ4, or Vδ6.3/2; Annexin V; and/or 7-aminoactinomycin D (BD Biosciences). To ensure intertube comparability, relative cell numbers were determined by adding 3000 True-Count beads (BD Biosciences) to each tube prior to flow cytometry analysis and counting 500 True-Count bead events per sample during flow cytometry acquisition. These beads serve as an independent and absolute counting control to ensure that equivalent volumes are counted for each tube (independent of cell composition or number).

**In vivo γδ T cell response and flow cytometry**

Mouse bone marrow–derived dendritic cells (BMDCs) were prepared by culturing bone marrow progenitors with 20 ng/ml GM-CSF (PeproTech) for 7 d following standard protocols. On day 7, BMDCs were washed, counted, and cultured with phospholipids or vehicle control (100 μg/ml) for 24 h. After being washed with PBS, phospholipid-pulsed BMDCs (5 × 10^5) were injected i.v. into mice. Concurrently, mice were injected i.p. with 1 mg BrdU (BD Pharmingen). Liver mononuclear cells positive for CD3 and γδ TCR (BD Pharmingen) were analyzed directly ex vivo for BrdU incorporation and activation markers CD25 and CD69 (BD Pharmingen) by flow cytometry. Cells were analyzed using an LSR II (BD Biosciences) and FlowJo software (Tree Star).

**Cytokine detection**

Supernatants of hepatic T cells stimulated with phospholipid (0.2 μM) or vehicle were harvested 48 h poststimulation, and a panel of 22 cytokines and chemokines was assayed using RayBio Mouse Cytokine Ab Array 1 (RayBiotech, Norcross, GA), according to the manufacturer’s instructions. Furthermore, supernatants were assayed for IFN-γ (BD Biosciences) and RANTES (R&D Systems, Minneapolis, MN) by ELISA, according to the manufacturer’s instructions.

**Results**

**Crystal structure of cardiolipin bound to mouse CD1d**

mCD1d is known to bind diacylated glycolipids and phospholipids (34), but its ability to bind tetra-acylated phospholipids has not been studied. Recently, CL, among other lipids, has been identified by mass spectrometry as a natural human CD1d ligand (6). However, CL does not appear to be an Ag for human invariant NKT cells, as it failed to activate a panel of human iNKT cell clones (15). To determine whether mCD1d is able to bind to CL, a tetra-acylated phospholipid with two negatively charged phosphate groups, we performed ligand binding studies with recombinant mCD1d and native (bovine heart) and synthetic (TM [14:0]) forms of CL. We also evaluated hCL to determine whether fatty acid chain saturation has an influence on CD1d binding. Bovine heart PC, which was derived from the same source as native CL and had similar fatty acid saturation, served as a control for mCD1d in the presence of a neutral phospholipid. Binding of CL to mCD1d was assessed by native IEF gel electrophoresis (Fig. 1A). Upon incubation of mCD1d with native bovine CL or synthetic TM-CL, but not fully saturated bovine hCL, two major bands were apparent (Fig. 1A, left panel). The lower band corresponds to mCD1d containing an uncharged endogenous spacer lipid derived from its expression in insect cells (net charge 0), whereas the upper fainter band corresponds to mCD1d–CL complexes (net charge −2). In addition, purified mCD1d also contains a faint double band above the major species of mCD1d. This double band is likely a result of endogenous ligands with different charge values bound to mCD1d, as it disappears upon loading with disulfatide (Fig. 1A, left panel, positive control). The mCD1d

![Image](http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/)

**FIGURE 1.** CL binds to mCD1d. A, Synthetic TM-CL (14:0) and native bovine CL can bind to mCD1d, as detected by native IEF gel electrophoresis. Analytical gel (left panel): mCD1d (negative control) was incubated with bovine heart PC (net charge 0), bovine heart CL (net charge −2), hCL (net charge −2), or TM-CL (net charge −2) for 16 h. Removal of the third (monolysoc-CL) and fourth (dilysoc-CL) alkyl chain from CL increases loading efficiency (middle panel). Preparative gel (right panel): mCD1d-TM–CL complexes can be fully separated from mCD1d molecules by anion exchange chromatography (purified CD1d/CL). A positive control is shown, where mCD1d was loaded with disulfatide (a nonphospholipid ligand with a net negative charge of −2). The preparative gel depicts the large-scale TM-CL loading and purification of mCD1d–TM–CL used for crystallographic studies. The data in A are representative of two to four independent experiments. B, Chemical structure of the variants of CL used in the binding studies. Interestingly, although bovine heart CL, TM-CL, monolysoc-CL, and dilysoc-CL bind to mCD1d, hCL fails to bind to mCD1d under these conditions. This is likely due to reduced solubility of hCL during loading as a result of the long and fully saturated C18 acyl chains. Note the faint upper double bands in the negative control lane (left panel), which are likely the result of endogenously bound charged lipids.
CARDIOLIPIN STIMULATES CD1d-RESTRICTED γδ T CELLS

preparation used for the lipid loading experiment is shown in the middle and right panels (Fig. 1A) and is more homogeneous and does not contain endogenously bound charged lipids. These findings demonstrate that recombinant mCD1d protein binds native bovine CL and TM-CL (band at ~2 net charge), but does not bind saturated bovine hCL. mCD1d incubated with buffer or neutrally charged bovine heart PC showed a major band for the CD1d endogenous spacer lipid (net charge 0), but no band with a net charge of ~2. Notably, binding of TM-CL and bovine CL occurred in the absence of lipid transfer proteins (none were present in the system), although binding was less efficient than that of the positive control (disulfatide with only two alkyl chains).

We evaluated whether mCD1d could also bind bovine CL that had one (monolysoc-CL) or two (dilysoc-CL) fatty acyl chains removed (Fig. 1A, middle panel). mCD1d bound both lyso forms of CL, and binding was maximal with dilysoc-CL and appeared to decrease with the addition of fatty acyl chains. These data indicate that maximal binding of CL to mCD1d occurs with only two alkyl chains and that additional alkyl chains are not required for binding to mCD1d, although they could be required for recognition.

mCD1d–TM–CL complexes, purified using anion exchange (MonoQ) chromatography, showed a single band on IEF gel electrophoresis (Fig. 1A, right panel), with the same migration as purified complexes of mCD1d and disulfatide (the control for mCD1d loaded with a nonphospholipid Ag having a net ~2 charge) and were used for subsequent crystallization. These findings suggest that CL (bovine CL and TM-CL), despite the presence of four alkyl chains (Fig. 1B), can still bind to mCD1d and form a stable mCD1d–CL complex. The chemical structure of TM-CL raises the question whether TM-CL can bind simultaneously to two mCD1d molecules by inserting two alkyl chains in each of the CD1d binding grooves. However, size-exclusion chromatography revealed that one TM-CL molecule is bound by only one mCD1d protein molecule (data not shown).

To identify how CL binds to mCD1d, we crystallized mCD1d–CL complexes and determined the crystal structure to 2.3 Å resolution (Fig. 2, Table I). No gross structural differences in mCD1d are observed when comparing mCD1d–TM–CL (PDB ID 3MA7) to other mCD1d–glycolipid complexes (8, 19, 35–37). Two of the four alkyl chains are inserted into the A’ and F’ pockets of the CD1d binding groove (Fig. 2A), whereas the other two alkyl chains are exposed to the solvent. The central phosphate–glycerol–phosphate moiety of TM-CL is located above the binding groove in a position similar to the head groups of other glycolipid ligands (8, 19, 35–37). No clear electron density is observed for the two solvent-exposed alkyl chains of TM-CL, indicating that these chains make no major contact with CD1d residues and are disordered. Therefore, these two solvent exposed alkyl chains were not built into the final structure and are not depicted in Fig. 2. It is important to note that we observed a cavity within the crystal lattice. This cavity is big enough to accommodate the third and fourth alkyl chains. Moreover, it is clear from our ligand binding and size-exclusion chromatography experiments (Fig. 1A and data not shown) that one intact tetra-acylated TM-CL molecule binds to one CD1d molecule and that fully loaded mCD1d–TM–CL complexes have been purified and crystallized. Interestingly, there are no CD1d residues capable of neutralizing the negative charge of both phosphate groups of TM-CL. Despite this, mCD1d residues Asp80, Asp153, and Thr156 interact with the polar center of TM-CL through hydrogen bonding (Fig. 2C), thereby stabilizing the polar core of TM-CL and giving rise to very well-ordered electron density (Fig. 2B). In addition, two water molecules pro-

FIGURE 2. Schematic representation of the mCD1d–CL complex. A, TM-CL (yellow) is bound in the hydrophobic binding groove between the α1 and α2 helices of the mCD1d H chain (gray), which noncovalently associates with β2-microglobulin (β2M, gray). Two N-linked glycosylation sites (N42 and N165) carry well-ordered carbohydrates (green sticks). A spacer lipid (C16, cyan) is present in the binding groove to complement the shorter C14-alkyl chains, as well as the connecting glycerol, are not ordered in the crystal structure, and only 11 carbons of the myristoyl chain that binds in the A’ pocket are ordered. C, Hydrogen-bond interactions between CD1d residues (gray) and the polar moieties of CL (yellow) are represented. In addition, two water molecules also hydrogen bond with the two phosphate groups of CL. D, Comparison between the presentation of TM-CL (yellow) and α-GalCer (cyan) by mCD1d. E, Comparison between the presentation of CL (yellow) and sulfatide (green) by mCD1d. The central phosphate–glycerol–phosphate moiety of CL is located farther above the binding groove as compared with the more intimate binding of α-GalCer (the predominant invariant NKT cell Ag). However, the polar groups of both CL and sulfatide occupy similar positions. Notably, the terminal phosphate of CL is in a similar position as the sulfate of sulfatide. Binding comparison between TM-CL (yellow sticks) and PIM2 (thin green sticks) (F) and between TM-CL and PC (thin cyan sticks) (G). Protein backbone is colored accordingly, and side chains are shown for Arg70 (R79) and Asp80 (D80) for orientation. Surface representation, with electrostatic potential (red, electronegative, and blue, electropositive, contoured from ~30 to +30 kT/e), is shown in D and E. Several amino acid residues, which are involved either in the shaping of the mCD1d-binding grooves (B, D, E) or in polar interactions with CL (C), are depicted using the single letter amino acid code with the relevant residue number. Individual atoms are colored as follows: carbon, yellow, green, or cyan; oxygen, red; nitrogen, blue; phosphorus, purple; and sulfur, orange.
provide additional hydrogen-bond interactions with both phosphate groups of TM-CL.

We next compared the mCD1d binding of TM-CL to that of other mCD1d ligands, including α-galactosyl ceramide (α-GalCer), which stimulates Vα14-invariant NKT cells (35, 38); sulfatide, which stimulates a minor subset of non-Vα14 NKT cells (19, 39, 40); and two phosphoglycerolipids, PIM2 and PC (7, 8). The comparative structures (Fig. 2D–G) reveal that the lipid backbone of the various Ags binds slightly differently inside mCD1d and therefore leads to a slightly different presentation of the polar epitopes. Although binding of α-GalCer to mCD1d shows greater differences because of its α-linked galactose, binding of TM-CL and sulfatide to mCD1d follows a similar pattern in which the terminal phosphate of TM-CL, or sulfate of sulfatide, is located just above a slightly electropositive patch formed by the Glu134 and Gly155 backbones of the α2-helix. When TM-CL binding to mCD1d is compared with either PIM2 or PC (Fig. 2F, 2G), the binding of all three phosphoglycerolipids appears quite similar. However, the binding of the diacylglycerolipid backbones, in general, appears less conserved than the binding of the glycosphingolipid backbones, which all superimpose very closely when bound by mCD1d (34, 41). The position of the proximal phosphate is slightly different in all three structures, and this orientation affects the presentation of the subsequent headgroup (Fig. 2F, 2G). In addition, the phosphoglycerolipids appear to bind in opposite orientations in the CD1d binding groove, leading to subtle differences in the binding orientation of the diacylglycerolipid backbone, similar to the binding of two α-galactosyl diacylglycerolipids from *Borrelia burgdorferi* to mCD1d (42). Although both TM-CL and PIM2 bind with the sn-1 linked fatty acyl in the A’ pocket and the sn-2 linked acyl chain in the F’ pocket, the binding orientation of PC is reversed (Fig. 2F, 2G).

### Table I. Data collection and refinement statistics for the CD1d–CL complex

<table>
<thead>
<tr>
<th>Statistics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data collection</td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>50.0–2.3 (2.38–2.30)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>95.4 (88.4)</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>37,815</td>
</tr>
<tr>
<td>Redundancy</td>
<td>1.9</td>
</tr>
<tr>
<td>R_{sym} (%)</td>
<td>9.1 (38.4)</td>
</tr>
<tr>
<td>L0 (%)</td>
<td>12.9 (1.9)</td>
</tr>
<tr>
<td>Refinement statistics</td>
<td></td>
</tr>
<tr>
<td>No. of reflections (f &gt; 0)</td>
<td>36,507</td>
</tr>
<tr>
<td>Maximum resolution (Å)</td>
<td>2.3</td>
</tr>
<tr>
<td>R_{free} (%)</td>
<td>23.2 (31.2)</td>
</tr>
<tr>
<td>R_{sym} (%)</td>
<td>27.3 (32.7)</td>
</tr>
<tr>
<td>No. of atoms</td>
<td>6,282</td>
</tr>
<tr>
<td>Protein</td>
<td>5,899</td>
</tr>
<tr>
<td>Glycolipid/Spacer lipid</td>
<td>92/36</td>
</tr>
<tr>
<td>N-linked carbohydrate</td>
<td>98</td>
</tr>
<tr>
<td>Water</td>
<td>157</td>
</tr>
<tr>
<td>Ramachandran statistics (%)</td>
<td>96.8</td>
</tr>
<tr>
<td>Favored</td>
<td></td>
</tr>
<tr>
<td>Disallowed</td>
<td>0</td>
</tr>
<tr>
<td>Root mean square deviation from ideal geometry</td>
<td></td>
</tr>
<tr>
<td>Bond length (Å)</td>
<td>0.013</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.5</td>
</tr>
<tr>
<td>Average B values (Å²)</td>
<td>46.9</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
</tr>
<tr>
<td>Glycolipid/Spacer lipid</td>
<td>66.7/48.3</td>
</tr>
<tr>
<td>Water molecules</td>
<td>26.0</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>57.3</td>
</tr>
<tr>
<td>Disordered residues</td>
<td>C89–C92, C110–111, C201–202</td>
</tr>
</tbody>
</table>

Data collection and refinement statistics for the CD1d–CL complex. The statistics have been calculated with the CCP4 program TLSANL (28).

Murine T cells proliferate in response to CL in a dose-dependent manner and produce IFN-γ

To determine whether CL-reactive T cells are found in the immune repertoire of healthy, untreated mice, we studied total spleen- and liver-derived T cells. Both splenic and hepatic T cells from C57BL/6 mice proliferated in response to the maximal concentration of native bovine CL (0.2 μM), but only hepatic T cells showed a dose-dependent response at lower concentrations of CL (i.e., ≤0.1 μM) (Fig. 3A). Moreover, the maximal proliferative response of hepatic T cells was >3-fold higher than that of splenic T cells (OD_{540} = 1.086 ± 0.110 and 0.333 ± 0.100, respectively).
This is consistent with the fact that the liver contains a higher percentage of CD1-reactive T cells, such as NKT cells, than the spleen (43, 44). In contrast, we observed no significant proliferative response to hCL, a completely saturated form of bovine CL (Fig. 3A), in agreement with the finding that hCL did not bind to mCD1d (Fig. 1A). Similarly, PC, from the same source (bovine heart) as CL and with a high degree of fatty acid unsaturation, also failed to stimulate T cell proliferation (Fig. 3A), consistent with its inability to bind to mCD1d (Fig. 1A). We also evaluated whether monolysos-CL or dilsyos-CL, potential breakdown products of bovine CL that lack one or two fatty acyl chains, respectively, could stimulate hepatic T cells. T cell proliferation was maximal with native CL and appeared to decrease with removal of fatty acyl chains (Supplemental Fig. 1A). These data indicate that maximal T cell proliferation occurs with the native form of the phospholipid and not with its breakdown products. To determine whether the T cell response was limited to bovine CL, we evaluated two other forms of CL: TM-CL (synthetic CL) and Escherichia coli CL (bacterial CL) (Supplemental Fig. 1B). TM-CL induced a low level of T cell proliferation, similar to that observed with dilsyos-CL. In contrast, E. coli CL induced marked levels of T cell proliferation that were statistically indistinguishable from those seen with bovine CL (p < 0.8). These results suggest that murine hepatic T cells can differentiate between different forms of CL, but recognize both eukaryotic (bovine heart) and prokaryotic (E. coli) CL.

We next studied the time course of the T cell response to CL by monitoring IFN-γ expression of CD3+ T cells by flow cytometry. Purified splenic and hepatic T cells were stimulated for different periods of time with CL or other phospholipids (0.1 and 0.2 μM). Approximately 35.4% of splenic T cells treated with CL produced IFN-γ at 48 h, but far fewer cells (14.3%) produced IFN-γ at 24 h (Fig. 3B). In contrast, little or no IFN-γ production was detected in T cells treated with hCL or PC. Hepatic T cells were particularly responsive to CL treatment, and >5-fold IFN-γ–producing T cells were found in hepatic, compared with splenic, T cell preparations after treatment with 0.2 μM CL for 48 h (Fig. 3C). Maximal stimulation was observed with 0.2 μM CL at 48 h, with approximately half-maximal stimulation observed after 24 h. At 72 h, <25% of the CL-responsive T cells observed at 48 h were still viable (data not shown). These findings demonstrate that T cells proliferate and produce IFN-γ rapidly in a dose-dependent manner to CL. CL-responsive T cells are found predominantly in the liver, but are also detected in the spleen of healthy C57BL/6 mice.

**CL-responsive T cells express γδ TCR**

To determine the TCRs expressed by CL-responsive T cells, we monitored γδ and γδ TCR positivity of phospholipid-stimulated hepatic and splenic CD3+ T cells by flow cytometry. Vehicle-treated splenic and hepatic T cells were largely γδ TCR positive and γδ TCR negative (96.4 and 97.1%, respectively; Fig. 4A, 4B). In contrast, CL stimulation resulted in a reduction in γδ TCR+ CD3+ T cells and an increase in γδ TCR+ CD3+ T cells. Reduction in γδ TCR+ T cells was due not to increased apoptosis (as detected by Annexin V and 7-aminoactinomycin D staining), but instead to a specific proliferation of γδ TCR+ CFSE-labeled T cells (20.2% for CL versus 2.0% for vehicle) (Supplemental Fig. 2). These changes were specific to native CL and were not observed with hCL or PC. Stimulation of γδ TCR-bearing T cells was maximal in both hepatic and splenic T cells at 48 h, but the relative cell numbers (as defined in Materials and Methods) differed substantially (6367 [hepatic] versus 1029 [splenic]) (Fig. 4C) and suggest that there are ~6-fold more CL-responsive γδ T cells in the liver than spleen. When hepatic CL-responsive γδ T cells were evaluated for Vγ2, Vγ3, Vγ4, and Vδ6.3/2 expression, only Vγ3 expression was found to be significantly increased (2.4% compared with 0.3% in vehicle-stimulated cells) (data not shown). Taken together, our data indicate that CL-responsive cells are γδ T cells located more predominantly in the liver than in the spleen and suggest that Vγ2, Vγ4, and Vδ6.3/2 are not expressed on the predominant γδ T cell subset that responds to CL.

**CL-responsive γδ T cell expansion is independent of αβ T cells**

To exclude the possibility that αβ TCR-bearing cells (e.g., NKT cells) provide a first signal that induces a secondary γδ T cell response, we compared the responses of T cells from TCRβ-deficient and wild-type (WT) (C57BL/6) mice. As shown in Fig. 5A, splenic and hepatic T cells from TCRβ-deficient mice demonstrated strong proliferative responses to CL, similar to those of T cells from WT mice. The relative number of CL-responsive, IFN-γ–producing T cells found in the liver of TCRβ-deficient mice was also similar to that observed in WT mice (Fig. 5B, 5C). These results demonstrate that the proliferation of γδ T cells in response to CL is independent of the αβ T cell population and not secondary to activation of an αβ T cell subset. Furthermore, we have observed CL-specific stimulation of γδ T cells purified from spleen and liver of WT mice in the absence of any other cells.
CL-induced expansion of γδ T cells was unchanged by the addition of anti-MHC I or anti-MHC II APCs derived from C57BL/6 CD1d−/− MHC class II (MHC II).

To confirm the CD1d restriction indicated by the Ab blocking experiments, we investigated whether CL-induced expansion of γδ T cells occurred in the presence of APCs derived from C57BL/6 CD1d−/− (CD1d knockout [KO]) mice. γδ T cells derived from WT mice were unresponsive to CL presented by APCs from CD1d KO mice, but responded normally to CL presented by WT APCs (Fig. 6C, 6D). Furthermore, CL-responsive γδ T cells were undetectable in CD1d KO mice, whether APCs from CD1d KO or WT mice were used (Fig. 6C, 6D). The findings were the same whether detected by flow cytometry (γδ TCR, IFN-γ positivity) (Fig. 6C) or BrdU proliferation assay (Fig. 6D). Together, these data demonstrate that CL-responsive γδ T cells are restricted by CD1d and that CD1d is required for presentation of CL.

**CD1-restricted γδ T cells express the activation marker CD25 and produce RANTES and IFN-γ in response to CL**

To characterize the activation state of CL-responsive γδ T cells, we determined their expression of two activation markers (CD69 and CD25) 48 h poststimulation with CL (Supplemental Fig. 4). Stimulation of hepatic T cells with CL resulted in γδ TCR+ cells that were >98% negative for the expression of the early activation marker CD69. In contrast, a proportion (17.4%) of these same γδ TCR+ cells expressed CD25. Notably, IFN-γ expression was observed in 37.2% of CD25+ γδ TCR+ cells compared with 5.2% of CD25− γδ TCR− cells. These data indicate that CL-responsive IFN-γ-producing γδ T cells are clearly activated by CL and express the later activation marker, CD25, after 48 h of stimulation. To investigate the potential biological effects of CL-responsive γδ T cells, we used an Ab array to examine the production of 22 cytokines and chemokines by hepatic T cells stimulated with CL, vehicle, or other phospholipids (Fig. 7A). Two cytokines, RANTES and IFN-γ, were significantly elevated (≥2-fold) in supernatants from CL-treated T cells compared with control cells (vehicle-, hCL-, or PC-treated). IL-4 was also detected in supernatants of vehicle-treated cells, but its presence was decreased by phospholipid treatment. Minimal levels of IL-13, IL-2, IL-12, soluble TNFR1, and TNF-α were detected, but did not correlate with CL treatment. IFN-γ and RANTES levels in the supernatants of treated cells were 61.4 ± 0.7 ng/ml and 1563.8 ± 25.1 pg/ml, respectively, after treatment with 0.2 μM CL. Lower, but significantly elevated, levels of both cytokines (34.0 ± 4.7 ng/ml for IFN-γ and 244.6 ± 25.9 pg/ml for RANTES) were also observed with 0.1 μM CL (Fig. 7B). In contrast, only background levels (4.6 ± 0.1 ng/ml and 45.0 ± 4.4 pg/ml, respectively) of these cytokines were detected in supernatants of cells treated with hCL or PC (0.1 or 0.2 μM) (Fig. 7B). In parallel studies, 17.4% of CL-responsive γδ T cells were shown to express the activation marker CD25 (data not shown). Taken together, our results show that hepatic γδ T cells are activated and secrete significant amounts of the cytokines IFN-γ and RANTES when stimulated by CL.

**CL activates γδ T cells in vivo**

To determine whether CL also stimulates γδ T cell in vivo, we injected mice that had received BrdU with BMDCs pulsed with CL or PC. Twenty-four hours later, we analyzed the expression of activation markers (CD25 and CD69) on CD3+ γδ TCR+ T cells in the liver. Injection of CL-pulsed BMDCs resulted in increased expression of CD25 on the proliferating BrdU+ γδ T cells in the liver (mean fluorescence intensity of 500, compared with 220 and 200 for vehicle and PC, respectively), whereas CD25 expression remained unaffected in the majority of the BrdU− cells (Fig. 8). In contrast, PC-pulsed BMDCs did not induce the expression of activation markers on BrdU+ cells. Interestingly, this upregulation was observed in the liver and not the spleen, and upregulation of CD69 was not observed (data not shown). Importantly, the percentage of γδ T cells proliferating remained unaltered regardless of the phospholipid (CL or PC) used to pulse BMDCs (data not shown).
and experiment of three independent experiments, whereas population was evaluated by flow cytometry using TruCOUNT beads (C phospholipid (PC, hCL, or CL), or vehicle, and APCs (from either WT or CD1 KO mice) for 48 h. Relative expansion of the CD3+, TCR-expressing CD3+ cell population was evaluated by flow cytometry using True-Count beads. Raw flow cytometry data, shown in A, are represented graphically in B. C and D, Purified hepatic T cells from either C57BL/6 (WT) or C57BL/6 CD1d−/− (CD1 KO) mice were incubated in vitro with 0.2 μM phospholipid (PC, hCL, or CL), or vehicle, and APCs (from either WT or CD1 KO mice) for 48 h. Relative expansion of the CD3+, γδ TCR+, IFN-γ+ cell population was evaluated by flow cytometry using TruCOUNT beads (C) or BrdU proliferation assay (D). A and B show data from a representative experiment of three independent experiments, whereas C and D show data from a representative experiment of two independent experiments. Error bars in B and D indicate SD of the means of duplicate (B) or triplicate (D) samples.

Discussion

There is a growing body of evidence showing that CD1-restricted T cells recognize diacylated phospholipids (10, 11, 13). We demonstrate in this study that CD1d interacts specifically with CL, a tetra-acylated phospholipid. Moreover, we show that γδ T cells from the normal immune repertoire (particularly the liver) of healthy mice are stimulated by CL in a CD1d-dependent manner, proliferate rapidly, and secrete IFN-γ and RANTES. Our data suggest that CL-reactive γδ T cells could play a role in host responses to bacteria and, potentially, to self-CL by promoting IFN-γ- and RANTES-mediated responses.

We present in this paper the structure of mCD1d complexed with CL, which is, to our knowledge, the first structure of CD1d with a tetra-acylated phospholipid. Although the structures of several CD1d-glycolipid complexes have been determined, the structures of only two CD1d–phospholipid complexes have been elucidated: endogenously bound PC (7) and exogenously added PIM-2 (8). PC and PIM-2 bind in opposite orientations in the lipid-binding groove, indicating a less stringent binding orientation for glycerolipids in general, as compared with the binding of ceramide-based ligands, such as sulfatide and α-GalCer. CL, like PIM-2, binds with the sn-1 fatty acid inserted into the A’ pocket and the sn-2 fatty acid in the F’ pocket. In line with previous data on glycerolipid Ags from B. burgdorferi (42), we postulate that variations in the fatty acid composition of naturally occurring phosphoglycerolipids, such as length and saturation of the fatty acid (C14:0, C16:0, C18:0, C18:1, or C18:2), could influence the overall binding orientation of the glycerolipid in the binding groove of mCD1d, leading to subtle structural changes in the exposed T cell epitope and, possibly, altered T cell recognition. Although both bovine CL and TM-CL interacted with mCD1d, hCL did not. Chemically, hCL differs from native bovine CL only in the saturation of its fatty acid chains. Intriguingly, the role of the third and fourth alkyl chain of CL in T cell activation is currently unclear. On a molecular level, both exposed alkyl chains are flexible and can be accommodated between the CD1–TCR interface, similar to the squashed model that has been proposed for the highly exposed iGb3 ligand (37). Monolyso-CL and dilyso-CL (bovine CL with one and two fatty acids, respectively, removed) bound to mCD1d in our binding studies. However, lipid loading into CD1d is directly linked to the solubility of the lipid in question. Our findings for CD1d binding are consistent with this, in that binding/loading of dilyso-CL > monolyso-CL > TM-CL. In contrast, in T cell proliferation studies, dilyso-CL was only minimally stimulatory, and monolyso-CL was not stimulatory (and appeared to be toxic to the cells). These data suggest that the solvent-exposed third and fourth alkyl chains of CL may be important for T cell recognition and activation.

CL was recognized predominantly by hepatic, but also by splenic, γδ T cells. T cell stimulation occurred in a dose-dependent and CL-specific manner. Indeed, treatment with neither hCL nor PC induced similar T cell stimulation. In contrast, CL from both prokaryotic and eukaryotic cells was able to induce hepatic T cell proliferation to the same degree. The only difference between CL from different species is the fatty acid composition. Both E. coli CL and bovine CL are of natural origin and, as such, contain fatty acids that are both common (palmitic acid and stearic acid) and unique to prokaryotes (cyclopropyl fatty acids). Our
samples.
ever, as unsaturation tends to lower the transition temperature

FIGURE 7. CD1d-restricted γδ T cells produce RANTES and IFN-γ in response to CL. Purified hepatic T cells from C57BL/6 mice were incubated for 48 h in vitro with vehicle or 0.2 μM phospholipid (CL, hCL, or PC) in presence of APCs. A, Supernatants of treated cells were tested for the presence of 22 cytokines and chemokines using an Ab array quantitated by densitometry. Values indicate the signal intensity (%) relative to the positive control (set at 100%) for each cell treatment. B, IFN-γ or RANTES in supernatants of treated cells was quantified by ELISA. The data in A and B show the mean ± SD of two independent experiments. A and B show data from a representative experiment of two independent experiments. Error bars in A and B indicate SD of the means of duplicate samples.

limited data suggest that CL-reactive hepatic γδ T cells are not sensitive to the differences in fatty acid composition between eukaryotic and prokaryotic forms of CL, unlike invariant Vε14 NKT cells that have been reported to be sensitive to the fatty acid composition of *B. burgdorferi* α-galactosyl diacylglycerolipids (45) at least in part as a result of the two opposite binding orientations of the lipids observed inside the CD1d groove (42). To fully address the question of fatty acid specificity of murine hepatic γδ T cells, a panel of synthetic versions of prokaryotic and eukaryotic CL is required. In contrast to similar recognition of *E. coli* and mammalian CL, murine hepatic γδ T cells clearly differentiated between saturated (hCL) and unsaturated (native CL) forms of mammalian CL. CL from different organisms are not characterized by unique degrees of fatty acyl chain length or unsaturation (dominant fatty acids may vary in length between 14 and 22 carbon atoms and contain between 0 and 6 double bonds) (46). In light of these data, unsaturation is unlikely to be essential for the biological function of CL. However, as unsaturation tends to lower the transition temperature of a phospholipid, it can affect lipid solubility and fluidity. We have previously shown that CD1d-restricted recognition of phosphatidylethanolamine by an NKT cell hybridoma (24.8.A) correlated with the degree of acyl chain unsaturation (13). Although it is tempting to speculate that CL induces γδ T cell activation because it is able to load more efficiently into the mCD1d molecule than hCL, it is equally possible that acyl chain unsaturation fine tunes the binding orientation of the phospholipid in the CD1d binding groove, leading to slight structural changes in the exposed T cell epitope, which, possibly, affects T cell recognition.

Our finding that CL is able to trigger the proliferation and activation of γδ T cells in vivo may play a biological role in several settings, especially in host responses to infection in the liver. The liver is an important site of visceral infection and mucosal immunity, and the low hepatic blood pressure and high surface area in contact between blood and parenchymal cells provide pathogens with an easy route of access. Immune surveillance for pathogens in the liver is an important, but poorly understood, process. CL-responsive, CD1d-restricted T cells are particularly well suited to hepatic immune surveillance because CD1d is expressed on hepatocytes, Kupffer cells, hepatic DCs, and endothelial cells of the sinusoid lining (48), all of which have significant Ag uptake capabilities (49). CL is a major prokaryotic phospholipid expressed by many genera of bacteria (46). Thus, bacterial infections may result in circulating CL that could
act as a danger signal and trigger protective γδ T cell responses. Indeed, transient increases of circulating human γδ T cells are observed during the early acute stage of several bacterial, parasitic, and viral infections (48, 50–53). In mice, the H3 variant of Coxackievirus B3 activates splenic γδ T cells that recognize CD1d expressed by Coxackievirus B3-infected myocytes, resulting in severe myocarditis (54).

Our results show that upon exposure to CL, γδ T cells are activated and secrete significant amounts of IFN-γ and RANTES. Both cytokines are important in amplifying the innate immune response and bridging the transition from innate to adaptive immunity. IFN-γ, a pleiotropic cytokine known to orchestrate many distinct cellular programs such as macrophage-mediated killing of intracellular pathogens and antiviral immunity, has wide-reaching effects that extend beyond conventional immune cells to nonprofessional host defense cells (e.g., endothelial cells, fibroblasts, and hepatocytes). In contrast, RANTES (or CCL5) is a chemokine that plays an active role in recruiting leukocytes to sites of inflammation. In the presence of cytokines such as IFN-γ, RANTES also induces the activation and proliferation of natural killer cells (55). Furthermore, RANTES is known to be secreted by certain T cell subsets in human cancer states. To date, there are no published data on human CL-reactive γδ T cells. However, peripheral blood-derived CD1-restricted γδ T cells recognize pollen phospholipids (phosphatidyl-ethanolamine and PC), produce both IFN-γ and IL-4, and provide help for IgE production (10, 14). Phosphatidylethanolamine and PC also activate human CD1-restricted duodenal γδ T cells, but the cytokines (IL-4 and TGF-β) they produce appear to be regulatory in nature (11). CL was not evaluated in the latter studies. Furthermore, little is known about the recognition of phospholipid by human splenic or hepatic γδ T cells.

In summary, we demonstrate that CL, a tetra-acylated phospholipid, is a natural ligand for mCD1d. Furthermore, we show that a subset of γδ T cells, found in the normal immune repertoire, is activated both in vitro and in vivo by CL in a CD1d-restricted manner and secretes IFN-γ and RANTES. We propose that pro-karyotic, and possibly also eukaryotic, CL may serve as a danger signal for mammalian T cells. We further propose that CD1d-bound CL induces a potent innate-like immune response, which, through the release of IFN-γ and RANTES, promotes the transition to an adaptive immune response. In this manner, CL-reactive γδ T cells may play a key role in immune surveillance for CL expression during infection or tissue injury.

Acknowledgments

We thank Drs. Peter van den Elzen and Rusung Tan for providing the CD1d-deficient mice (originally derived by Drs. Steven Balk and Mark Exley) and Drs. Michel Lafleur and Gerhard Wingender for careful review and comments about the manuscript. We also thank Rebecca Subang and David Salem for technical assistance and Maria Da Silva Martins and Ekaterina Yurchenko for expert advice, assistance, and discussion in the later phase of the project. This paper is dedicated by Dr. Mélanie Dieudé to the memory of Rita Larochelle Boucher.

Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure Legends

Supplementary Figure 1. Murine hepatic T cells proliferate in response to various forms of CL. Purified hepatic T cells from C57BL/6 mice were incubated with vehicle or phospholipid (monolysocardiolipin [monolysoCL], dilysocardiolipin [dilysoCL], cardiolipin [CL], phosphatidylcholine [PC]), hydrogenated CL [hCL], tetramyristoyl CL [TM-CL], or E. coli CL [E. coli CL]) in presence of antigen-presenting cells in vitro. Panel a compares lyso forms of CL with native CL, and panel b compares CL from different sources (CL [bovine heart], TM-CL [synthetic], and E. coli CL [bacteria]). Phospholipids were used at the following final concentrations: 0.2 µM (panel a), and 0.1 or 0.2 µM (panel b). Proliferation was evaluated by BrdU incorporation during the last 24 h of treatment, and values show the mean values (OD_{450}) ± standard deviation (SD) of triplicate samples from a representative experiment of two independent experiments. Other controls (not shown) included concanavalin A and cell medium, which had OD_{450} (± SD) values of 1.194 (0.037) and 0.063 (0.004), respectively.

Supplementary Figure 2. CL induces proliferation, but not apoptosis, of γδ T cells. Purified hepatic T cells from C57BL/6 mice were incubated with vehicle or 0.2 µM phospholipid (cardiolipin [CL], hydrogenated CL [hCL], or phosphatidylcholine [PC]) in presence of antigen-presenting cells for 48 h in vitro. (a) Cell proliferation was assessed using CFSE-labeled T cells, which were evaluated for CFSE, CD3⁺, and γδ TCR⁺ staining by flow cytometry; and (b)
\(\alpha\beta\) TCR\(^+\) cell apoptosis was assessed by staining with annexin V and 7-AAD. The percentage of cells in each quadrant is indicated, and the data are from a representative experiment of two independent experiments.

**Supplementary Figure 3.** Purified murine splenic and hepatic \(\gamma\delta\) T cells proliferate in response to CL and express IFN-\(\gamma\). \(\gamma\delta\) T cells, purified from the spleen (left panels) or liver (right panels) of C57BL/6 mice, were incubated with vehicle or 0.2 \(\mu\)M phospholipid (cardiolipin [CL], hydrogenated CL [hCL], or phosphatidylcholine [PC]) in presence of antigen-presenting cells *in vitro*. IFN-\(\gamma\) production was evaluated by flow cytometry in treated \(\gamma\delta\) T cells at 0 h, 24 h, or 48 h. The data are from a representative experiment of two independent experiments.

**Supplementary Figure 4.** CD1-restricted \(\gamma\delta\) T cells express activation marker CD25, but not CD69. Purified hepatic T cells from C57BL/6 mice were incubated with vehicle or 0.2 \(\mu\)M phospholipid (cardiolipin [CL], hydrogenated CL [hCL], or phosphatidylcholine [PC]) in presence of antigen-presenting cells for 48 h *in vitro*. CD69 (a) or CD25 (b) expression by CD3\(^+\) \(\gamma\delta\) TCR\(^+\) cells was evaluated by flow cytometry. IFN-\(\gamma\) expression by CD3\(^+\) \(\gamma\delta\) TCR\(^+\) and CD3\(^+\) \(\gamma\delta\) TCR\(^+\) cells was also evaluated for CL-stimulated cells (see lower panels indicated by arrows). The percentage of cells in each quadrant is indicated, and the data are from a representative experiment of two independent experiments.
Supplementary Figure 1

a

![Graph a](image)

b

![Graph b](image)
Supplementary Figure 3

Vehicle
Counts
IFN-γ
0h 3.54
24h 6.32
48h 3.21

Spleen
Counts
IFN-γ
0h
24h
48h

Liver
Counts
IFN-γ
0h
24h
48h

PC
5.87
5.03

hCL
6.36
6.71

CL
8.79
37.12

4.07
3.97

2.46
3.14

56.78
62.94