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Recognition of Lysophosphatidylcholine by Type II NKT Cells and Protection from an Inflammatory Liver Disease

Igor Maricic,* Enrico Girardi,[†] Dirk M. Zajonc,[†] and Vipin Kumar*

Lipids presented by the MHC class I–like molecule, CD1d, are recognized by NK T (NKT) cells, which can be broadly categorized into two subsets. The well-characterized type I NKT cells express a semi-invariant TCR and can recognize both α- and β-linked glycolipids, whereas type II NKT cells are less well studied, express a relatively diverse TCR repertoire, and recognize β-linked lipids. Recent structural studies have shown a distinct mode of recognition of a self-glycolipid sulfatide bound to CD1d by a type II NKT TCR. To further characterize Ag recognition by these cells, we have used the structural data and screened other small molecules able to bind to CD1d and activate type II NKT cells. Using plate-bound CD1d and APC-based Ag presentation assay, we found that phospholipids such as lysophosphatidylcholine (LPC) can stimulate the sulfatide-reactive type II NKT hybridoma Hy19.3 in a CD1d-dependent manner. Using plasmon resonance studies, we found that this type II NKT TCR binds with CD1d-bound LPC with micromolar affinities similar to that for sulfatide. Furthermore, LPC-mediated activation of type II NKT cells leads to anergy induction in type I NKT cells and affords protection from Con A–induced hepatitis. These data indicate that, in addition to self-glycolipids, self-lysophospholipids are also recognized by type II NKT cells. Because lysophospholipids are involved during inflammation, our findings have implications for not only understanding activation of type II NKT cells in physiological settings, but also for the development of immune intervention in inflammatory diseases. *The Journal of Immunology*, 2014, 193: 4580–4589.

N atural killer T cells are innate-like and generally reactive to lipid Ags presented by CD1d MHC class I–like molecules (1–3). NKT cells can play an important immunoregulatory role in inflammatory conditions, including autoimmune diseases, infectious diseases, and cancer (4–8). NKT cells are comprised of two main subsets, type I and type II. Type I NKT cells express a semi-invariant TCR encoded predominantly by a germline invariant Vα gene (Vα14-Jα18 in mice and Vα24-JαQ in humans) and more diverse nongermline Vβ chain genes (Vβ8.2/7/2 in mice and Vβ11 in humans) (1, 3). Owing to their predominance in mice as well as the ability of type I NKT cells to recognize a marine sponge-derived glycolipid, αGalCer, this subset has been well studied. In contrast, type II NKT cells that use a relatively diverse TCR repertoire are less abundant in mice and are less well studied with regard to their physiological role and Ag recognition.

Recently, one of the major subsets of type II NKT cells has been shown to be reactive to a self-glycolipid sulfatide (9, 10). The sulfatide/CD1d tetramer^{+} cells express an oligoclonal TCR repertoire with predominant usage of Vα3/Vα1-Jα7/Jα9 and Vβ8.1/Vβ3.1-Jβ2.7 gene segments (9). Only ~14% of TCR Vα and 13–27% of TCR Vβ chains in sulfatide-reactive type II NKT cells are exclusively encoded by germline gene segments. The semi-invariant TCR on type I NKT cells binds to CD1d in a parallel configuration that mainly involves the α-chain. At least one type II NKT TCR contacts its ligands primarily via its β-chain rather than α-chain, suggesting that the TCR Vβ chain contributes significantly to Ag fine specificity (11, 12). The mechanism of binding of type II NKT TCRs to Ags uses features of TCR binding shared by both type I NKT cells and conventional T cells (9, 11, 12). Thus, type I and type II NKT cell subsets display distinct modes of recognition.

Type I NKT cells respond to both α- and β-linked glycolipids, whereas type II NKT cells have been shown to recognize β-linked glycolipids. Unlike αGalCer, most microbial lipids and other self-Ags, including isoglobotrihexosylceramide, do not stimulate type I NKT cells very effectively. Similarly, lipids recognized by the type II NKT cells, including sulfatides, βGlcCer and βGalCer, as well as some pollen-derived lipids, are not as potent in activating them, as αGalCer is in activating type I NKT cells. In this regard, it is notable that, although the binding affinity of the TCR of type I NKT cells to CD1d-presented αGalCer is very high (K_d of 11–30 nM) (13), the affinity to microbial ligands is in the micromolar range (0.7–6 μM) (14) comparable to that of typical peptide–MHC I interactions (1–100 μM) (15). Recently, it has been shown that lysophosphatidylethanolamine (LPE) induced following hepatitis B viral infection may be a self-Ag for a subset of type II NKT cells (16). Type II NKT cells have been shown to be regulatory, as their activation with the self-glycolipid sulfatide results in protection from autoimmune diseases by downregulation of inflammatory responses elicited by type I NKT cells as well as conventional MHC-restricted CD4^{+} and CD8^{+} T cells (7, 10, 17–19). To further characterize the type II NKT subset and their physiological role in immune regulation, it is important to identify additional lipid Ags recognized by them. In this study, we have found that murine type II NKT cells are also reactive to self-phospholipids, including lysophosphatidylcholine (LPC), lysos-

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The online version of this article contains supplemental material.

Abbreviations used in this article: ALT, alanine aminotransferase; EAE, experimental autoimmune encephalomyelitis; LPAF, lyso platelet-activating factor; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LSM, lysosphingomyelin; WT, wild-type.

phingomyelin (LSM), and lyso platelet-activating factor (LPAF). Furthermore, as with sulfatide, LPC-mediated activation of type II NKT cells induces anergy in type I NKT cells. Because LPC levels are physiologically controlled during inflammatory conditions, these findings have important implications in understanding biology of type II NKT cells as well as in the development of potential therapeutics for the inflammatory diseases.

Materials and Methods

Animals

C57BL/6J female mice (7–10 wk) were purchased from The Jackson Laboratory (Bar Harbor, ME). CD1d$^{-/-}$ and Jα18$^{-/-}$ BL/6 mice, originally generated in the laboratories of L. Van Kaer (Vanderbilt University, Nashville, TN) and M. Taniguchi (Chiba University, Chiba, Japan), were provided by M. Kronenberg (La Jolla Institute for Allergy and Immunology). All mice were bred and maintained in specific pathogen-free conditions in the Torrey Pines Institute for Molecular Studies animal facility. Treatment of animals was in compliance with federal and institutional guidelines and approved by the Torrey Pines Institute for Molecular Studies Animal Care and Use Committee.

Lipid Ags

Synthetic lipids were acquired from different sources, as follows: LPC (C16:0), LSM, LPAF (C18:0), and N-palmitoyl dopamine from Cayman Chemicals (Ann Arbor, MI); LPC (C18:0), LPC (C18:1 9Z), LPC (C18:1 1oleyl), LPC (C24:0), or 1-lignoceroyl-2-hydroxy-sn-glycero3-phosphocholine from Avanti Polar Lipids (Alabaster, AL); (±)-erythro-aleuritic acid from Alfa Aesar (Ward Hill, MA); and LSF and lysoβGluCer from Matreya (Pleasant Gap, PA). Synthetic αGalCer was provided by Kirin Brewery (Tokyo, Japan). All the lipids were dissolved in vehicle (0.5% Tween 20 and 0.9% NaCl solution) and further diluted in PBS.

Ag presentation assay using APCs to stimulate NKT cells

APC-based presentation assays were performed essentially as described earlier (10), with Vα1$^+$ sulfatide-reactive (Hy19.3 or XV19) (20) as well as nonsulfatide-reactive Vα3$^+$ (Hy24 or VIII24, HyIF4, or IF4) (20) or Vα8$^+$ (HyB11 or TCB11, Hy49, or VII49) (21) type II NKT hybridomas or an αGalCer-reactive type I NKT hybridoma (Hy1.2) in the presence of different lipids and irradiated splenocytes. Type II NKT hybrids Hy19.3, Hy24, HyIF4, Hy49, and HyB11 were provided by the laboratories of S. Cardell (University of Gothenburg, Gothenburg, Sweden) and A. Bendelac (University of Chicago, Chicago, IL). The type I NKT Hy1.2 was provided by M. Kronenberg. T cell hybridoma cells (5 × 10^4/well) were cocultured with irradiated splenocytes (4 × 10^5/well) in triplicates from 7- to 10-wk-old wild-type (WT) BL/6, or CD1d1$^{-/-}$ mice in the presence of graded concentrations of glycolipids. Supernatants from these 16-h cultures were used in a typical sandwich ELISA to measure IL-2 production. Proliferative responses in splenic cell proliferation assays (6 × 10^5 cells/well) from naive mice were measured in RPMI medium 1640 with 10% FBS (Atlas Biologicals, Fort Collins, CO) and by addition of 1 μCi ^{3}H-thymidine (MP Biomedicals, Santa Ana, CA) per well for the final 18 h of a 5-d in vitro culture in the presence of different lipids and CD1d molecules.

Ag presentation assay using CD1d-coated plates to stimulate NKT cells

Flat-bottom 96-well plates were coated for 18 h with 1 μg/well soluble mouse CD1d or CD1d mutants isolated and purified using a baculovirus expression system at the concentration of 1.0 μg at 4°C in 50 mM Na-acetate buffer (pH 5), essentially as described earlier (22). Coated plates were washed three times with the same buffer and then incubated for another 4 h at 37°C with graded concentrations of lipids. After washing three times with PBS, 5 × 10^4/well T cell hybridomas (Hy19.3 or Hy1.2) were added in triplicate. Supernatants were collected after 16 h to measure IL-2 release by sandwich ELISA (eBioscience, San Diego, CA).

Surface plasmon resonance analysis for LPC-CD1d binding to the Hy19.3 TCR

Surface plasmon resonance measurements were performed with purified proteins, essentially as described earlier (11). Briefly, recombinant mouse CD1d and β$_2$-microglobulin were coexpressed in Sf9 cells and purified by affinity, ion exchange, and size exclusion chromatography. The Hy19.3 TCR was expressed as V/mouse-C/human chimeric constructs in *Escher-*

ichia coli. The expressed inclusion bodies were then refolded and purified by ion exchange and size exclusion chromatography. Lipid Ags were loaded overnight in the presence of 10 mM HEPES (pH 7.5) and 150 mM NaCl and further purified by size exclusion chromatography. Biotinylated Hy19.3 TCR (300–500 resonance units) was immobilized on a CAPture chip on a Biacore 3000 instrument (GE Healthcare). A range of concentrations of each mCD1d-lipid complex was then passed on the surface to measure association and dissociation rates and equilibrium constants by fitting with a 1:1 Langmuir model in the Biaevaluation software.

Flow cytometry analysis for monitoring type I NKT cells

Splenocytes were suspended in FACS buffer (PBS containing 0.02% NaN$_3$ and 2% FCS), blocked (anti-mouse FcRγ; BD Biosciences, San Diego, CA), and stained with αGalCer-loaded mCD1d tetramer PE, and FITC-labeled anti–TCR-β mAb (eBioscience), as described earlier (18). Flow cytometric analysis was performed using FlowJo (version 9.3.1) software (Ashland, OR).

Induction of Con A–induced hepatitis, serum alanine aminotransferase, histopathology, and cytokine analysis

To induce autoimmune hepatitis, mice were injected i.v. with 10 mg/kg Con A. Lipids, including LPC (100 μg), were administered i.p. immediately after Con A injection. Control mice were injected with PBS/vehicle (200 μl/mouse). Mice were sacrificed, and serum was collected 24 h after the injections. Alanine aminotransferase (ALT) serum levels were measured using ALT Infinity Liquid Stable Reagent from Thermo Scientific (TR71121). For histopathology, liver tissues were fixed in 10% formaldehyde solution and kept at room temperature until use. H&E staining of the liver sections was performed by Scripps Research Institute Histology Core Laboratory. Serum cytokines were determined in different groups of mice 24 h after Con A injection using BD cytometric bead array mouse Th1/Th2/Th17 cytokine kit (BD Biosciences), per manufacturer's protocol.

Determination of anergy induction in type I NKT cells

Groups of BL/6 mice were injected with LPC (C18:0) or LPC (C16:0) 100 μg/mouse, or LSF 80 μg/mouse, or PBS. One day later, all treated mice were administered a single injection of αGalCer (2 μg/ml). Mice were sacrificed on day 5 (3 d after αGalCer injection), and single-cell suspensions of spleen cells were stained with anti–TCR-β FITC and αGalCer/CD1d tetramer PE ex vivo and analyzed, as mentioned above.

In another experiment, groups of BL/6 mice injected as above with LPC (C18:0) or LPC (C16:0), LSF, or PBS were sacrificed after 19 h, and splenocytes were cultured (6 × 10^5/well) in 96-well plates in the presence of αGalCer (10 ng/ml). After an initial 72 h of in vitro culture, ^{3}H-thymidine was added, and, after another 18 h, cells were harvested, and incorporated radioactivity was measured using a beta plate counter, as described earlier (18, 23).

Statistical analysis

Data are expressed as mean ± SEM for each group. Statistical differences between groups were evaluated by the unpaired one-tailed Student t test using GraphPad Prism software (version 5.0a; GraphPad Software, La Jolla, CA).

Results

Activation of a sulfatide-reactive type II NKT hybridoma by lysophospholipids using a CD1d plate-bound assay

Recently, we and others have provided a snapshot of type II NKT TCR-mediated recognition of sulfatide or LSF by determining the ternary structure of a type II NKT TCR (Vα1Jα26-Vβ16Jβ2.1) in complex with LSF-CD1d or C24:1 sulfatide-CD1d (11, 12). Based upon the similarities of these structures, we have used computer-based lead generation technology (ScieIntl, Greensboro, NC) to predict small molecules other than glycolipids that may bind to CD1d in a similar manner and could be potentially recognized by the type II NKT cells. In this study, we have used a plate-bound CD1d assay to determine whether any of the six molecules (for structures, see Fig. 1) can stimulate a type II NKT hybridoma (Hy19.3). As shown in Fig. 2A, two synthetic lysophospholipids, LPC and LPAF, are efficient in stimulating type II NKT cells. Interestingly, in earlier experiments (11), purified egg lysolecithin,
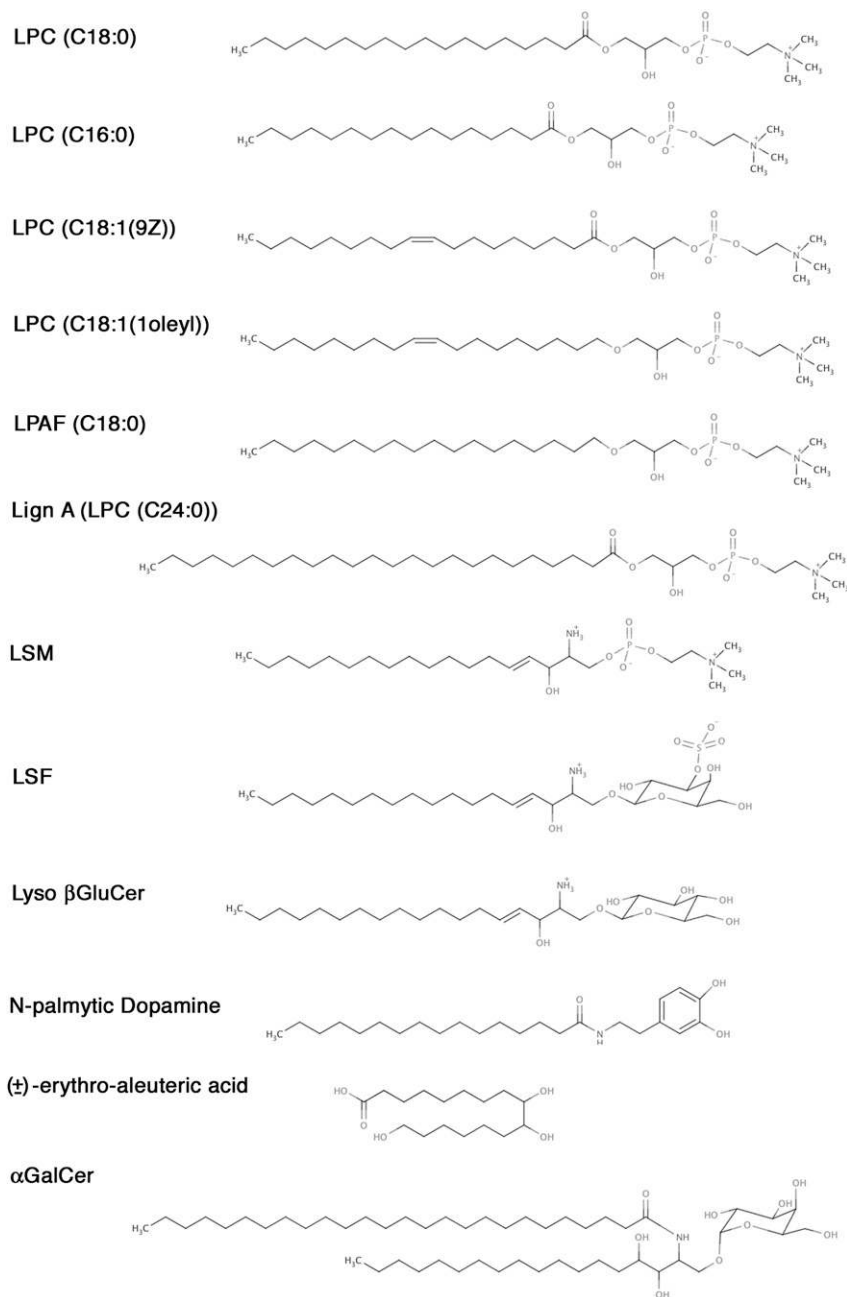
FIGURE 1. Detailed chemical structures of different lysophospholipids as well as glycolipids used in this study.

a mixture of LPC isoforms, was unable to stimulate Hy19.3 effectively owing to perhaps suboptimal loading/presentation of a single species. Additionally, N-palmitoyl dopamine also stimulated Hy19.3, albeit less efficiently compared with lysophospholipids. As expected and shown earlier (11, 24), both LSF and lyso βglucosylceramide were able to stimulate Hy19.3 and were used as positive controls. Two other molecules, 1-lignoceroyl-2-hydroxy-sn-glycero-3-phosphocholine (or LPC C24:0) and (±)-erythro-aleuritic acid, were unable to activate the type II NKT Hy19.3. In contrast, none of these molecules was able to stimulate a type I NKT hybridoma Hy1.2 (Fig. 2B). However, αGalCer, a ligand for the type I NKT cells, stimulated Hy1.2, but not Hy19.3. In this study, we have chosen to further analyze the activity of LPC to activate type II NKT cells both in vitro as well as in in vivo assays. A dose titration of curve of stimulation of Hy19.3 with LPC in typical CD1d plate-bound assays is shown in Fig. 2C. It is interesting that the concentration of LPC required to stimulate Hy19.3 is similar to that of LSF (22).

Stimulation of a type II NKT cell hybridoma by lysophospholipids in the presence of CD1d$^{+/+}$ but not CD1d$^{-/-}$ APCs

Ag presentation of lipid molecules by CD1d may involve endocytic pathways as well as processing and loading with the help of the lipid-binding proteins. In this study, we determined whether, in a typical Ag presentation assay with irradiated splenocytes from the WT C57BL/6 (CD1d$^{+/+}$) or the CD1d-deficient (CD1d$^{-/-}$) mice, lysophospholipids can be appropriately presented to stimulate Hy19.3. Fig. 3A clearly shows that both LPC and LPAF are able to stimulate the type II NKT Hy19.3 in the presence of CD1d$^{+/+}$, but not CD1d$^{-/-}$ splenocytes. Other lipids, including N-palmitoyl dopamine, did not stimulate type II NKT cells. As expected, Hy19.3 and Hy1.2 were stimulated by their respective ligands LSF and αGalCer, respectively, in the presence of CD1d$^{+/+}$ splenocytes, but not CD1d$^{-/-}$ APCs. A typical dose titration curve of Hy19.3 stimulation with LPC is shown in Fig. 3B and is similar to that of LSF (22).
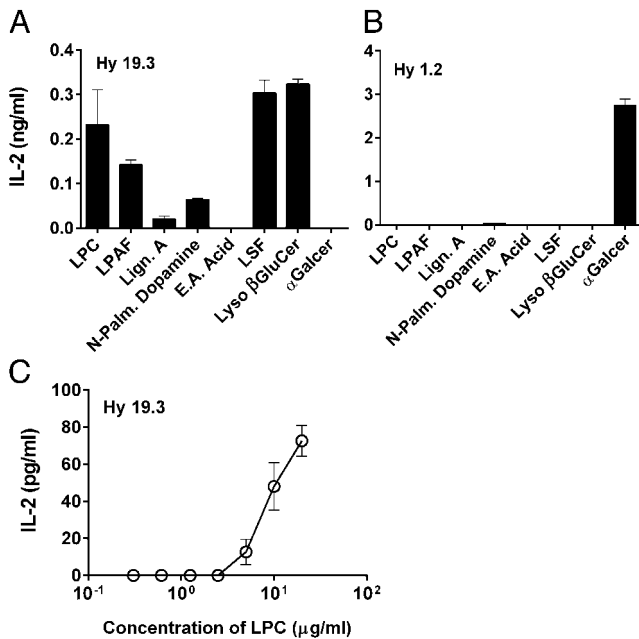
FIGURE 2. Stimulation of a type II NKT cell Hy19.3 by self-lyso-phospholipids in an APC-free CD1d-coated Ag presentation assay. Plates were coated with the recombinant CD1d protein in PBS and incubated at 4°C overnight. Next day, plates were washed and loaded with indicated lipids for 5–6 h at 37°C. IL-2 release at an optimum concentration (5 μg/ml) following incubation with 5×10^4 cells/well of a type II NKT cell Hy19.3 (A) or a type I NKT cell Hy1.2 (B) is shown. A typical dose titration curve of stimulation of Hy19.3 with various concentrations (0.6–20 μg/ml) of LPC is shown in (C). There was no significant amount of IL-2 secretion (0.01–0.03 ng/ml) with 1 μg/well plate-coated CD1d only in these assays. These data are representative of four different experiments.

Binding of different forms of LPC with a type II NKT TCR

We recently reported that the Hy19.3 TCR binds to the mouse CD1d-LSF complex with micromolar affinities (11). In this study, we determined the binding affinities of different isoforms of LPC-CD1d complexes (LPC C18:0, LPC C16:0, LPC C18:1 9Z, LPC C18:1 1oleyl) to the Hy19.3 TCR using surface plasmon resonance. As shown in Fig. 4, this type II NKT TCR binds to CD1d-LPC with micromolar affinities (~3–9 μM) similar to CD1d-LSF binding (~6 μM). The binding was characterized by relatively slow on rates, followed by fast off rates, similar to that in the case of LSF-CD1d (11). This is also similar to what has been reported for the weak type I NKT cell lipid ligands, such as microbial glycolipids or self-Ags (25). Interestingly, LPC C18:0 appears to bind with the higher affinity in comparison with other LPC isoforms examined in this study (Fig. 4).

Ability of different LPC isoforms to stimulate type II NKT cells

Different isoforms of LPC are predominantly enriched in different tissues, and, among them, C16 and C18 are the most abundant forms (26). In this study, we have examined the ability of different LPC isoforms to stimulate the sulfatide-reactive Vα1+ Hy19.3 as well as a panel of other nonsulfatide-reactive Vα3+ and Vα8+ type II NKT hybridomas in a typical Ag presentation assay. Fig. 5 clearly shows that LPC stimulated only the sulfatide-reactive Hy19.3, but none of the other type II NKT cell hybridomas. Furthermore, LPC C18:0 is most efficient in stimulating Hy19.3 compared with other LPC isoforms, such as C16:0, C18:1 9Z, C18:1 1oleyl, or C24:0 (Fig. 5). Notably, another phospholipid, LSM, similar in structure to that of LPC, was also able to stimulate the sulfatide-reactive Hy19.3 hybridoma in this assay.
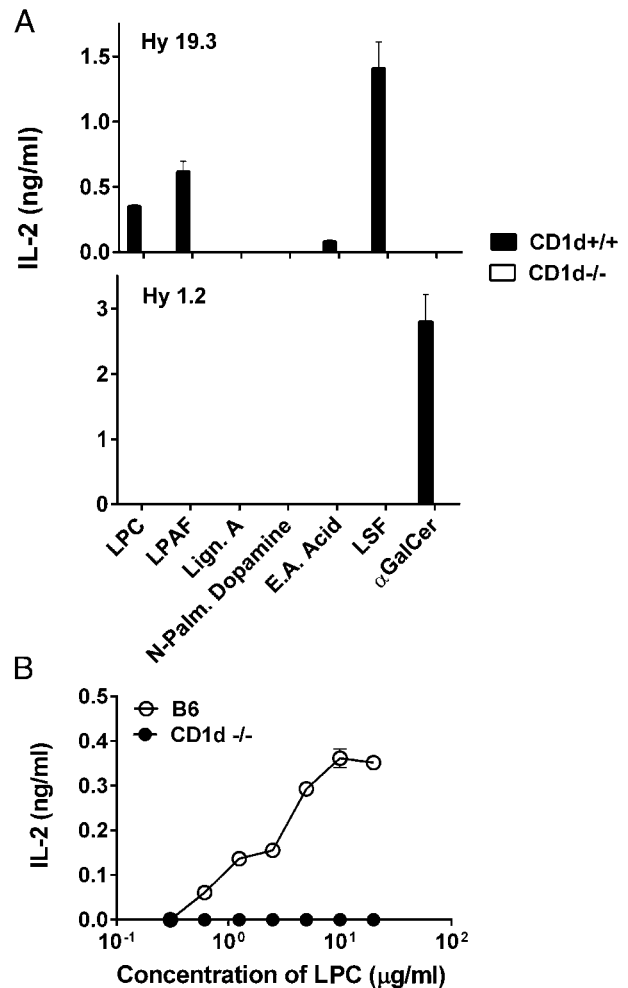

FIGURE 3. CD1d-dependent stimulation of a sulfatide-reactive type II NKT cell by self-lysophospholipids. (A) A type II NKT Hy19.3 or a type I NKT Hy1.2 was stimulated in the presence of APCs (irradiated splenocytes) from either the WT C57BL/6 (CD1d+/+) or CD1d-deficient (CD1d−/−) mice in the presence of different concentration of lipids (0.6–20 μg/ml). IL-2 release at optimum concentration (5 μg/ml) of indicated lipids is shown. (B) A typical dose titration of Hy19.3 stimulation in the presence of APC and LPC (0.6–20 μg/ml) is shown. These data are representative of at least three independent experiments.

Substitutions of the A′ pocket on CD1d results in loss of LPC recognition by the type II NKT Hy19.3

From two recent structural studies, it is clear that the docking of the Hy19.3 type II NKT TCR on the surface of CD1d molecule is distinct from that of the type I NKT TCR (11, 12). In this study, we determined whether some of the key residues on CD1d involved in the binding of the LSF-CD1d complex to the type II NKT TCR are also important for LPC recognition using a CD1d plate-bound Ag presentation assay. Fig. 6A shows that perturbations of the area around the A′ pocket (Phe^{10}Ala, Gly^{155}Trp) of CD1d resulted in the loss of recognition of LPC by the type II NKT Hy19.3. However, substitutions around the F′ pocket (Asp^{153}Y/Leu^{150}Ile, Leu^{84}Phe) did not result in loss of stimulation of the Hy19.3 hybridoma. These residues are involved in the recognition of lipid Ags by the type I NKT TCR (11, 12). These data suggest that residues in the CD1d molecule critically involved in the recognition of LSF are also similar for the recognition of LPC by this type II NKT TCR.

It is clear from Fig. 5 that none of the other nonsulfatide-reactive type II NKT hybridomas examined in this study were reactive to LPC. Thus, we were not able to determine reactivity of

	K_D (μM)
LPC C18:0	3.85 ± 0.15
LPC C16:0	9.75 ± 0.25
LPC C18:1 9z	8.50 ± 0.60
LPC C18:1 1oleyl	7.70 ± 4.30

FIGURE 4. Binding of a type II NKT TCR to mCD1d/LPC complexes. Sensograms showing mCD1d-LPC complexes binding to the Hy19.3 TCR. The association and dissociation constants measured show a slightly higher binding affinity of this ligand for the TCR, compared with mCD1d-LSF complexes. Affinity measurements for the lysophospholipid variants measured for this study. Average and SEM of two independent measurements are reported.
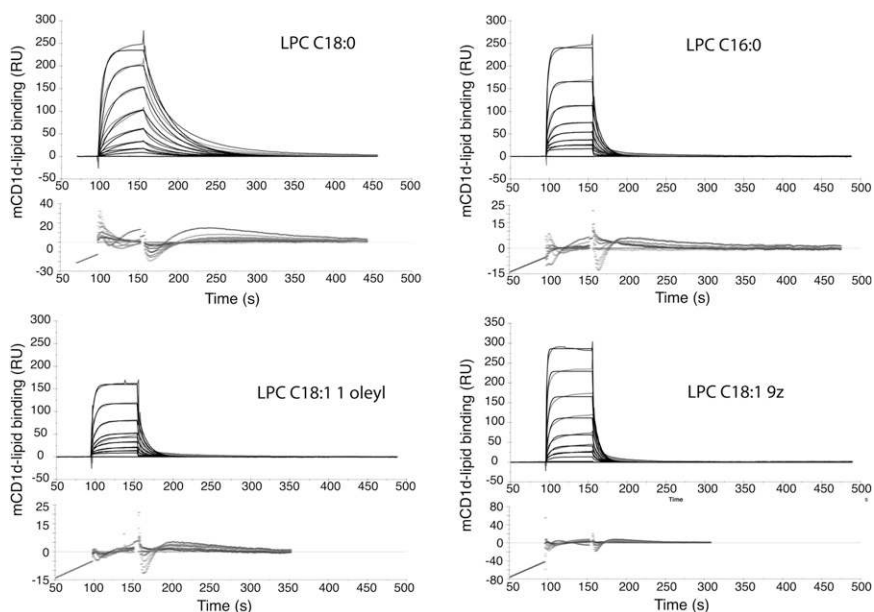
LPC to other type II NKT cells at the monoclonal level. In this work, we determined LPC recognition at the polyclonal level using a plate-bound CD1d Ag presentation assay with polyclonal spleen cells from the WT (CD1d$^{+/+}$) or CD1d-deficient (CD1d$^{-/-}$) BL/6 mice in the presence of plate-bound CD1d molecules (Fig. 6B, WT or mutant CD1d as in Fig. 6A). As shown in Fig. 6B, although the reactivity is low at the polyclonal level using naive spleen cells, the stimulation pattern (stimulation index >2) of LPC with the WT CD1d as well as some of the mutant CD1d molecules was similar to that of the Hy19.3. These data collectively suggest the presence of LPC-reactive type II NKT cells in a polyclonal population with an overlapping repertoire similar to that of sulfatide-reactive type II NKT cells.

Induction of type I NKT anergy following LPC administration

Recently, we have identified a dominant immunoregulatory pathway in which activation of CD1d-restricted type II NKT cells by sulfatide leads to anergy induction in type I NKT cells (17, 18). In this study, we determined whether type I NKT cells are also anergized following activation of type II NKT cells by LPC. Groups of BL/6 mice were administered LPC, and, the following day, anergy induction in type I NKT cells was examined by in vivo expansion of αGalCer/CD1d tetramer$^+$ cells (Fig. 7A, 7B), as well as by proliferation (as measured ^{3}H-thymidine incorporation) in response to an in vitro challenge with αGalCer (Fig. 7C). As shown in Fig. 7A and 7B, type I NKT cells (αGalCer/CD1d tetramer$^+$) expand in spleen following αGalCer administration in PBS/vehicle-treated mice (0.8–4.9%). However, in mice treated with LPC (C16:0 or C18:0) or LSF, the ability of type I NKT cells to expand in vivo in response to αGalCer injection was significantly inhibited (Fig. 7A, 7B). In another assay shown in Fig. 7C,

a significant inhibition of the proliferative response of type I NKT cells was found in splenocytes from LPC- or LSF-treated mice in comparison with that in control or PBS-injected mice. This inhibition of proliferation of type I NKT cells can be reversed by the addition of IL-2 (17, 18) (data not shown). These data suggest that type I NKT cells are anergized following activation of type II NKT cells by LPC in C57BL/6 mice.

LPC administration leads to inhibition of Con A–induced hepatitis

Anergy induction in type I NKT cells following activation of type II NKT cells with sulfatide has been shown recently to limit inflammation-induced damage in liver diseases mediated by these cells (17, 18). In this study, we examined whether LPC-mediated anergy induction in type I NKT cells is able to prevent Con A–induced hepatitis in C57BL/6 mice. As shown in Fig. 8A, treatment of BL/6 mice with LPC C16:0 or LPC C18:0 significantly inhibited liver damage, as determined by the serum ALT levels. Furthermore, H&E staining of liver sections (Fig. 8B) showed that liver damage or necrosis was significantly blunted in BL/6 mice treated with LPC C16:0 compared with those treated with PBS/vehicle in the control group. Because Con A–induced liver injury is mediated by type I NKT cells (27–29), Jα18$^{-/-}$ animals deficient in type I NKT cells or CD1d$^{-/-}$ mice lacking both type I and type II NKT cells are protected. Accordingly, LPC C16:0 administration did not further alter liver damage in Jα18$^{-/-}$ or CD1d$^{-/-}$ mice, as indicated by liver histology (Fig. 8B), as well as by serum levels of ALT (Fig. 8A) and proinflammatory cytokines (Fig. 8C). These data suggest LPC-mediated regulation of type I NKT cells can be used to regulate inflammatory liver diseases.
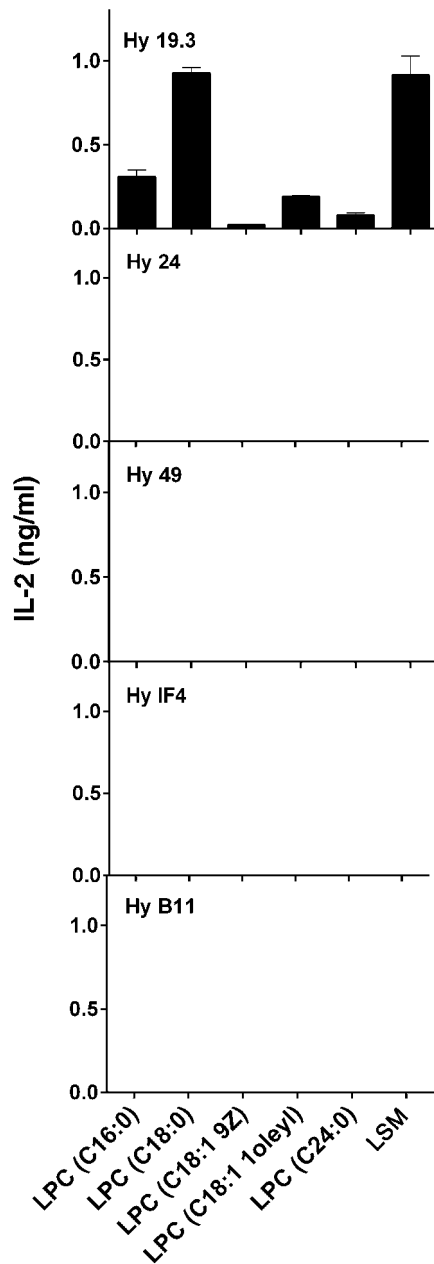
FIGURE 5. Recognition of different isoforms of LPC and LSM by a sulfatide-reactive type II NKT cell, but not other type II NKT hybridomas. A sulfatide-reactive type II NKT Hy19.3 as well as other nonsulfatide-reactive type II NKT hybridomas were used in a typical IL-2 release assay in the presence of APC and indicated lipids. IL-2 release at an optimum concentration (5 μg/ml) of lipids is shown. All other hybridomas showed IL-2 release (ranges from 0.7 to 1.3 ng/ml) in response to the plate-bound anti-CD3 stimulation. These data are representative of two independent experiments.

Discussion

The data presented in this work demonstrate that lysophospholipids are recognized by CD1d-restricted type II NKT cells. Accordingly, a self-glycolipid sulfatide-reactive type II NKT hybridoma is activated by LPC, LPAF, and LSM in a CD1d-dependent manner. The CD1d-bound LPC binds to a type II NKT TCR with affinity similar to that observed for the CD1d-LSF. A similar recognition mode of LPC and LSF is also reflected by the finding that modification of critical residues around the A′ but not the F′ pocket in CD1d has a relatively similar influence on the ability of both lipids to stimulate type II NKT cells at the monoclonal and polyclonal level. Notably, similar to the immunoregulatory pathway follow-
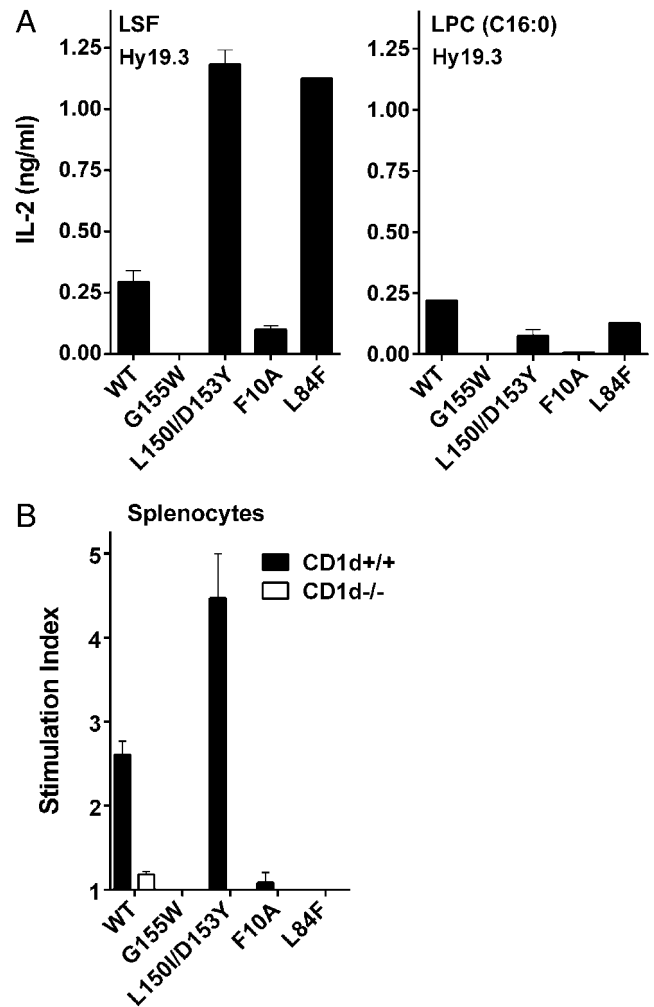

FIGURE 6. Modification of residues in CD1d molecules around the A′ but not F′ pocket inhibits recognition of LPC by a type II NKT cell hybridoma. (**A**) In a CD1d-coated plate assay, Hy19.3 was stimulated in the presence of WT CD1d or mutant CD1d molecules with modifications in residues, as indicated. IL-2 release at an optimum concentration of either 2 μg/ml LSF or 5 μg/ml LPC is shown. These data are representative of three independent experiments. (**B**) A proliferative response of spleen cells from naive WT C57BL/6 (CD1d$^{+/+}$) or CD1d-deficient (CD1d$^{-/-}$) mice in response to a typical CD1d-coated plate assay as above in the presence of an optimum concentration of LPC (5 μg/ml) is shown. Stimulation index was calculated by dividing cpm in the presence versus absence of the lipid. These data are representative of two independent experiments.

ing CD1d-dependent activation of sulfatide-reactive type II NKT cells, LPC administration also leads to anergy induction in type I NKT cells and protection from type I NKT-mediated Con A–induced hepatitis. Collectively, our data suggest that at least a portion of type II NKT cells is reactive to lysophospholipids, and their activation can play an important role in immune regulation.

Lysophospholipids are generated from their respective phospholipids by the action of phospholipases that cleave the acyl-ester bonds at sn-1 and sn-2 positions, leaving only a single fatty acid chain. Recent studies have shown that LPC and LPE are associated with the CD1d molecules (30, 31). Human type II NKT cells from myeloma patients (32) and some of the human type I NKT cell clones can also recognize LPC bound to CD1d (33). Furthermore, LPE generated in hepatocytes following hepatitis B infection can also stimulate type II NKT cells in murine liver (16). Interestingly, although mouse type I NKT cells do not recognize lysophospholipids (16, 34), type I NKT-mediated lipid presentation is com-

FIGURE 7. Induction of anergy in type I NKT cells following LPC administration in C57BL/6 mice. (**A**) Flow cytometric analysis of splenocytes from groups of C57BL/6 mice (n = 3–5) treated i.p. with PBS/vehicle or indicated lipids, LPC (C16:0), LPC (C18:0), or LSF, at 100 µg/mouse. An in vivo expansion of type I NKT cells (αGalCer/CD1d tetramer+) was measured in splenocytes following administration with αGalCer (2 µg) using two-color staining with αGalCer/CD1d tetramer and anti–TCR-β and flow cytometry. Numbers indicate percentage of tetramer+ cells in total spleen lymphocytes. (**B**) A summary of the data from two independent experiments indicating LPC-mediated inhibition of type I NKT cell expansion in BL/6 mice (n = 3–5) is shown. Average percentage of αGalCer/CD1d tetramer-positive cells for each group from (A) (mean ± SEM) is shown. *p < 0.05, **p < 0.01. (**C**) Inhibition of type I NKT cells in response to an in vitro challenge with αGalCer in splenocytes isolated from the groups (n = 3) of control (PBS/vehicle) or different lipid-injected mice. Proliferation following a 90-h culture in the presence of αGalCer among indicated groups is shown as ^{3}H-thymidine incorporation. The frequency of αGalCer tetramer+ cells in the beginning of the culture was similar in all the groups. ***p < 0.001.
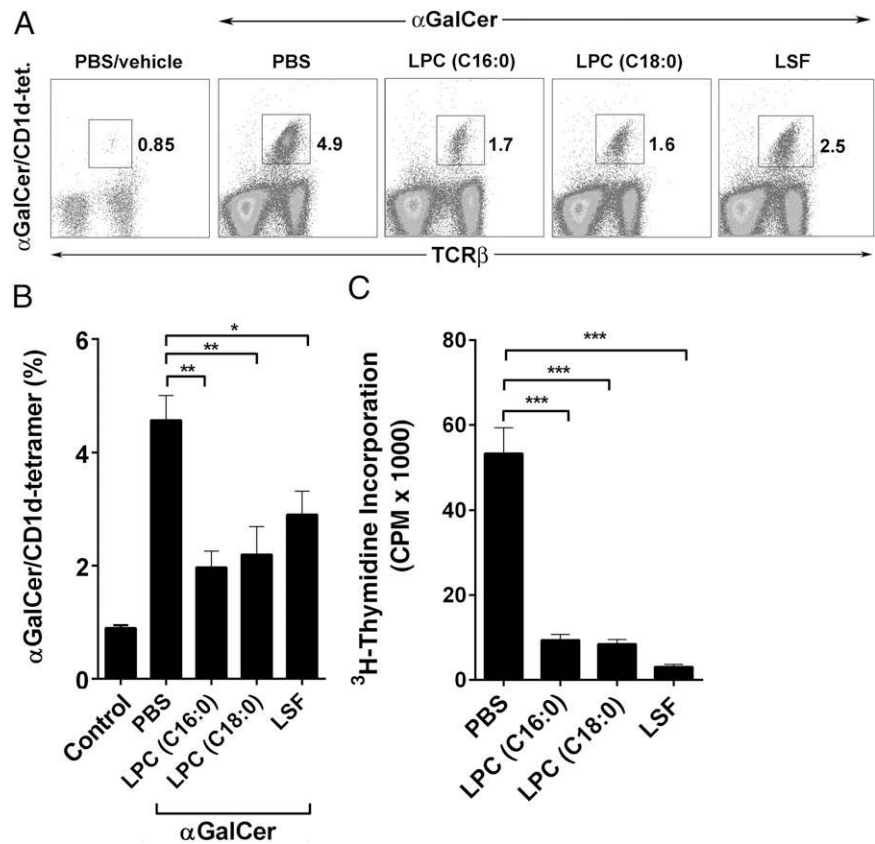
promised in mice deficient in the lysosomal phosphatase A2 (35). These data suggest that lysophospholipids, in addition to being directly recognized by type II NKT cells, may also be involved in Ag loading or CD1d-mediated presentation. Additionally, both microbial and mammalian phospholipids, such as phosphatidyl-glycerol, phosphatidylinositol, and cardiolipin, have been shown to activate murine type II NKT cell hybridomas (36). Thus, both self-lysophospholipids and phospholipids are potential Ags for type II NKT cells.

The levels of self-lysophospholipids, including LPC and lyso-phosphatidic acid (LPA), are altered during inflammation, viral infections, and cancer (26, 37). Additionally, the enzymes that participate in LPC/LPA metabolism play a central role in regulating their levels and form important signaling axes, the phospholipase A(2) superfamily/LPC and autotaxin/LPA for regulating inflammation (26, 37). Accordingly, decreased level of LPC correlates with sepsis-related mortality, and the beneficial effects of synthetic LPC administration in experimental sepsis have recently been shown (38). A similar therapeutic effect of a LPC analog has been shown in the treatment of chronic-relapsing experimental autoimmune encephalomyelitis (EAE) (39). It is interesting that a decrease in serum levels of C16 and C18 isoforms of LPC also has been found in inflammatory liver diseases, including patients following alcoholic consumption, chronic HBV infection, as well as nonalcoholic steatohepatitis (40, 41). It will be important to examine whether LPC-reactive CD1d-restricted T cells shown to be potentially anti-inflammatory in this work (Fig. 8) also play a regulatory role in these inflammatory conditions and whether these cells can be targeted for disease intervention.

Recent insights from the crystal structure of a type II NKT cell TCR that recognizes sulfatide and LSF suggest the presence of a distinct recognition motif for TCR recognition between the type I and type II NKT cell subsets (11, 12). Although the type I NKT TCR binds above the CD1d F′ pocket, the Hy19.3 type II NKT

TCR binds above the A′ pocket. This results in the TCRs approaching the Ag, which is generally bound at the center of the binding groove between the two pockets, from opposite directions. It therefore appears that two different binding strategies evolved to allow recognition of ligands, for example, LPC, by NKT cells (11, 12, 42). In particular, the type II NKT TCR binds sulfatide and LSF exclusively with its β-chain by pinning them against the CD1d surface. This binding mode would allow binding of different lipid moieties, such as LSF/sulfatide and LPC (see Supplemental Fig. 1), while maintaining a conserved TCR-docking footprint on CD1d. It is interesting that, although LSF and isoforms of LPC-CD1d complexes bind to TCR (Fig. 4) with similar binding affinities (∼6 and 3–9 mM, respectively), two of them, LPC [C18:1(9Z)] and LPC [C18:1(1oleyl)], containing unsaturation do not stimulate Hy19.3 effectively (Fig. 5). Further studies are needed to address whether unsaturation may result in differential binding, for example, in A′ pocket and an altered orientation resulting in loss of TCR signaling.

Understanding Ag specificity and TCR repertoire of NKT cell subsets is critical for the manipulation of their function. Although our data suggest some redundancy and overlap in TCR repertoires among type II NKT cells that recognize self-glycolipids as well as self-phospholipids, a detailed structural study of the TCR repertoire from several type II NKT cell clones/hybridomas is needed to further clarify this. It is becoming clear that type I NKT cells can also recognize self-lipids (43–45), and some of these can activate both type I and type II NKT cells. For example, βGluCer as well as LPC and LSM can activate at least a portion of cells in both subsets (43–45). Although only a small number of human type I NKT cell clones can be in vitro stimulated with LPC (33, 42), frequency of LPC-reactive type II NKT cell clones has been shown to be increased significantly in lymphoma patients (32). As shown in this study (Fig. 2) and earlier (11, 24), βGluCer is also recognized by sulfatide-reactive type II NKT hybridomas.

FIGURE 8. Inhibition of Con A–induced hepatitis in mice treated with LPC. (**A**) Serum ALT levels from groups of BL/6, Jα18$^{-/-}$, or CD1d$^{-/-}$ mice immunized i.v. with Con A (10 mg/kg body weight) and treated i.p. with PBS or indicated LPC C:16 (100 μg/ mouse) or naive control are shown. ***$p < 0.001$, ****$p < 0.0001$. (**B**) Inhibition of histological severity of Con A–induced liver damage following treatment with LPC as in (A). Groups of BL/6, Jα18$^{-/-}$, or CD1d$^{-/-}$ mice were sacrificed 24 h after the injection, and livers were removed and fixed in 10% formalin solution until H&E staining. H&E-stained histological sections at original magnification ×100 are shown. (**C**) A fold increase in serum cytokines in Con A or Con A plus LPC-treated BL/6, Jα18$^{-/-}$, or CD1d$^{-/-}$ mice as in (A) is shown. Basal values (pg/ml) for IL-6, IFN-γ, TNF-α, IL-17A, and IL-10 were 11.0, 2.6, 2.9, 2.3, and 2.1, respectively.
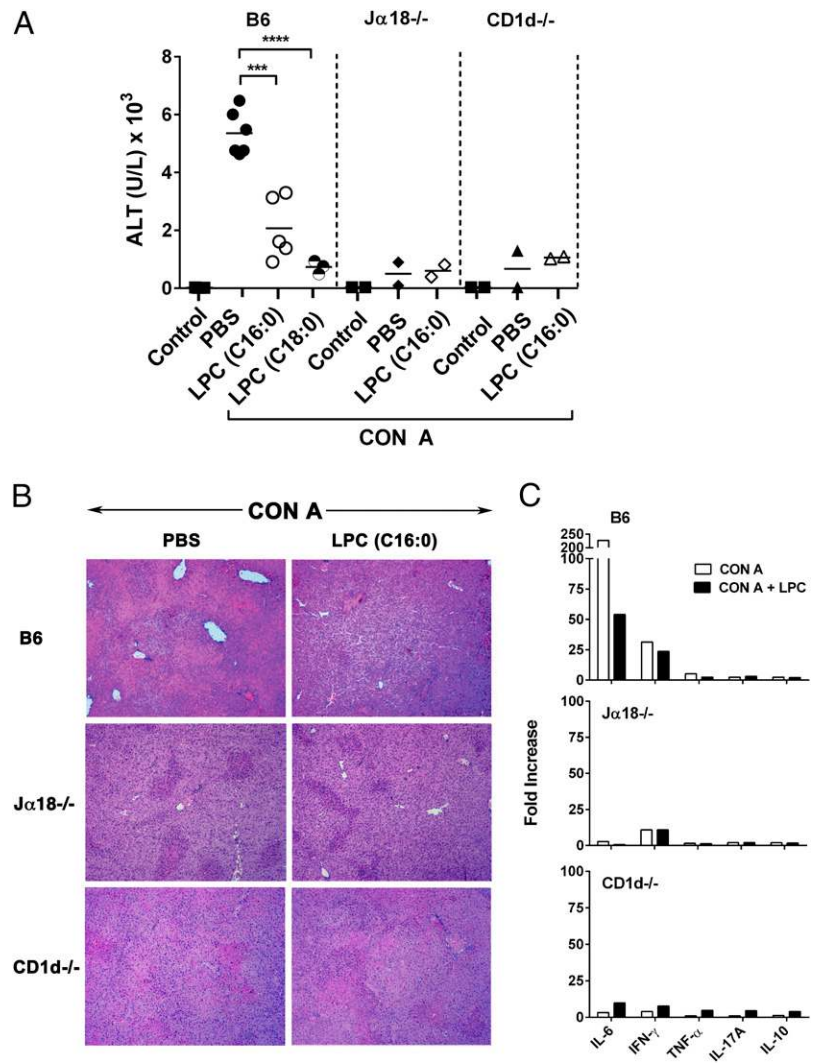
It is important to point out that, whereas some Ags are able to activate both type I and type II NKT cells, the two lineages are not completely redundant as αGalCer, as well as some microbial lipids (46) are not recognized by the type II NKT cells. It is not yet known whether the semi-invariant TCR provides a bias the recognition of microbial Ags by type I NKT cells.

Generally, it seems that self-glycolipids with a long acyl chain are better at activating type II NKT cells (19, 47). For example, sulfatide isoforms with long acyl chains are more efficient in activating type II NKT cells and accordingly in regulating EAE and type 1 diabetes in NOD mice. Thus, C24:1 and C24:0 sulfatides were better than C16:0 or C18:0 isoforms in treating EAE and type 1 diabetes, respectively (19, 47, 48). Interestingly, self-phospholipid LPC C18:0 is better than other isoforms, including LPC C16:0, in activating type II NKT cells (Fig. 5). Surprisingly, LPC C24:0 is less efficient than LPC C18:0 in stimulating the Hy19.3. It is not yet known whether this is due to a higher binding affinity to CD1d or the TCR, or both, or whether C24:0 binds in the A′ pocket. Interestingly, unlike the synthetic C24:1 sulfatide (47), both LSF and LPC (C16:0, C18:0) are inefficient in forming stable CD1d tetramers that do not stain Hy19.3.

It is clear that activation of type II NKT cells with sulfatide controls inflammatory liver or kidney diseases, Ag-induced and spontaneously arising autoimmune diseases, as well as antitumor immunity in a CD1d-dependent manner (4, 6, 7, 49). Mechanisms by which NKT cell subsets modulate immunity depend on their interactions with other immune cells following activation by their respective lipid Ags, for example, αGalCer and sulfatide for type I and type II NKT cell subsets, respectively. It is notable that dendritic cells not only play a crucial role in the activation of NKT cells, but also are central to their role in the regulation of immune responses (6, 49). This type II NKT-mediated immuno-regulatory pathway results in the following: 1) inactivation of type I NKT cells that now function as regulatory T cells; 2) tolerization of conventional dendritic cells; 3) tolerization of microglia in the CNS; and 4) inhibition of the effector functions of pathogenic MHC-restricted CD4$^+$ T cells (6, 10, 17–19, 48). Consistent with this, LPC-mediated activation of type II NKT cells also leads to inactivation of type I NKT cells (Fig. 7) and ultimately to regulation of inflammatory liver disease. Thus, activation of type II NKT cells with sulfatide or LPC appears to follow a similar mechanism of immune regulation. Recently, CpG (50)-, alum (51)-, or IL-25 (52)–activated type II NKT cells also have been shown to play important roles in antitumor or humoral immunity or in regulating inflammation in adipose tissue/obesity. It is becoming clear that activation of type II NKT cells has major consequences in regulating immune responses in a variety of conditions.

The CD1d-dependent Ag recognition pathway is highly conserved from mice to humans, and several key features of NKT cell subsets are shared between them. Interestingly, type II NKT cells occur more frequently than type I NKT cells in humans, thereby facilitating their further characterization using appropriate lipid

ligands such as LPC. A detailed characterization of LPC-reactive type II NKT cells in mice and in humans will have important implications for their appropriate manipulation for intervention in inflammatory diseases, including autoimmunity and cancer.

Acknowledgments

Disclosures

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