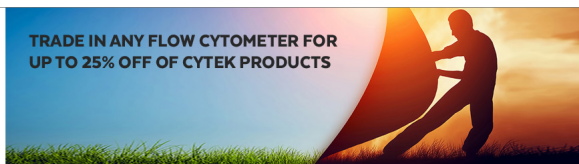




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Autoreactivity to Sulfatide by Human Invariant NKT Cells

Annelein M. Stax,^{*,†} Jessica Tuengel,^{*,†} Enrico Girardi,[‡] Naoki Kitano,^{*,†} Lenka L. Allan,^{*,†} Victor Liu,^{*,†} Dongjun Zheng,^{*,†} William J. Panenka,[§] Joren Guillaume,[¶] Chi-Huey Wong,^{||} Serge van Calenbergh,[¶] Dirk M. Zajonc,^{‡,#} and Peter van den Elzen^{*,†}

Invariant NKT (iNKT) cells are innate-like lymphocytes that recognize lipid Ags presented by CD1d. The prototypical Ag, α -galactosylceramide, strongly activates human and mouse iNKT cells, leading to the assumption that iNKT cell physiology in human and mouse is similar. In this article, we report the surprising finding that human, but not mouse, iNKT cells directly recognize myelin-derived sulfatide presented by CD1d. We propose that sulfatide is recognized only by human iNKT cells because of the unique positioning of the 3-*O*-sulfated β -galactose headgroup. Surface plasmon resonance shows that the affinity of human CD1d-sulfatide for the iNKT cell receptor is relatively low compared with CD1d- α -galactosylceramide (K_D of 19–26 μ M versus 1 μ M). Apolipoprotein E isolated from human cerebrospinal fluid carries sulfatide that can be captured by APCs and presented by CD1d to iNKT cells. APCs from patients with metachromatic leukodystrophy, who accumulate sulfatides due to a deficiency in arylsulfatase-A, directly activate iNKT cells. Thus, we have identified sulfatide as a self-lipid recognized by human iNKT cells and propose that sulfatide recognition by innate T cells may be an important pathologic feature of neuroinflammatory disease and that sulfatide in APCs may contribute to the endogenous pathway of iNKT cell activation. *The Journal of Immunology*, 2017, 199: 97–106.

Lipid Ags are recognized by T cells when presented by the MHC class I-like molecule CD1. Among the CD1-restricted T cells, invariant NKT (iNKT) cells are the most well characterized, having innate-like features and the ability to influence multiple arms of the immune response (1). iNKT cells are defined by their expression of a conserved TCR α -chain (V α 14 in mice and V α 24 in human) and their recognition of the CD1d-presented glycosphingolipid, α -galactosylceramide (α GalCer), originally derived from a marine sponge. These innate-like T cells can be activated exclusively through TCR stimulation by strong glycolipid Ags, via a combination of a weak TCR stimulus plus innate signals, or exclusively by innate signals, in the case of human CMV infection. Although endogenous self-lipids were originally believed to mediate their activation, several exogenous microbial Ags have now been identified, including α GalCer-like compounds

derived from pathogens and, more recently, α GalCer itself from a prominent member of the gut microbiota (2–5). Together, these studies illustrate the requirement of Ag presentation by CD1d and TCR signaling for iNKT cell activation. Significant homology exists between mouse CD1d (mCD1d) and human CD1d (hCD1d) and between their invariant TCRs. The conserved recognition of α GalCer by mouse and human iNKT cells has led to the presumption that the same natural lipid Ags are recognized by both species' iNKT cells, although there are notable exceptions (6–12).

Beyond iNKT cells, more diverse T cell populations recognizing CD1-bound lipids exist, in humans restricted by CD1a, CD1b, CD1c, and CD1d. In mice, where only CD1d is expressed, iNKT cells (also termed type I NKT cells) are a dominant component of the CD1-restricted T cell repertoire, whereas diverse CD1d-restricted T (also known as type II NKT [dNKT]) cells recognize Ags distinct from α GalCer (13, 14). Sulfatide, one of the most abundant lipids in the myelin sheath, has been shown to be a CD1d-presented Ag for mouse and human type II NKT cells (15–21), whereas humans also harbor sulfatide-specific CD1a-, CD1b-, and CD1c-restricted $\alpha\beta$ T cells (22), as well as CD1d-restricted $\gamma\delta$ T cells (23, 24).

In this study, we made the surprising finding that human, but not mouse, iNKT cells recognize myelin-derived sulfatide and cerebrospinal fluid–derived apolipoprotein E (apoE)-bound sulfatide in the context of CD1d, as well as endogenous sulfatide derived from APCs. This represents a significant divergence between mouse and human iNKT cell recognition of self-lipids that has many implications for innate T cell biology.

Materials and Methods

Cells and reagents

K562 cells transfected with CD1d (and controls) were a kind gift from Dr. De Jong (Harvard Medical School), and RMAS cells transfected with murine CD1d were a gift from Dr. Behar (Harvard Medical School). Murine iNKT cell hybridomas 24.7 and DN32 were kindly provided by Dr. Gumperz and Dr. Bendelac, and the sulfatide-reactive dNKT cells XV19 were kindly provided by Dr. Cardell. The human iNKT cell clone BM2a.3 and NKT cell line M0 were used as described previously (25, 26). The iNKT cell clones J3N.5, J3N.1, and GG1.2 were kind gifts from

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Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; apoE, apolipoprotein E; DC, dendritic cell; dNKT, diverse CD1d-restricted T (type II NKT); α GalCer, α -galactosylceramide; hCD1d, human CD1d; iNKT, invariant NKT; LCL, lymphoblastoid cell line; mCD1d, mouse CD1d; MLD, metachromatic leukodystrophy; MS, multiple sclerosis; Tg, transgenic; TLC, thin-layer chromatography.

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Dr. Gumperz, and the human iNKT cell lines/clones NN1 and NN2 were kindly provided by Dr. Lavoie (University of British Columbia). The CD1b-restricted T cell clone DS1C9b was kindly provided by Dr. DeLibero. SH and SL lines were generated in the van den Elzen laboratory by expanding circulating iNKT cells with sulfatide and selecting these with α GalCer-loaded tetramer. Lymphoblastoid cell lines (LCLs) from meta-chromatic leukodystrophy (MLD) or healthy controls were obtained from the NIGMS Coriell Cell Repositories.

α GalCer (kindly provided by Dr. Ovaa), GalGalCer (kindly provided by Dr. Ilarionov), and synthetic sulfatides C26:0 and C24:1 were obtained from C.-H.W. (27) and lysosulfatide was prepared by S.v.C. Cerebroside and mixed gangliosides were from Matreya; mixed sulfatide was from Sigma or Matreya; CD1b blocking Ab (clone BCD1b3.1) and CD1d blocking Ab (clone 24.1.1.1) were from Dr. Brenner; and mouse IgG₁ was from AbLab.

Lipid isolation from myelin

Whole myelin was prepared from bovine brain by homogenization in 0.32 M sucrose, followed by ultracentrifugation in a discontinuous 0.32/0.88 M sucrose gradient (28). Myelin lipids were extracted in chloroform/methanol (3:4 v/v) and further fractionated using Supelco Supelclean LC-NH2 (Sigma) and C18 (Waters) columns. Fractions were spotted on TLC silica gel plates (Merck) and run in chloroform/methanol/0.25% KCl (50:40:10 v/v/v). Glycolipids were visualized with orcinol (0.1%) staining.

T cells assay

APCs were cultured overnight in 96-well round-bottom plates with NKT cells, as described previously (16). Where indicated, serum-free medium (AIM-V; Invitrogen) was used. Human IFN- γ or mouse IL-2 was measured in supernatant by ELISA.

Cell free plate assay

hCD1d-Fc β 2-microglobulin single-chain protein was generated as described previously (29). Briefly, CD1d-Fc (0.5 μ g/ml) and anti-LFA-1 (50 ng/ml; Serotec) were coated on 96-well flat-bottom plates, pulsed with sonicated lipids, and cultured with BM2a.3 (50,000 cells per well) overnight.

Flow cytometry

PBMCs were isolated from buffy coats, as previously described (30). PBMCs were stimulated with α GalCer (1 μ g/ml) or mixed sulfatide (10 μ g/ml), and splenocytes from V α 14-transgenic (Tg) mice (kindly provided by Dr. Brenner and Dr. Brigl, with permission of Dr. Bandelac) were stimulated with α GalCer (200 ng/ml) or mixed sulfatide (10 μ g/ml). On days 1, 2, and 4, cells were stained with α GalCer-loaded CD1d tetramer (National Institutes of Health), CD3, 7-aminoactinomycin D (7AAD; eBioscience), CD19, or B220 and CD69 (BD Pharmingen). 7AAD[−]CD19[−]CD3⁺CD1d tet⁺ cells were analyzed for CD69 expression levels using an LSR II flow cytometer (BD).

Generation of monocyte-derived dendritic cells

Cells were positively selected from PBMCs using CD14 MACS MicroBeads (Miltenyi Biotec) and cultured with GM-CSF (100 ng/ml; GenScript), IL-4 (50 ng/ml; eBioscience), and human serum for 4 d.

Hydrolysis of sulfatide

The sulfate group of sulfatide was hydrolyzed as described previously (22). Briefly, mixed sulfatide or GalGalCer was treated with 1 ml of 0.05 M HCl diluted in methanol for 16 h.

apoE isolation from cerebrospinal fluid and thin-layer chromatography separation of apoE-bound lipids

apoE was isolated from human cerebrospinal fluid using an immunoaffinity column, as previously described (31). Briefly, 4 ml of pooled cerebrospinal fluid was concentrated to 1 ml using Amicon filters (Millipore) and loaded on the immunoaffinity column. PBS and sodium thiocyanate were used to elute apoE-negative and apoE-positive fractions, respectively. Collections were washed with PBS. Lipids extracted from apoE-positive and apoE-negative fractions were separated on thin-layer chromatography (TLC) plates. Sulfatide was visualized by immunostaining using the ABC kit (Vector Laboratories) and O4 mAb (Millipore), as described previously (32).

Protein expression and purification

The fully glycosylated mouse and human CD1d/ β 2-microglobulin heterodimers were expressed and purified as described previously for mCD1d

(16). Human V α 24V β 11 iNKT TCR (CDR3 β : CASSDPNEQFF, cDNA kindly provided by Dr. Kronenberg) with a C-terminal birA-tag sequence (LHHILDAQKMVWNHR) on the V β 11 chain was cloned in pET22b (V α 24 chain) and pET30a (V β 11 chain) *Escherichia coli* expression vectors. An interchain disulfide bond was introduced by mutating to cysteine Thr⁴⁸ (TRBC 48) on the C α domain and Ser⁵⁷ on the C β domain. An additional cysteine residue (Cys⁷⁵) on the C β domain was mutated to serine to avoid improper oligomerization. The two chains were expressed, refolded, and purified with the method reported for the mouse V α 14V β 8.2 iNKT TCR (33). Purified human TCR was biotinylated with a commercially available kit (Avidity).

Glycolipid loading

A 3-fold molar excess of synthetic sulfatide (compound #7, dissolved in DMSO) was incubated overnight with hCD1d in the presence of 100 mM Tris-Cl (pH 7). Excess lipid was removed by size-exclusion chromatography on a Superdex 200 10/300 column (GE Healthcare) equilibrated in HBS buffer (10 mM HEPES, 150 mM NaCl, 3.0 mM EDTA [pH 7.4]).

Surface plasmon resonance

Surface plasmon resonance studies using a refolded and biotinylated V α 24V β 11 TCR were carried out as previously reported (33) by immobilizing 500–700 RU of biotinylated human TCR or mCD1d (as a negative control) on a CAPture chip (GE Healthcare). Serial dilutions of hCD1d-sulfatide in HBS buffer were injected in increasing concentrations (0.39–100 μ M). Experiments were carried out at 25°C with a flow rate of 20 μ l/min and were performed at least two times. Kinetic parameters were calculated using a simple Langmuir 1:1 model in BIAevaluation software version 4.1.

Model of hCD1d-sulfatide-iNKT TCR complex

A dictionary file for C24:1 sulfatide was generated with the Dundee PRODRG server (34). The hCD1d-sulfatide complex was generated by automatic docking in AutoDock Vina (35) using the mCD1d-sulfatide structure (PDB 2AKR) as a positive control and reference structure. The ternary complex was manually modeled in Coot (36) by superimposing the hCD1d-sulfatide structure onto the hCD1d- α GalCer-TCR structure (PDB 2PO6). The sulfo-galactosyl headgroup was further manually reoriented to prevent clashing with the CDR1 α loop of the TCR, while maximizing potential TCR contacts. Figures were prepared with PyMOL (Schrödinger, New York, NY).

Results

Myelin extracts and sulfatides activate human iNKT cells

We tested the hypothesis that the lipid-rich myelin sheath, which is the target of autoimmunity in multiple sclerosis (MS), may contain Ags recognized by iNKT cells. Lipid extracts of bovine myelin were fractionated by aminopropyl chromatography to separate the lipids based on their hydrophobicity and charge. Of the six fractions generated, the most polar fraction (fraction 6) demonstrated activity for human iNKT cells (Fig. 1A, 1B). Fraction 6 consists mainly of charged lipids, including sulfatides and gangliosides. No detectable activity was found in the other fractions, including fraction 4, where neutral glycosphingolipids, such as galactosylceramides, would elute. We then assessed the response of iNKT cells to purified commercial preparations of sulfatides, gangliosides, and galactocerebrosides and found that only sulfatides elicited responses from iNKT cells (Fig. 1C). To establish that activation was due to sulfatide and not a contaminant in these mixtures, we tested synthetic sulfatides, including C26:0 sulfatide and C24:1 sulfatide (27), as well as lysosulfatide, which was independently synthesized for this study (Fig. 1D). These synthetic sulfatides all induced activation of human iNKT cells, consistently showing the order of potency of lysosulfatide > C26:0 sulfatide > C24:1 sulfatide.

Human iNKT cells directly recognize sulfatide bound to CD1d

Dendritic cells (DCs) pulsed with mixed sulfatides were able to stimulate various V α 24⁺ NKT cells, including multiple monoclonal and oligoclonal lines (BM2a.3, J3N.5, J3N.1, GG1.2, M0,

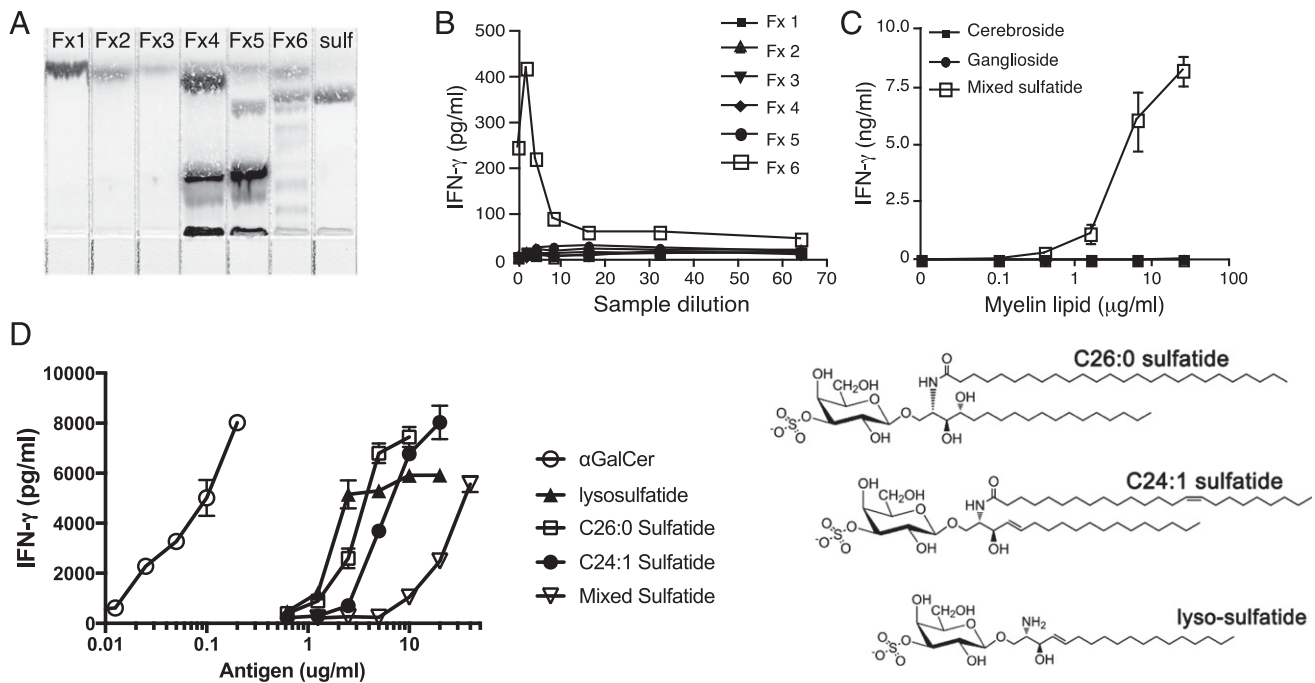


FIGURE 1. Myelin extracts and sulfatides activate human iNKT cells. **(A)** Myelin lipids were isolated and extracted from bovine brain, separated using aminopropyl and C18 columns, and visualized on a TLC plate stained with orcinol, showing increasingly polar fractions (Fx) and mixed sulfatide (sulf). **(B)** Fx were tested for reactivity by coculturing with CD1d-transfected K562 cells and the iNKT cell clone BM2a.3, and reactivity was measured by IFN- γ release using ELISA. **(C)** Cerebroside, ganglioside, or mixed sulfatide was cultured with CD1d-transfected K562 cells and BM2a.3 cells. **(D)** Synthetic sulfatides [C24:1 and C26:0 sulfatide from (27)] and lysosulfatide (from S.v.C.) were incubated with CD1d-transfected K562 cells and cultured overnight with BM2a.3.

NN1, NN2) generated in our laboratories (25, 26) (Fig. 2A, data not shown). To confirm that sulfatides activate iNKT cells in a CD1d-dependent manner, we tested the ability of DCs to stimulate iNKT cells in the presence of anti-CD1d blocking Abs. CD1d blockade abolished iNKT cell activation compared with the control anti-CD1b Ab, with the latter only inhibiting activation of CD1b-restricted T cells DS1C9b (Fig. 2A). In addition, activation was seen in the presence of CD1d-transfected, but not untransfected, K562 cells used as APCs (Fig. 2B).

To show that iNKT cells are activated by intact sulfatide bound to CD1d, we used an APC-free system consisting of plate-bound CD1d, loaded with C26:0 sulfatide or α GalCer. Sulfatide elicited significant iNKT cell activation (Fig. 2C), albeit with ~ 10 -fold less potency than for α GalCer. To further confirm that sulfatide was being directly recognized and that the sulfate group was necessary for recognition of the Ag, we pretreated the Ag with acid, which cleaves the sulfate group from sulfatide, yielding β -galactosylceramide (37). Acid treatment completely abolished the reactivity of sulfatide but had no effect on α GalCer or GalGalCer used as controls (Fig. 2D). Importantly, this finding rules out the possibility that small amounts of α GalCer-like contaminants may account for the activity we see in our various sulfatide preparations [as was previously seen in other contexts (38, 39)] because these compounds are not acid labile.

Sulfatide is not cross-presented to murine iNKT cells

It is also known that α GalCer strongly activates mouse iNKT cells and that mouse and human CD1d can cross-present α GalCer to the other species' invariant TCR. Thus, we assessed whether sulfatides could similarly be cross-presented (Fig. 3). First, we confirmed previous results (15, 40) (E. Girardi, P. van den Elzen, and

D.M. Zajonc, unpublished observations) showing that sulfatide did not stimulate murine iNKT cells using V α -14 TCR-Tg mice. These mice express an invariant TCR α -chain with variable β -chains, thus representing a wide range of possible iNKT TCR rearrangements; however, no reactivity was detected compared with the positive controls α GalCer and PHA (Fig. 3A). We then tested the mouse hybridomas XV19 (type II, sulfatide reactive) and 24.7 (type I, α GalCer reactive) for reactivity using murine bone marrow-derived DCs and found that sulfatide stimulated only XV19, and α GalCer stimulated only 24.7 (Fig. 3B). We further tested two mouse iNKT cell hybridomas (24.7 and DN32) for their ability to respond to mouse or human APCs (RMAS-mCD1d or K562-hCD1d) in the presence of α GalCer or sulfatide and reciprocally tested two human iNKT cell lines (BM2a.3 and M0) with the same APCs (Fig. 3C). α GalCer could be cross-presented by mouse or human APCs to mouse or human iNKT cells, whereas sulfatide could only be presented by hCD1d to human iNKT cells. Thus, sulfatide is uniquely recognized by the human invariant TCR in the context of hCD1d. Importantly, we can also conclude that the antigenic activity that we observe is unlikely to be due to a small amount of α GalCer-like contamination, because these products would be cross-presented like α GalCer.

Peripheral blood iNKT cells recognize sulfatide

We next wished to determine what proportion of iNKT cells from healthy donors recognize sulfatide ex vivo. Thus, we cultured PBMCs in the presence of mixed sulfatides or α GalCer as a control. As expected, iNKT cells from all donors showed a robust response to α GalCer compared with the medium control (Fig. 4B, 4D). Most α GalCer-CD1d tetramer-positive cells disappeared as a result of TCR downregulation (data not shown). Sulfatide also stimulated CD69 expression on tetramer-positive cells in most

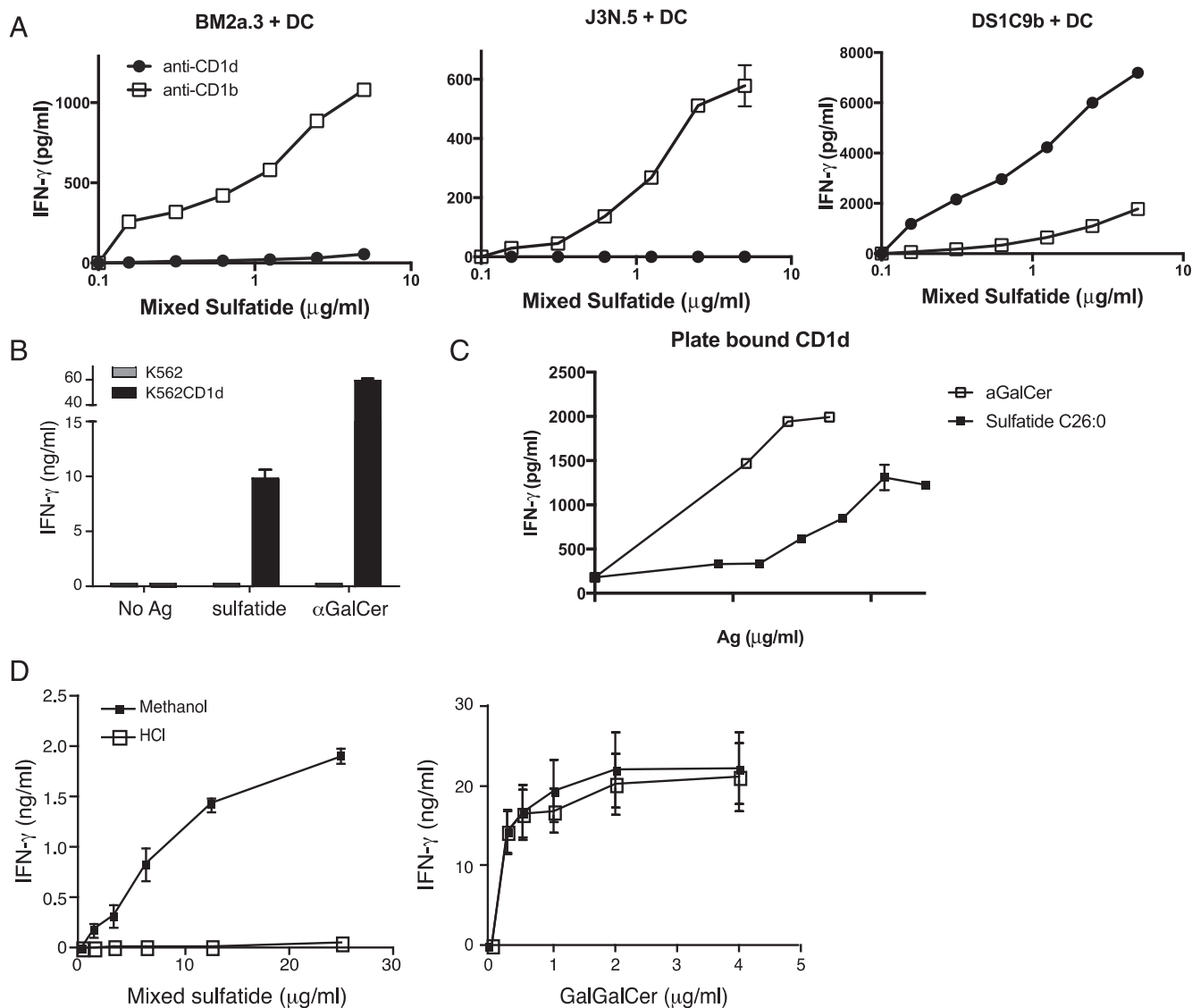


FIGURE 2. (A) Mixed sulfatides were cultured with DCs and two iNKT cell clones (BM2a.3, J3N.5) or CD1b-restricted T cells as a control (DS1C9b) in the presence of blocking CD1b or CD1d Abs (10 μ g/ml). (B) K562 or K562-CD1d cells were cultured with BM2a.3 and medium, 10 μ g/ml mixed sulfatide, or α GalCer. (C) Plate-bound CD1d-Fc loaded with synthetic sulfatide (C26:0) or α GalCer were cultured with BM2a.3, and IFN- γ production was measured in the supernatant by ELISA. Data in (A)–(C) show the mean \pm SEM of one representative experiment of at least three independent experiments. (D) Sulfatide was hydrolyzed using 0.05 M HCl in methanol or methanol only as a control (left). The unsulfated GalGalCer was used as a control (right). After treatment, lipids were cultured with K562-CD1d and iNKT cells (BM2a.3), and IFN- γ was measured in the supernatant by ELISA. Similar results were obtained for α GalCer. Complete hydrolysis was confirmed by visualizing a shift in mobility on a TLC plate (data not shown). Data show the mean \pm SEM of three independent experiments.

samples (five of six healthy donors tested), albeit with slower dynamics and a lower proportion of cells than for α GalCer (Fig. 4C versus media control Fig. 4D). We did not observe TCR downregulation with sulfatide stimulation (data not shown). There was considerable donor-to-donor variability, with some not showing CD69 upregulation until day 4 (e.g., donor 4) and some showing stimulation at day 2, even at lower doses of sulfatide (100 or 1000 ng/ml; donors 2, 5, and 6, Fig. 4E). Thus, a sizeable proportion of human peripheral blood iNKT cells recognize sulfatide in most individuals.

Interaction of hCD1d-sulfatide with the invariant TCR

Next, we measured the strength of interaction between immobilized invariant TCRs and soluble CD1d-sulfatide using surface plasmon resonance (Fig. 5A). The iNKT TCR bound to CD1d-sulfatide complexes with an equilibrium dissociation binding

constant (K_{Deq}) of 25.9 ± 1.6 μ M, in good agreement with the value obtained by binding kinetics (18.8 ± 1.8 μ M). TCR binding is characterized by an association rate of $2.08 \pm 0.03 \times 10^3$ $M^{-1} s^{-1}$ and a dissociation rate of 0.04 ± 0.003 s^{-1} . Compared with a TCR-binding affinity ~ 1 μ M for α GalCer-CD1d (41), the binding affinity for sulfatide is ~ 20 -fold weaker; however, overall, the affinity is similar to conventional TCR-peptide-MHC interactions (42).

One of the important differences that may account for the ability of human, but not mouse, CD1d-bound sulfatide to interact with the iNKT TCR is the presence of the side chain of Trp¹⁵³ (43). It has previously been demonstrated that the species-specific CD1d residue Trp¹⁵³ (equivalent to Gly¹⁵⁵ in mCD1d) affects recognition of several microbial Ags, without affecting cross-presentation of α GalCer (33). To address this point, we modeled the interaction of sulfatide in the hCD1d-TCR complex. Two possible

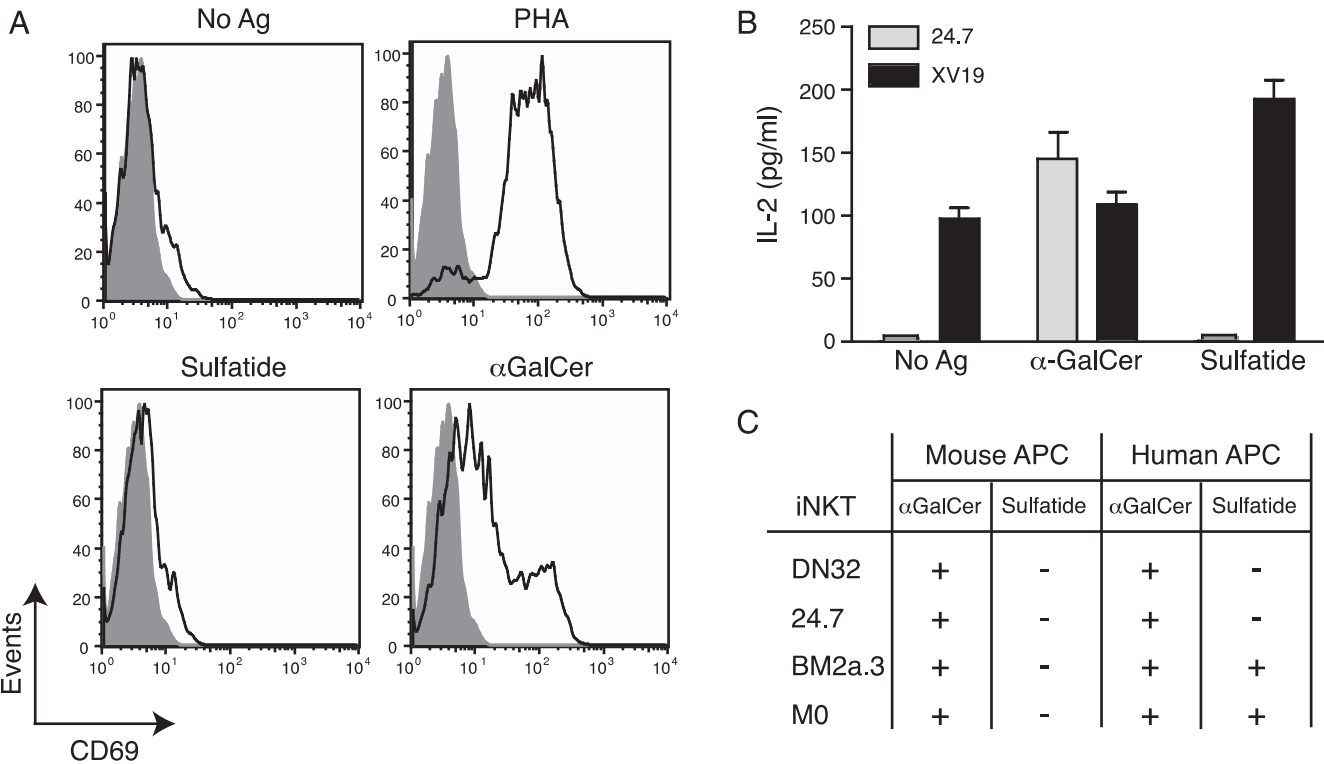


FIGURE 3. Sulfatide activates human, but not mouse, iNKT cells. **(A)** Splenocytes from Vα14-Tg mice were stimulated with sulfatide (10 μg/ml), αGalCer (200 ng/ml), or PHA. CD69 expression levels were measured on αGalCer-CD1d tetramer+ iNKT cells. Data are representative of three independent experiments. **(B)** Murine iNKT (24.7) and dNKT (XV19) hybridomas were cultured with αGalCer (100 ng/ml) or with sulfatide (5 μg/ml) pulsed bone marrow–derived DCs. IL-2 production was measured in the supernatant by ELISA. Data shown are the mean ± SEM of one experiment representative of three independent experiments. **(C)** Mouse (DN32 and 24.7) or human (BM2a.3 and M0) iNKT cells were cultured with K562-hCD1d (human APCs) or RMA5-mCD1d (mouse APCs) and αGalCer (1 μg/ml) or sulfatide (6 μg/ml). Mouse IL-2 or human IFN-γ production by iNKT was measured in the supernatant by ELISA, as determined in independent experiments performed a minimum of three times. The plus sign (+) indicates statistically significant responses ($p < 0.01$, ANOVA).

orientations of the sugar moiety were modeled (Fig. 5B, 5C). The sulfate group and the sugar moiety are involved in polar contacts with CD1d and the TCR. The importance of the interaction between the sulfate group and the TCR was demonstrated by the loss of activity of desulfated sulfatide compared with control sulfatide (Fig. 2D). Our model shows that the sulfate and the sugar moiety are bound between Trp¹⁵³ and the CDR1α (Ser³⁰) and CDR3α loops of the TCR. Because of the absence of Trp¹⁵³ in mouse (Gly¹⁵⁵ in mouse), a large conformational change is required to force the sulfatide molecule in the conformation recognized by the mouse TCR (Asn³⁰), which would come at a high energetic cost. Therefore, the species-specific CD1d residue Trp¹⁵³ may provide one possible rationale for the lack of observed reactivity of the mouse iNKT TCR for sulfatide. However, there may also be differences within the TCR that prevent mouse iNKT cells from recognizing human or mouse CD1d with sulfatide.

Cerebrospinal fluid apoE carries sulfatide that is recognized by iNKT cells

In addition to its abundant presence in the myelin sheath, sulfatide has been shown to be carried by the lipid transporter (apoE) in cerebrospinal fluid (30). Because apoE is the predominant lipid carrier in the CNS, and because we have previously demonstrated the delivery of lipid Ags by apoE (31), we isolated apoE from pooled human cerebrospinal fluid using an immunoaffinity column. The apoE-positive fraction stimulated iNKT cells, and this activity was blocked by anti-CD1d Abs (Fig. 6A, 6B). Extracts of the apoE-positive and apoE-negative fractions were

analyzed by immuno-TLC using an anti-sulfatide Ab (O4). The apoE-positive fraction contained readily detectable sulfatide compared with the apoE-negative fraction (Fig. 6C). The TLC band corresponding to sulfatide was then scraped, and the lipids were extracted and used to activate iNKT cells (Fig. 6D). Thus, in addition to recognition of bovine myelin-derived sulfatide, human iNKT cells recognize sulfatide derived from a human source, cerebrospinal fluid–derived apoE, which can then be acquired by APCs and presented to iNKT cells in the context of CD1d.

Endogenous sulfatide recognition by iNKT cells in metachromatic leukodystrophic APCs

Because iNKT cells are often activated by APCs presenting lysosomally derived self-glycosphingolipids, we wished to determine whether endogenously produced sulfatide could activate iNKT cells. We obtained EBV-immortalized LCLs from patients with MLD, whose cells accumulate sulfatide (and lysosulfatide) as the result of an enzyme defect in arylsulfatase A (44, 45). We previously demonstrated that LCLs are effective APCs for iNKT cells when treated with retinoic acid analogs (e.g., AM580, all-*trans* retinoic acid), which reverses the downregulation of CD1d associated with EBV infection (46). LCLs from MLD patients potentially activated iNKT cells compared with healthy control LCLs in the absence of added Ag (Fig. 6E, open bars). CD1d levels were upregulated to a similar degree in both cell lines (data not shown), and both could activate iNKT cells with αGalCer to a similar extent (Fig. 6E, filled bars). Thus, endogenous, lysosomally derived sulfatide is capable

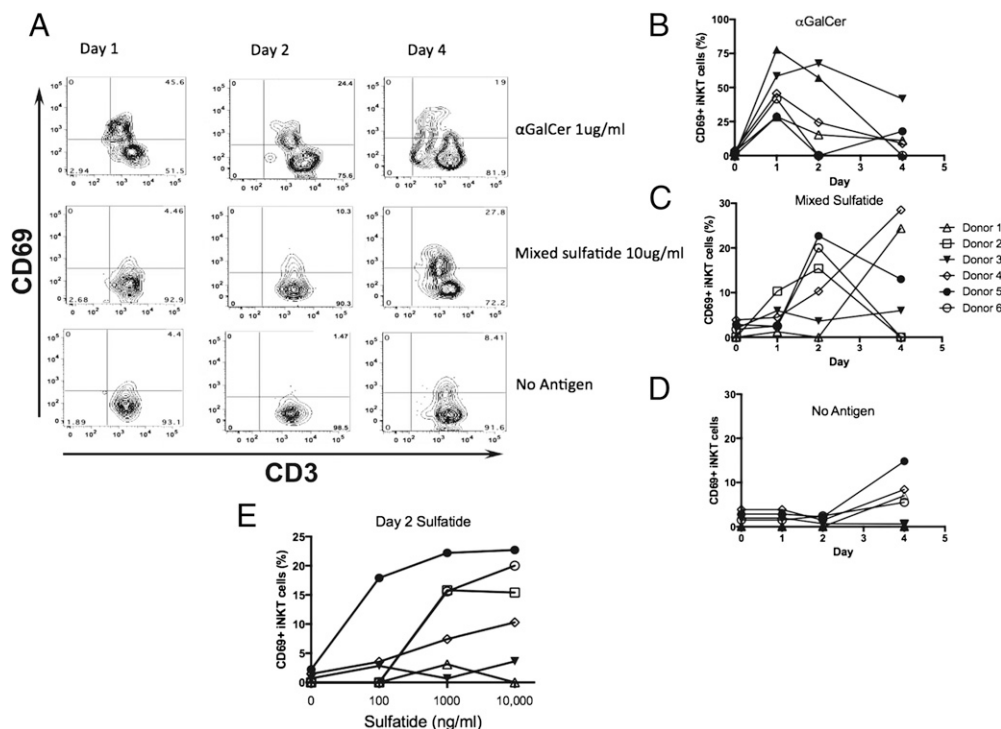


FIGURE 4. Sulfatide activation of peripheral blood iNKT cells. PBMCs were stimulated by α GalCer (1 μ g/ml) or mixed sulfatide (10 μ g/ml). On days 1, 2, and 4, cells were harvested, and CD19⁺7AAD⁺CD1d⁺CD3⁺ cells were analyzed for CD69 expression levels by flow cytometry. **(A)** Representative flow cytometry plots of one donor (donor 4). A summary of six donors is shown for α GalCer (1 μ g/ml) **(B)**, mixed sulfatide (10 μ g/ml) **(C)**, and no Ag **(D)**. **(E)** Dose-response curve for sulfatide stimulation on day 2.

of activating iNKT cells and may contribute to the innate activation of iNKT cells.

Discussion

We have made the unexpected finding that human, but not mouse, iNKT cells recognize sulfatide presented by CD1d. The results are unexpected because mouse and human iNKT cells generally have conserved recognition of Ags (e.g., α GalCer, iGb3), whereas sulfatide has been shown to be recognized by distinct (noninvariant) populations of human CD1-restricted T cells and mCD1d-restricted (type II) NKT cells (15–17, 22). Also, it has been recently shown that the majority of T cells stained by hCD1d-sulfatide tetramers were $\gamma\delta$ T cells (23), as well as that human iNKT cells failed to be stained under standard conditions using sulfatide-CD1d tetramers (47). The estimated affinities of the $\gamma\delta$ TCR with the sulfatide/CD1d complex were 5–9 μ M (24), significantly higher than our estimation for the invariant TCR-sulfatide/CD1d interaction (19–26 μ M). This may account for the lack of staining of iNKT cells with sulfatide-loaded CD1d (47) (data not shown), although poor loading of sulfatide into tetramers may also be a factor. Similar affinities occur (as do difficulties with tetramer staining) with many MHC-peptide-TCR interactions, which, nonetheless, have clear physiologic relevance.

Another concern that we considered in our studies was whether the activity we saw for iNKT cells was due to small amounts of α GalCer-like contaminants in the natural extracts of myelin, the commercial products, or the synthetic compounds. This was especially a concern in light of recent reports of β -glucosylceramides being capable of stimulating iNKT cells, whose activity was subsequently reported to be due to contamination with α -anomers (38, 39, 48). We feel that this is highly unlikely for our studies. First, in our tests on myelin fractions separated by aminopropyl chromatography, α GalCer-like compounds and most

other known iNKT cell Ags would have eluted in fraction 4, where neutral glycosphingolipids elute. iNKT activity eluted only with charged lipids corresponding to sulfatides and gangliosides (fraction 6; Fig. 1A, 1B). Second, we demonstrate that the antigenic activity of mixed sulfatides for iNKT cells is completely abolished by acid treatment, which hydrolyzes the sulfate group from sulfatide. Acid treatment does not affect α GalCer or GalGalCer, known iNKT cell Ags and, thus, would presumably not have affected our sample's activity if it had been due to such contaminants. Further, if sulfated α GalCer had been present, the acid treatment would have yielded α GalCer, which of course would have retained its activity. Third, we demonstrate that our sulfatide mixture is unable to be cross-presented by mCD1d to human iNKT cells or indeed to stimulate mouse iNKT cells by human or mouse CD1d. However, α GalCer is capable of being cross-presented in these situations (Fig. 3C) and so too would any small amount of contaminant having similarly potent activation. Thus, we conclude that sulfatides, and not α GalCer-like contaminants, are stimulating human iNKT cells in our studies.

The recognition of sulfatide by the invariant TCR is unique to the human proteins. Because hCD1d uses Trp¹⁵³ to elevate the galactose headgroup of α -anomeric Ags and present them in a rather diagonal orientation to the human TCR, recognition of β -anomeric Ags is energetically more favorable than in the mouse, because they require less molding by the TCR. In mCD1d, Gly¹⁵⁵ leaves any α -anomeric Ag completely flat, and β -anomeric Ags would have to undergo an energetic penalty to be molded into that position for recognition, whereas the human TCR would only have to mold into a diagonal orientation. The orientation of the sulfate in our model cannot be uniquely identified. Both orientations depicted (Fig. 5B, 5C) would allow the sulfate group to form polar interactions with the TCR. The finding that lyso-sulfatide is a stronger Ag for human iNKT cells can be explained by the apparent flexibility of this Ag as a result of the loss of the second

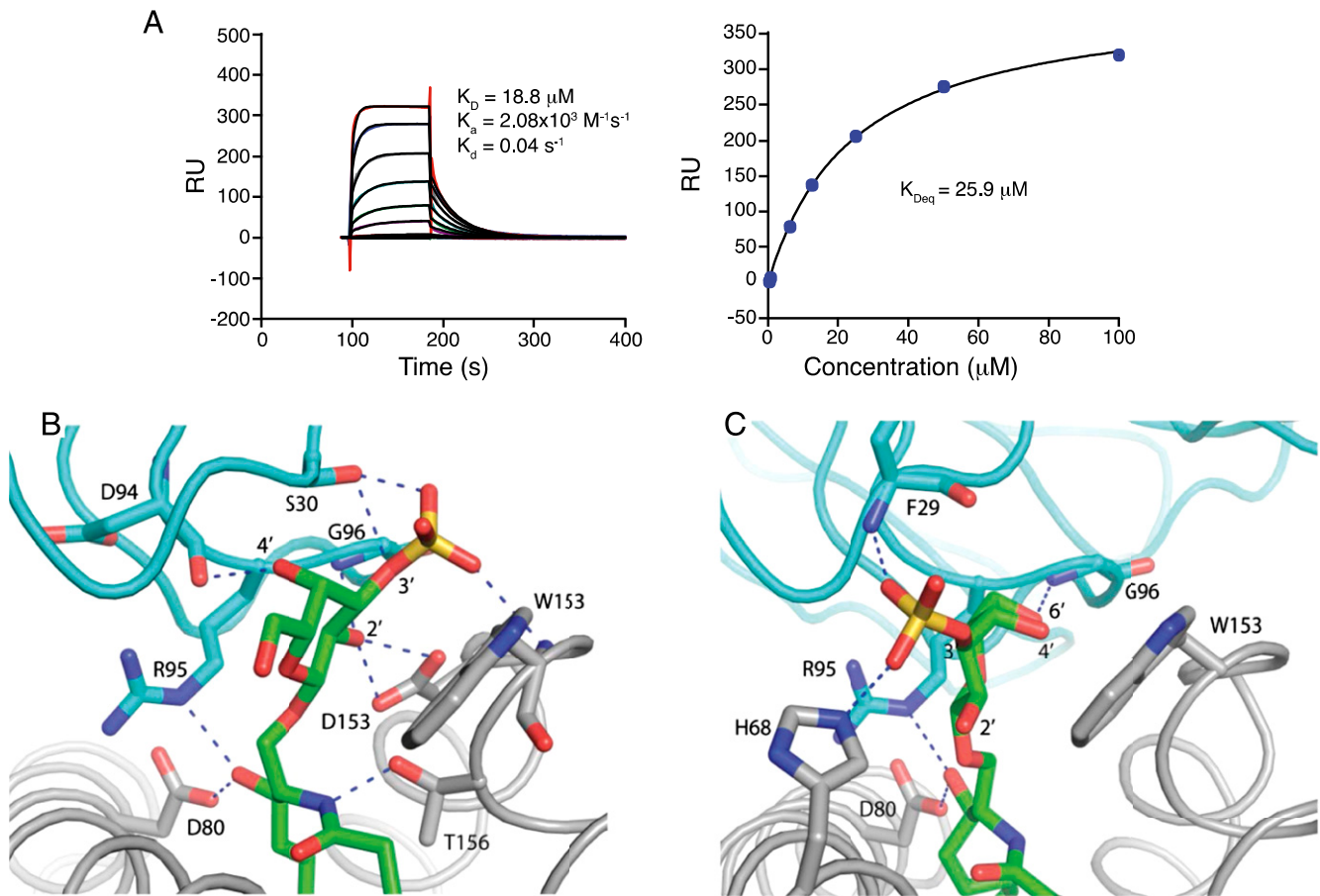


FIGURE 5. (A) Binding response of hCD1d-sulfatide complexes to immobilized human iNKT TCR (left panel). Steady-state analysis of the binding affinity of the hCD1d-sulfatide-TCR interaction (right panel). (B and C) Two possible models of the hCD1d-sulfatide-TCR interaction. (B) Sulfatide is bound between the side chain of Trp¹⁵³ of CD1d and the CDR1 α and CDR3 α loops of the iNKT TCR, with the sulfate group projecting above the $\alpha 2$ helix of CD1d. (C) An alternate model in which the galactose is rotated 180°. The sulfate group is now pointing toward the $\alpha 1$ helix to make contact with hCD1d and the CDR1 α loop. The relative distance between Trp¹⁵³ and the opposing residue on the TCR CDR1 α (Ser³⁰ in human, Asn³⁰ in mouse) remains constant in the two species because of the different length of the TCR residue side chain (Ser being shorter than Asn). This, in turn, results in an ideal binding site for the sugar moiety present on glycolipid Ags for which ligand specificity is determined by contacts between the ligand and both CD1d and TCR residues. Furthermore, the presence of the bulky side chain of Trp¹⁵³ in the human protein results in an upright tilt of the bound Ags, compared with mouse (16).

acyl chain. Any structural rearrangement within the Ag requires less energy by the human TCR when using a single alkyl chain Ag.

Sulfatide recognition by human iNKT cells has important implications for neuroinflammatory conditions. Sulfatide is the second most abundant lipid species in the myelin sheath, and, as we and other investigators have shown, it is carried by the lipid carrier apoE in the cerebrospinal fluid (Fig. 6A–D) (30). We have also previously demonstrated that apoE enhances the antigenicity of CD1-presented lipid Ags (31). Any disorder in which blood–brain barrier disruption would allow exposure of apoE-sulfatide to iNKT cells may be impacted by this recognition. In MS, there are several reports of perturbed iNKT cell numbers and/or function (49–54), consistent with the possibility that they are activated in vivo by sulfatide. Beyond MS, other neurologic conditions characterized by inflammation and involving apoE and/or sulfatide, including Alzheimer's disease (55) and traumatic brain injury (56, 57), may be affected by iNKT activation.

We also propose that iNKT cell recognition of sulfatide may occur more broadly in physiological and pathophysiological conditions. Sulfatide has been reported in the insulin-secreting granules of pancreatic β cells (58), suggesting a mechanistic link for the influence that iNKT cells have on autoimmune diabetes (59). Of broader relevance, TLR or bacterial stimulation of human DCs has been shown to induce endogenous sulfatide

synthesis leading to the activation of diverse CD1a-, CD1b-, and CD1c-restricted clones (60). We have now shown that B cell-derived LCLs from MLD patients can similarly present endogenously produced sulfatide. Because one of the hallmarks of iNKT function is the innate self-recognition of endogenous lipids presented by CD1d on APCs (6, 60, 61), sulfatide may play a role in the physiological activation of iNKT cells, either peripherally or in thymic selection.

The unique recognition of sulfatide by human, but not mouse, iNKT cells is a significant divergence between mouse and human innate T cell biology. This divergence will necessitate a reassessment of the role of iNKT cells in a number of diseases for which mouse models have served as a major basis for insight into human immunopathology, including experimental autoimmune encephalomyelitis for MS, NOD mice for type 1 diabetes, and murine models of Alzheimer's or traumatic brain injury. It is of interest to note that the phenotype of ARSA-deficient mice is considerably milder than seen in ARSA-deficient humans with MLD, with considerably less demyelination and inflammation than the human counterpart (62, 63). Whether the difference between mouse and human is due to the ability of human iNKT cells to recognize the accumulated sulfatide is a question that demands attention.

In conclusion, we have shown that sulfatide from myelin or from cerebrospinal fluid apoE readily activates human, but not

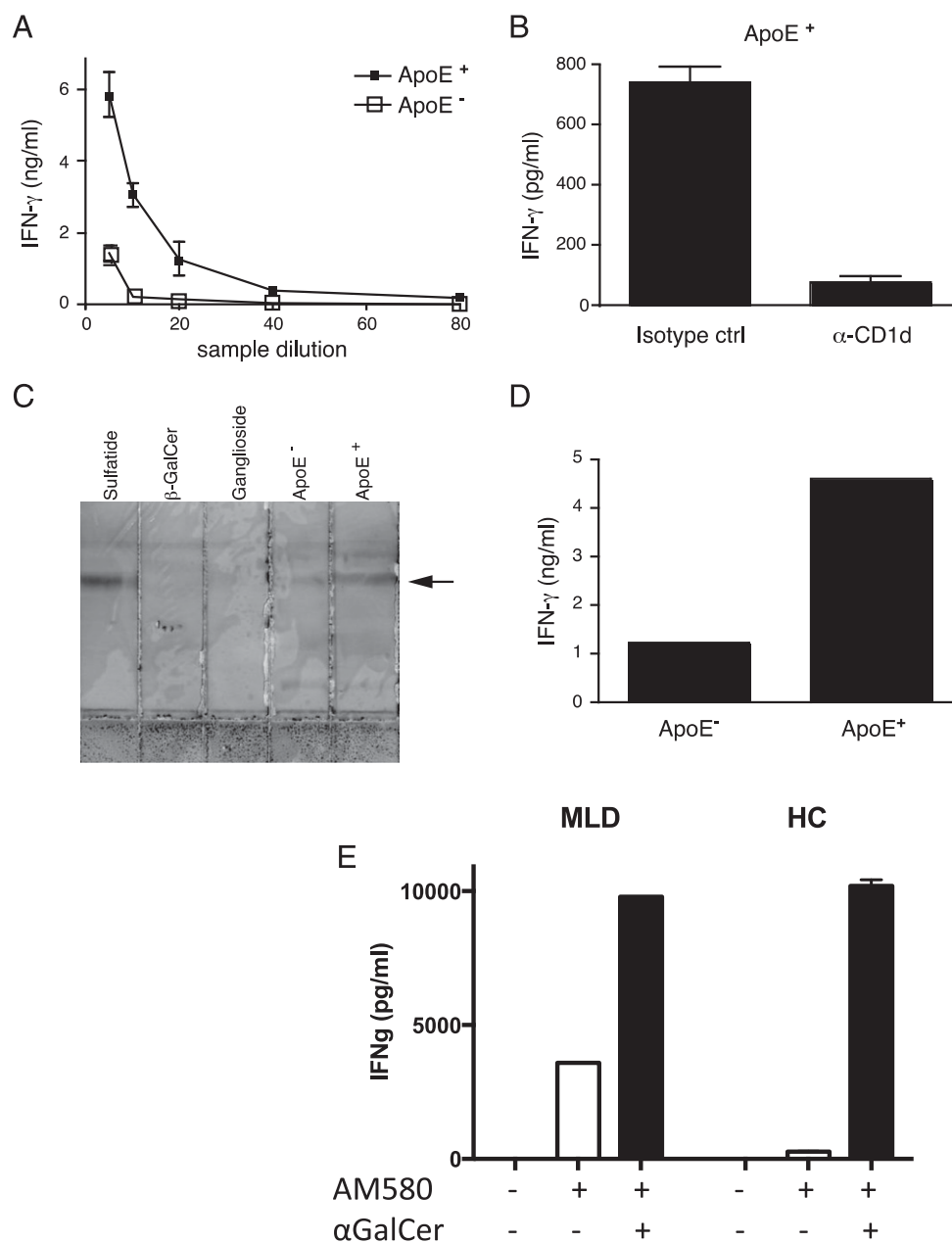


FIGURE 6. Human cerebrospinal fluid contains apoE-bound sulfatide, which is recognized by iNKT cells. **(A)** apoE-positive (eluted) and apoE-negative (unbound) fractions isolated from cerebrospinal fluid were cultured with K562-CD1d and M0. **(B)** apoE-positive fraction cultured with blocking CD1d or isotype control Abs (10 μ g/ml). **(C)** Lipids from apoE-positive and apoE-negative fractions and controls were separated by TLC and visualized using the O4 Ab. Bands indicated by the arrow were scraped. **(D)** Extracted lipids from the scraped bands were cultured with K562-CD1d and BM2a.3. IFN- γ production was measured in the supernatant by ELISA in these experiments. **(E)** EBV-immortalized B cells (LCLs) were treated with the retinoic acid analog AM580 where indicated for 48 h, as described (46, 64), and CD1d upregulation was confirmed by FACS (data not shown). α GalCer (100 ng/ml) was added where indicated, all samples were incubated with iNKT cells (clone BM2a.3) overnight, and IFN- γ production was measured in the supernatant by ELISA. All data are representative of at least three independent experiments. HC, healthy controls.

mouse, iNKT cells. The recognition of a myelin lipid Ag by this ubiquitous, innate-like lymphocyte has direct implications for diseases such as MS, in which myelin is the target of autoimmunity, and for other neurologic conditions in which inflammation or blood-brain barrier disruption would allow exposure of apoE-bound sulfatide to iNKT cells. The broader role of sulfatide in iNKT cell physiology remains to be determined.

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Disclosures

The authors have no financial conflicts of interest.

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