

Recognition of microbial and mammalian phospholipid antigens by NKT cells with diverse TCRs

Raju V. V. Tatituri^a, Gerald F. M. Watts^a, Veemal Bhowruth^b, Nathaniel Barton^c, Alissa Rothchild^{a,e}, Fong-Fu Hsu^f, Catarina F. Almeida^g, Liam R. Cox^d, Lothar Eggeling^h, Susanna Cardelliⁱ, Jamie Rossjohn^j, Dale I. Godfrey^g, Samuel M. Behar^a, Gurdyl S. Besra^{b,1}, Michael B. Brenner^{a,1}, and Manfred Brigl^{c,1}

^aDivision of Rheumatology, Immunology and Allergy and ^cDepartment of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115; Schools of ^bBiosciences and ^dChemistry, University of Birmingham, Birmingham B15 2TT, United Kingdom; ^eDivision of Medical Sciences, Harvard Medical School, Boston, MA 02115; ^fDivision of Endocrinology, Metabolism and Lipid Research, Washington University, St. Louis, MO 63110; ^gDepartment of Microbiology and Immunology, University of Melbourne, Parkville, VIC 3010, Australia; ^hInstitut für Bio- und Geowissenschaften, D-52425 Jülich, Germany; ⁱDepartment of Microbiology and Immunology, Institute of Biomedicine, University of Gothenburg, S-405 30 Gothenburg, Sweden; and ^jDepartment of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, Clayton, VIC 3800, Australia

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CD1d-restricted natural killer T (NKT) cells include two major subgroups. The most widely studied are V α 14J α 18⁺ invariant NKT (iNKT) cells that recognize the prototypical α -galactosylceramide antigen, whereas the other major group uses diverse T-cell receptor (TCR) α - and β -chains, does not recognize α -galactosylceramide, and is referred to as diverse NKT (dNKT) cells. dNKT cells play important roles during infection and autoimmunity, but the antigens they recognize remain poorly understood. Here, we identified phosphatidylglycerol (PG), diphosphatidylglycerol (DPG, or cardiolipin), and phosphatidylinositol from *Mycobacterium tuberculosis* or *Corynebacterium glutamicum* as microbial antigens that stimulated various dNKT, but not iNKT, hybridomas. dNKT hybridomas showed distinct reactivities for diverse antigens. Stimulation of dNKT hybridomas by microbial PG was independent of Toll-like receptor-mediated signaling by antigen-presenting cells and required lipid uptake and/or processing. Furthermore, microbial PG bound to CD1d molecules and plate-bound PG/CD1d complexes stimulated dNKT hybridomas, indicating direct recognition by the dNKT cell TCR. Interestingly, despite structural differences in acyl chain composition between microbial and mammalian PG and DPG, lipids from both sources stimulated dNKT hybridomas, suggesting that presentation of microbial lipids and enhanced availability of stimulatory self-lipids may both contribute to dNKT cell activation during infection.

type II natural killer T cell | CD1 antigen presentation | innate immunity | adaptive immunity

Natural killer T (NKT) cells recognize lipid antigens presented by CD1d (1, 2). Two major subsets of NKT cells have been described in both mice and humans based on the utilization of T-cell receptor (TCR)- α and β genes: the widely studied type I or invariant NKT (iNKT) cells express a semi-invariant V α 14J α 18 TCR α -chain in mice and V α 24J α 18 in humans paired with a limited set of β -chains (V β 7, V β 8, and V β 2 in mice and V β 11 in humans). iNKT cells can recognize the prototypical α -galactosylceramide (α -GalCer) antigen; several glycosylated microbial ceramides and diacylglycerol lipids; and the self-antigens β -glucosylceramide (β -GluCer), isoglobotrihexosylceramide, phospholipids, and lysophospholipids (3). iNKT cells display an effector/memory phenotype and rapidly secrete large amounts of cytokines following primary stimulation. In contrast, CD1d-restricted NKT cells that use diverse TCR α - and β -chains have also been identified, although recurrent use of certain germ-line segments has been observed (4–8). These NKT cells are referred to as type II or diverse NKT (dNKT) cells.

The TCR diversity of dNKT cells suggests antigen specificities that are distinct from those of iNKT cells. Indeed, dNKT cells do not recognize α -GalCer or any of the α -glycan-linked microbial lipid antigens that are recognized by iNKT cells, and the self-lipid sulfatide, which is recognized by a small subset of mouse dNKT cells, is not recognized by iNKT cells (9, 10). Furthermore,

the self-antigen β -GluCer, which stimulates most or all human and mouse iNKT cells, stimulates only a small subset of mouse dNKT cells (11, 12). A number of studies have documented, or inferred, a function for dNKT cells during infection (13, 14), yet the antigens that stimulate dNKT cells during infection remain unknown. Studies using transgenic or sulfatide-specific dNKT cells revealed that a substantial fraction of dNKT cells have a naïve phenotype (CD44^{lo}, CD62L^{hi}, CD69^{neg/lo}) and express chemokine receptors, integrins and effector molecules distinct from iNKT cells (7, 15, 16). Thus, utilization of diverse TCRs by dNKT cells correlates with distinct antigen specificity, phenotype, and functional attributes compared with iNKT cells.

In humans, CD1a-, b-, and c-reactive T cells use diverse $\alpha\beta$ TCRs. The pattern of intracellular trafficking and localization of human CD1b is strikingly similar to that of mouse CD1d (17). Human CD1b is known to present lipid antigens from *Mycobacterium tuberculosis* (*Mtb*), and CD1b-restricted T cells expand following *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) immunization and *Mtb* infection in humans (14). Furthermore, immunization with *Mtb* lipids protected guinea pigs that harbor CD1b and CD1b-restricted T cells from subsequent *Mtb* infection (18). Based on the similarities between CD1b and CD1d and the utilization of diverse TCRs by both human CD1b-restricted T cells and mouse dNKT cells, we hypothesized that dNKT cells may also recognize mycobacterial lipid antigens.

Here, we found that *Mtb*-infected antigen-presenting cells (APCs) and several mycobacterial phospholipids stimulated dNKT hybridomas. dNKT hybridomas displayed distinct, albeit partially overlapping, antigen specificities for different lipid antigens. Interestingly, the mammalian counterparts of stimulatory microbial phospholipids, although composed of structurally distinct acyl chains, were also stimulatory for dNKT hybridomas, suggesting that the foreign-lipid reactive repertoire of dNKT cells is shaped by cross-reactivity with self-lipid antigens.

Results

***Mtb* Lipids Stimulate dNKT Cells.** To investigate whether CD1d-restricted dNKT cells recognize microbial lipid antigens, we studied eight dNKT cell hybridomas that were previously identified based on their recognition of CD1d expressing APCs and the use of V α 14-negative TCRs. Of the eight hybridomas, the XV19 and 14S.15 hybridomas recognize sulfatide and the XV19 and VII68

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¹To whom correspondence may be addressed. E-mail: mbrigl@rics.bwh.harvard.edu, mbrenner@rics.bwh.harvard.edu, or g.besra@bham.ac.uk.

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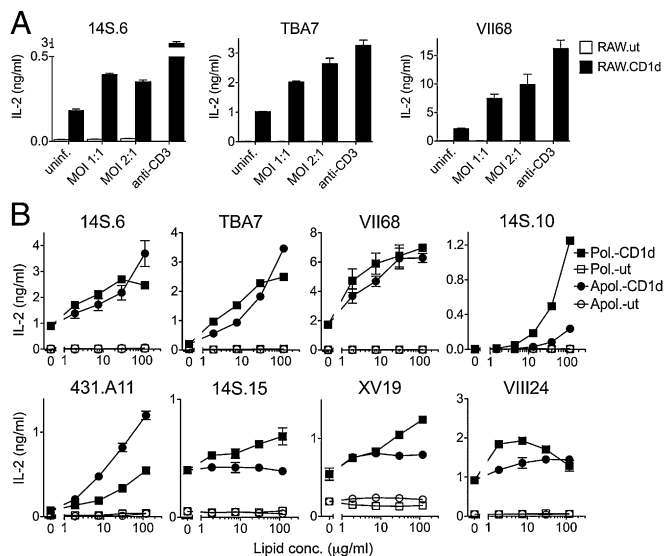


Fig. 1. *Mtb* infected APCs and *Mtb* lipids stimulate dNKT hybridomas. (A) CD1d-transfected or untransfected RAW cells were infected with virulent *Mtb* H37Rv and cocultured with dNKT hybridomas. (B) *Mtb* polar or apolar lipids stimulate dNKT hybridomas in the presence of CD1d-transfected or untransfected RAW cells. IL-2 in culture supernatants was measured by ELISA. Data show mean \pm SEM and are representative of two independent experiments.

hybridomas recognize β -GluCer. Seven of the hybridomas use diverse $V\alpha$ and $J\alpha$ TCR gene segments, whereas the 431.A11 hybridoma uses a recurrent $V\alpha 3.2/J\alpha 9^+$ TCR. To investigate whether the dNKT cell hybridomas are activated by *Mtb*-infected APCs, we used an in vitro infection system with dNKT hybridomas and a CD1d-transfected macrophage cell line (RAW cells). After incubation with RAW cells infected with virulent *Mtb* H37Rv dNKT hybridomas produced IL-2 in a CD1d-dependent manner (Fig. 1A).

We next tested whether lipids isolated from *Mtb* bacteria were stimulatory for dNKT hybridomas. Lyophilized *Mtb* was extracted in different organic solvents to obtain lipid fractions extracted in apolar solvents (petroleum ether) or polar solvents (chloroform/methanol). All of the dNKT hybridomas tested were stimulated in cocultures with RAW.CD1d cells in the presence of either polar or apolar *Mtb* lipids (Fig. 1B). Four out of eight dNKT hybridomas (14S.10, 14S.15, XV19, VIII24) secreted more IL-2 in response to polar *Mtb* lipids compared with apolar *Mtb* lipids, whereas three hybridomas (14S.6, TBA7, VII68) showed comparable responses. Interestingly, the 431.A11 hybridoma responded more strongly to apolar than to polar *Mtb* lipids. In contrast, iNKT hybridomas, characterized by expression of the semi-invariant $V\alpha 14J\alpha 18^+$ TCR, were not stimulated in the presence of *Mtb* lipids but secreted IL-2 following stimulation with α -GalCer (Fig. S1A and B). Thus, dNKT hybridomas were stimulated by *Mtb*-infected APCs or *Mtb* lipids in a CD1d-dependent manner.

Next, to identify the *Mtb* lipid(s) that stimulated dNKT hybridomas, we performed extensive lipid separations and purifications and tested the purified lipids in our dNKT hybridoma coculture assay. To track the biological activity of the purified lipids, we used the 14S.10 dNKT hybridoma that displayed very low autoreactivity to CD1d-transfected RAW cells and preferentially recognized *Mtb* polar lipids (Fig. 1B). First, we separated *Mtb* polar lipids into 10 fractions (semipreps 1–10) using ion-exchange chromatography with diethylaminoethyl (DEAE) cellulose columns and salt gradient elution. Stimulation of the 14S.10 hybridoma with *Mtb* semipreps 1–10 in the presence of RAW.CD1d cells resulted in strong IL-2 secretion only with semiprep 4, whereas most other fractions did not simulate or only weakly simulated (Fig. 2A). To isolate the stimulatory lipid(s) from the mixture of lipids contained in semiprep 4, we further separated

the lipids by preparative TLC into nine fractions (Fig. S2A). Stimulatory activity for the 14S.10 hybridoma was detected in fraction 4.3 (Fig. S2B). Based on the detection of multiple lipids in fraction 4.3 by one-dimensional (1D) and 2D TLC analysis (Fig. S2A and C), we next separated 10 different lipids from fraction 4.3 by preparative 2D TLC (Fig. S2C). Stimulatory activity for the 14S.10 hybridoma was detected in fraction 4.3.2 (Fig. 2B). Phosphate staining was positive and α -naphthol staining was negative for lipid 4.3.2, indicating the presence of a phosphate moiety and the absence of carbohydrate moieties, respectively (Fig. S2C). Analysis of the highly purified 4.3.2 lipid by electrospray ionization–MS revealed dominant $[M-H]^-$ ions with a mass/charge (m/z) ratio of 763.5, 761.5, 747.5, and 745.5 (Fig. 2C) and was consistent with the lipid being phosphatidylglycerol (PG; Fig. 2D). Additional fatty acid analysis by tandem MS of the purified lipid confirmed the presence of molecular PG species containing C19:0/C16:0, C19:0/C16:1, C18:1/C16:0, and C18:1/C16:1 acyl chain combinations (Fig. 2C). Thus, our isolation and purification revealed *Mtb* PG to stimulate a dNKT hybridoma in a CD1d-dependent manner.

***Corynebacterium glutamicum* Lipids Stimulate dNKT Cells.** Because of the difficulty of generating large quantities of highly pure lipids from virulent *Mtb*, we established a system using *Corynebacterium glutamicum* (*Cg*) for the purification of large amounts of stimulatory mycobacterial lipids. *Cg* expresses a lipid profile that is similar, but less complex, than that of *Mtb* and is widely used to investigate mycobacterial lipid synthesis pathways. Following purification of *Cg* polar or apolar lipids, dNKT hybridomas were tested, and, similar to the responses observed with *Mtb* lipids, all hybridomas tested secreted IL-2 in response to *Cg* lipids in our in vitro RAW cell coculture assay (Fig. 3A). Seven of the hybridomas showed stronger responses to *Cg* polar lipids than to *Cg* apolar lipids, and similar to the responses seen with *Mtb* lipids, the 431.A11 hybridoma responded stronger to apolar than to polar *Cg* lipids. iNKT hybridomas tested did not respond to *Cg* lipids (Fig. S3A). Next, we separated and purified 15 individual polar *Cg* lipids by preparative 2D TLC (Fig. S3B). *Cg* lipids p6

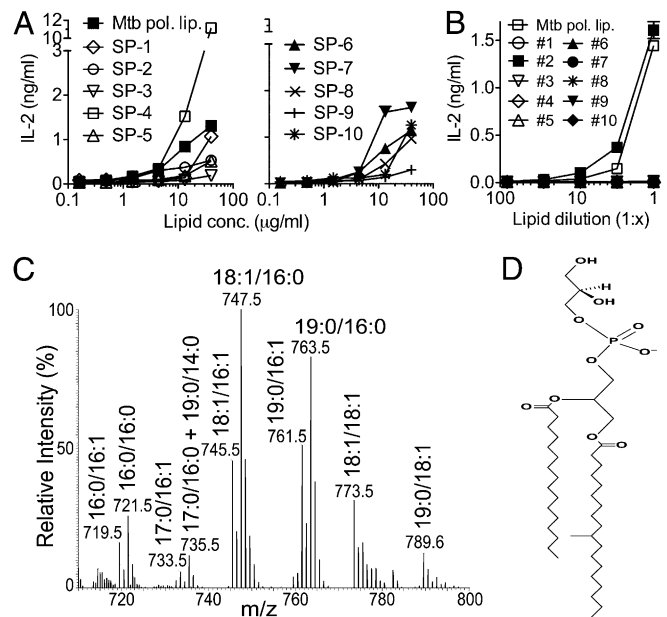


Fig. 2. Isolation of stimulatory *Mtb* lipids. (A) Using DEAE cellulose columns, *Mtb* polar lipids were separated into 10 semipreps (SP) that were tested for stimulation of 14S.10 cells. (B) Lipids from fraction 4.3 were further separated by preparative 1D and 2D TLC, resulting in the stimulatory 4.3.2 *Mtb* lipid. (C and D) MS analysis revealed lipid 4.3.2 (C) to be PG with various molecular species (D). Data are representative of two independent experiments.

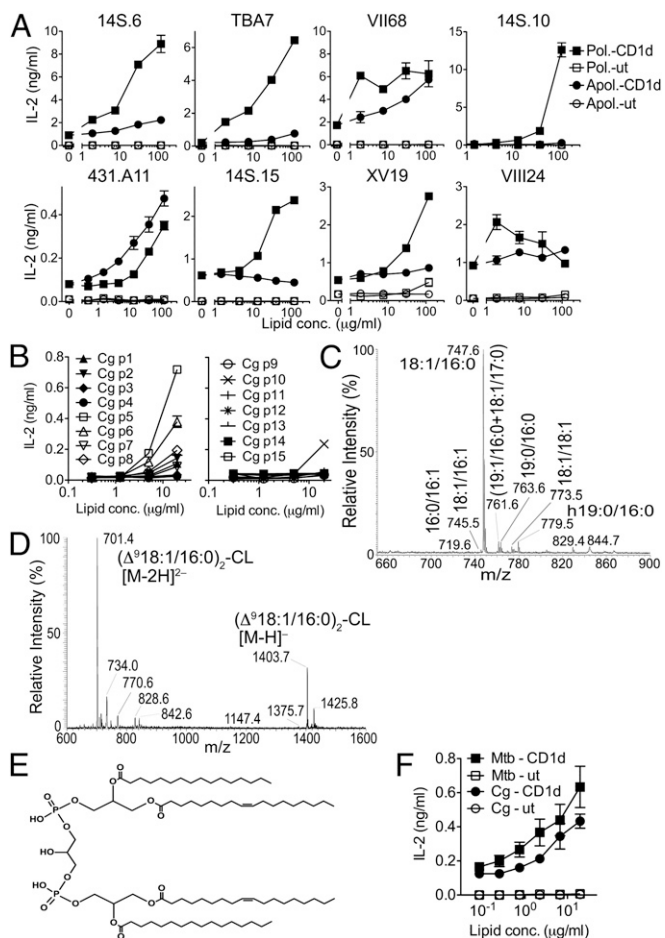


Fig. 3. *Cg* lipids stimulate dNKT hybridomas. (A) Polar or apolar *Cg* lipids were tested for reactivity of dNKT hybridomas as in Fig. 1B. (B) *Cg* polar lipids were separated by 2D TLC and analyzed for reactivity with 14S.10 cells. (C–E) MS analysis revealed (C) the stimulatory lipid p6 to be PG (C) and the active lipid p5 (D) to be DPG (or cardiolipin; CL) (E). (F) Stimulation of 14S.10 cells in the presence of RAW.CD1d or RAW.ut cells and purified *Mtb* or *Cg* PG. Data are representative of two independent experiments.

and p5 were stimulatory in our 14S.10 hybridoma assay (Fig. 3B). Structural analyses by mass spectrometry and tandem mass spectrometry revealed the stimulatory *Cg* lipids p6 and p5 to be PG (Fig. 3C and Fig. S3C and D) and diphosphatidylglycerol (DPG; or cardiolipin; Fig. 3D and E), respectively. For *Cg* PG, the dominant ion at m/z 747.6 corresponds to C18:1/C16:0 and less abundant ions at m/z 763.6 and 773.5 represent C19:0/16:0 and C18:1/C18:1, respectively, in which the double bond of the C18:1 fatty acid substituent is located at Δ^9 (Fig. S3D). When compared directly, PG isolated from *Mtb* and *Cg* stimulated the 14S.10 hybridoma in a similar manner (Fig. 3F). *Cg* DPG showed a dominant $[M-H]^-$ ion at m/z 1,403.7 corresponding to a (Δ^9 C18:1/C16:0)₂-DPG (Fig. 3D). Thus, in addition to the isolation of PG from *Mtb*, independent purification of lipids from *Cg* polar lipids revealed *Cg* PG and the structurally related DPG as stimulatory lipids recognized by the 14S.10 dNKT hybridoma.

Phosphatidylglycerophosphate Synthetase A-Deficient *Cg* Lacks Expression of PG and DPG. To confirm that PG and DPG indeed are stimulatory lipids produced by *Cg*, we generated mutant *Cg* bacteria that are deficient in PG and DPG synthesis. Phosphatidylglycerophosphate synthetase A (*pgsA*) is critical for the biosynthesis of PG and DPG in several bacterial species. We therefore deleted the *pgsA* gene in *Cg* (Fig. S4) and, as a control, generated *pgsA*-deficient strains with reconstituted *pgsA* expression. *pgsA*-

deficient *Cg* was viable, and TLC analysis of polar lipids showed loss of expression of PG and DPG and increased abundance of a phospholipid that was not detected in WT *Cg* (Fig. S5A), whereas *pgsA*-deficient *Cg* that had complemented *pgsA* expression showed a lipid profile that was similar to WT *Cg* (Fig. S5B). Surprisingly, despite the absence of PG and DPG, total polar lipids isolated from *pgsA*-deficient *Cg* stimulated 14S.10 cells comparable to polar lipids from wild-type (WT) *Cg* or *pgsA*-deficient *Cg* with reconstituted *pgsA* expression (Fig. S5C). To determine whether *pgsA*-deficient *Cg* had specifically lost the stimulatory capacity from PG and DPG, we separated lipids isolated from *pgsA*-deficient *Cg* by 2D TLC from regions corresponding to the location of PG and DPG found with WT *Cg* lipids (Fig. S5A). We detected no stimulatory activity from the expected locations for both PG and DPG isolated from *pgsA*-deficient *Cg* (Fig. S5D). Thus, deletion of the *pgsA* enzyme in *Cg* clearly resulted in a loss of PG and DPG recognized by 14S.10.

To determine whether the apparent compensatory increase in production of the phospholipid by *pgsA*-deficient *Cg* might be responsible for stimulating the 14S.10 hybridoma when total polar lipids were used, we isolated this lipid. Our functional analysis revealed the lipid to be stimulatory for the 14S.10 hybridoma (Fig. S5E), and structural analysis by MS revealed this lipid to be phosphatidylinositol (PI) with a dominant ion with m/z of 835.5 corresponding to a C18:1/C16:0 fatty acid composition (Fig. S5F and G). Together, these results demonstrate that *Cg* PG, DPG, and PI, in addition to *Mtb* PG, stimulate the 14S.10 dNKT hybridoma and suggest that an individual dNKT TCR can recognize structurally distinct microbial phospholipids.

Range of Reactivities to Microbial Lipids Among dNKT Cells. Our data suggested that several microbial phospholipids are recognized by the 14S.10 dNKT hybridoma, and, in addition, that several other dNKT hybridomas are stimulated by *Cg* polar lipids (Fig. 3A). To identify the lipids that specifically stimulate other dNKT hybridomas, we performed a large-scale preparative 2D TLC separation of *Cg* polar lipids (Fig. S3B). Then, we tested the reactivities to these purified lipids with additional dNKT hybridomas. Both 14S.6 and TBA7 hybridoma cells were stimulated by *Cg* PG (p6) and DPG (p5), with 14S.6 hybridoma cells responding to *Cg* DPG > PG, whereas TBA7 cells responded to *Cg* PG > DPG (Fig. 4A and B). In contrast, the VII68 hybridoma recognized *Cg* PG, DPG,

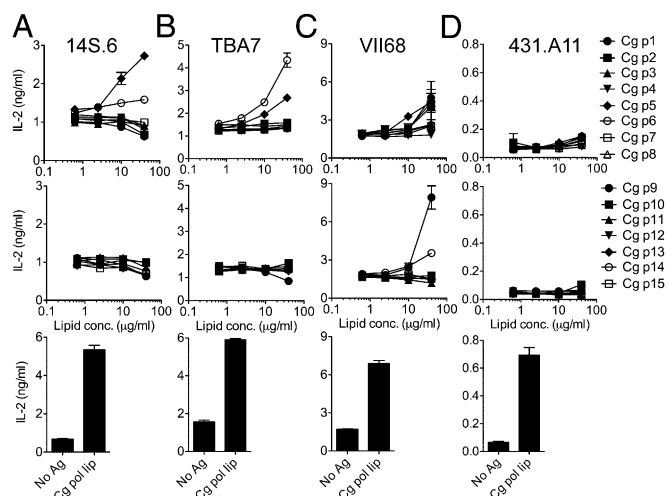


Fig. 4. Range of reactivities to microbial lipids among dNKT hybridomas. dNKT hybridomas were stimulated with lipids isolated from *Cg* polar lipids (as in Fig. 3B) in the presence of RAW.CD1d cells. (A) 14S.6. (B) TBA7. (C) VII68. (D) 431.A11. *Cg* lipid p6 is PG and p5 is DPG (Fig. 3). Table 1 shows TCR- α and β gene utilization and summarizes reactivities. Data are representative of two independent experiments.

and several other lipid fractions only weakly, but strongly responded to Cg lipid p9, which failed to stimulate any of the other hybridomas (Fig. 4C). The identity of the lipid(s) contained in Cg fraction p9 remains to be determined. Interestingly, 431.A11, the hybridoma that was stimulated by Cg apolar > Cg polar lipids, did not respond to any of the 15 purified polar lipids (Fig. 4D), suggesting that this hybridoma recognizes distinct apolar lipid species. Together, these data suggest that the use of TCR α - and β -chains with extensive junctional and germ-line diversity by dNKT cells results in the recognition of distinct microbial lipids, with partially overlapping and occasionally unique antigen specificities.

Antigen Uptake and/or Processing, but Not Toll-Like Receptor-Mediated Signaling, Is Required for dNKT Cell Activation by Microbial Lipids. iNKT cells can be stimulated by microbes either by direct recognition of microbial antigens or indirectly through APC-mediated activation that results in secretion of stimulatory cytokines such as IL-12, -18, or type I IFNs and CD1d presentation of stimulatory self-lipids. To bias our in vitro coculture assay toward the discovery of microbial lipids that stimulate dNKT cells directly and to allow possible processing of lipids, we used dNKT hybridomas in combination with RAW.CD1d cells as APCs. IL-2 production by T-cell hybridomas is expected to be primarily the result of TCR-mediated rather than cytokine-mediated activation. To confirm that activation of 14S.10 dNKT hybridomas was not dependent on Toll-like receptor (TLR)-mediated signaling by APCs to induce cytokine secretion or self-lipid synthesis, we stimulated the 14S.10 hybridoma with *Mtb* polar lipids in the presence of MyD88-deficient bone marrow-derived dendritic cells (BMDCs) that are defective in TLR-mediated signaling. IL-2 production by the 14S.10 hybridoma in response to *Mtb* polar lipids in the presence of MyD88-deficient BMDCs was not different from stimulation with WT BMDCs (Fig. 5A). As a control, stimulation of an iNKT hybridoma with α -GalCer also was comparable in the presence of WT and MyD88-deficient BMDCs (Fig. 5A). In contrast, stimulation of a primary iNKT cell line with the TLR agonist LPS resulted in production of IFN- γ only in the presence of WT, but not MyD88-deficient, BMDCs, showing that, unlike hybridomas, primary iNKT cells are cytokine responsive (Fig. 5A and Fig. S6). Thus, stimulation of the 14S.10 dNKT hybridoma did not require TLR-mediated APC activation, suggesting that this dNKT hybridoma may directly recognize CD1d-presented *Mtb* lipids.

We next determined whether antigen uptake and processing by APCs was required for activation of the 14S.10 hybridoma following stimulation with *Mtb* polar lipids. RAW.CD1d cells were either pulsed with *Mtb* polar lipids for 2 h and subsequently fixed with glutaraldehyde ("pulse \rightarrow fix") or fixed before incubation with *Mtb* lipids ("fix \rightarrow pulse") and then cocultured with 14S.10 hybridomas. Pulse \rightarrow fix-treated, but not fix \rightarrow pulse-treated, RAW cells stimulated IL-2 secretion by 14S.10 cells (Fig. 5B). Control experiments were performed with α -GalCer, an iNKT cell antigen

Table 1. Reactivity of dNKT hybridomas to Cg lipids

Hybridoma Source	V α /J α genes	V β /J β genes	Cg polar	Cg lipid	Cg apolar
14S.10	Ref. 5 V α 11.3/J α 4	V β 8.1/J β 2.7	++	DPG, PG, PI	0
14S.6	Ref. 5 V α 17/J α 32	V β 14.1/J β 2.1	+++	DPG, PG	+
TBA7	Ref. 6 V α 1/J α 18*	V β 8.2/J β 2.6	+++	PG, DPG	0/+
VII68	Ref. 4 V α 4/J α 32*	V β 11/J β 2.5	+/+	p9	+/+
14S.15	Ref. 5 V α 4/J α 38*	V β 5.1/J β 2.4	+/+	None	0
431.A11	Ref. 6 V α 3.2/J α 9	V β 8.2/J β 16*	+	None	++
XV19	Ref. 4 V α 1/J α 26	V β 16/J β 2.1	+++	NT	0
VIII24	Ref. 4 V α 3.2/J α 26*	V β 9/J β 1.4	0/+	NT	0

TCR- α and β gene use was determined for dNKT hybridomas used in this study. +++Very strong; ++robust; +weak; 0, SI < 3; NT, not tested.

*Gene use differs from reference.

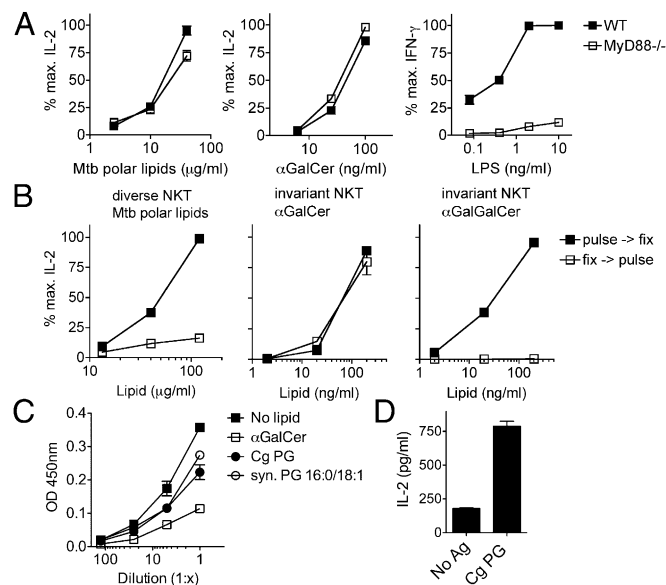


Fig. 5. Microbial lipids activate dNKT cells independent of APC TLR signaling and require uptake and/or processing. (A) 14S.10 dNKT hybridoma cells (Left) and 24.9 iNKT hybridoma cells (Center) or an iNKT cell line (Right) were stimulated with *Mtb* polar lipids, α -GalCer, or LPS, respectively, in the presence of WT or MyD88^{-/-} BMDCs. (B) RAW.CD1d cells were either pulse \rightarrow fix or fix \rightarrow pulse treated with lipids and cultured with 14S.10 dNKT or 24.9 iNKT hybridoma cells. (C) α -GalCer, Cg PG (contains C18:1/16:0), and synthetic C16:0/18:1 PG (representing mammalian PG) compete with biotin-PE binding to CD1d. (D) Stimulation of 14S.10 cells with Cg PG presented by plate-bound CD1d in the absence of APCs. Data are representative of two independent experiments.

known to be able to load onto CD1d molecules on the cell surface, and α -GalGalCer, an iNKT cell antigen known to require intracellular cleavage of the second galactose to result in generation of the stimulatory α -GalCer (19). Pulse \rightarrow fix and fix \rightarrow pulse treatment was similar for stimulation of an iNKT hybridoma with α -GalCer, but only pulse \rightarrow fix treatment resulted in stimulation of the iNKT hybridoma with α -GalGalCer (Fig. 5B). Thus, stimulation of the 14S.10 hybridoma with *Mtb* lipids required lipid uptake and/or processing by APCs, and together, these results suggest that the 14S.10 dNKT TCR directly recognizes *Mtb* lipid antigen(s) presented by CD1d.

Formation of CD1d-Lipid Complexes and Their Recognition by dNKT Cells.

To determine whether the stimulatory microbial phospholipids bind directly to CD1d molecules, we used an established competition assay (11). We incubated biotin-phosphatidylethanolamine (PE)/CD1d antigen complexes with microbial Cg PG and, as controls, synthetic C16:0/C18:1 PG (representing mammalian PG) and α -GalCer. Both microbial Cg PG and synthetic mammalian PG displaced biotin-PE, as did the control iNKT cell antigen α -GalCer (Fig. 5C), suggesting that microbial as well as mammalian PG can bind to CD1d and form lipid-CD1d complexes. Stimulation of NKT cells by CD1d-antigen complexes in the absence of APCs is considered strong evidence that a lipid is directly recognized as a cognate antigen. Indeed, recombinant CD1d molecules loaded with Cg PG stimulated 14S.10 cells (Fig. 5D). Together, these results suggest that Cg PG binds to CD1d and that dNKT cells directly recognize Cg PG/CD1d complexes to result in their activation.

Microbial and Mammalian PG and DPG Stimulate dNKT Cells. We next examined the structural features of phospholipid antigens that determine dNKT cell activation. The dominant ions detected in the stimulatory *Mtb* and Cg PG corresponded to C19:0/C16:0 and C18:1/C16:0 (Figs. 2C and 3C). In contrast to microbes,

mammalian cells express C16:0/18:1 PG that differs from microbial PG in the position of the fatty acids at sn-1 and -2 of the glycerol backbone (compared to C18:1/C16:0 and C19:0/C16:0 in *Cg* and *Mtb* PG). To elucidate the structural requirements for stimulating the 14S.10 hybridoma, we next examined the ability of a synthetic C16:0/18:1 PG (representing the mammalian sn-1/sn-2 fatty acid orientation) to stimulate the 14S.10 hybridoma. For iNKT cell lipid antigens, subtle lipid tail modifications, such as the sn-1/sn-2 orientation of two different fatty acids or the position of a double bond, can profoundly impact iNKT cell activation (20, 21). Surprisingly, the synthetic C16:0/18:1 PG, similar to the microbial *Cg* PG, stimulated 14S.10 cells in a CD1d-dependent manner (Fig. 6A). We complemented these experiments with a synthetic chemistry approach and synthesized two PG molecules that only differed in the sn-1/sn-2 position of the C18:1 and C16:0 fatty acids at the glycerol backbone. The synthetic C18:1/16:0 (microbial) as well as the C16:0/18:1 (mammalian) PG stimulated 14S.10 hybridoma cells in a CD1d-dependent manner (Fig. 6B). Next, we examined whether mammalian DPG, in addition to microbial *Cg* DPG, stimulated 14S.10 hybridoma cells. Bovine DPG, containing dominantly (C18:2/C18:2)₂ species, as well as microbial DPG, containing (C18:1/C16:0)₂ species, stimulated 14S.10 cells in a CD1d-dependent manner (Fig. 6C). Together, these data suggest that microbial as well as mammalian PG and DPG are recognized by and stimulate CD1d-restricted dNKT hybridomas.

Discussion

dNKT cells play an increasingly appreciated role in antimicrobial immunity, yet the antigens they recognize remain poorly defined. Here, we tested lipids from *Mtb* and *Cg*, closely related bacterial species, for their ability to stimulate a panel of dNKT cell hybridomas that exhibited a spectrum of TCR V α and V β use, and we identified several microbial phospholipids and other lipid fractions that activated dNKT cells. dNKT cell activation by microbial lipids was CD1d-dependent, occurred in the absence of TLR or IL-12 signaling by APCs, and required lipid uptake and/or intracellular processing by APCs. Furthermore, plate-bound microbial PG/CD1d complexes were sufficient to stimulate dNKT cells. Together, these findings demonstrate that the stimulatory microbial lipids activate dNKT cells through direct cognate interaction between the dNKT TCR and lipid antigen/CD1d complexes.

Characterization of the stimulatory lipids revealed a unique pattern of distinct and overlapping reactivities for microbial antigens among several dNKT cell hybridomas. Three dNKT cell hybridomas recognized microbial PG, DPG, and PI, with differential hierarchies in their responses to these antigens. Other dNKT hybridomas did not recognize those lipids, but instead had unique antigen specificities. For example, the VII68 hybridoma recognized *Cg* lipid p9, that has not yet been identified, and the

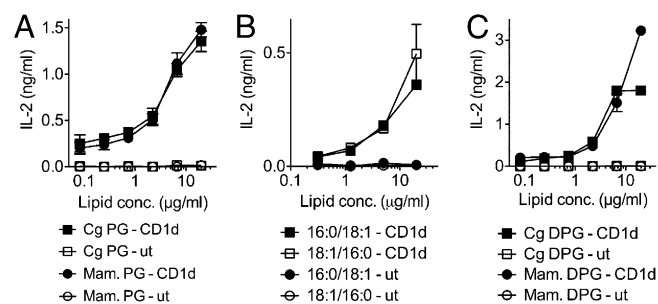


Fig. 6. Microbial and mammalian phospholipids stimulate dNKT cells. (A) Microbial *Cg* PG (containing C18:1/16:0) or mammalian PG (isolated from murine skin, containing C16:0/18:1) was added to RAW.CD1d or RAW.ut cocultures with 14S.10 hybridoma cells. (B) Synthetic versions representing the dominant microbial PG (C18:1/16:0) and mammalian PG (C16:0/18:1) species stimulated 14S.10 cells. (C) *Cg* and bovine DPG stimulated 14S.10 cells. Data are representative of two independent experiments.

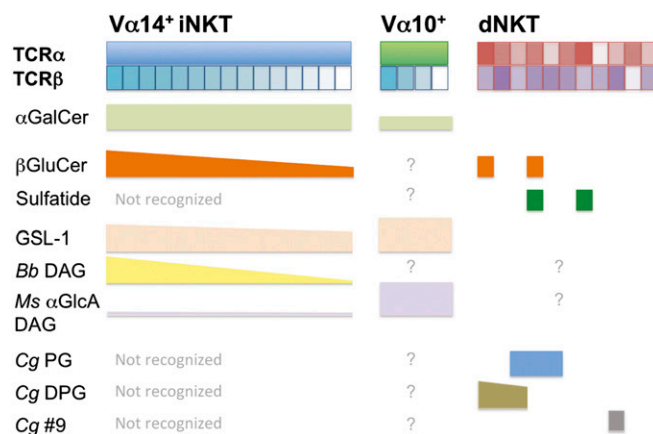


Fig. 7. Antigen specificities of V α 14⁺ iNKT, V α 10⁺ NKT, and dNKT cells. GSL-1 is found in *Sphingomonas* spp., Bb DAG in *Borrelia burgdorferi*, and Ms α -GlcA DAG in *Mycobacterium smegmatis* (3).

431.A11 hybridoma did not react to any of the isolated *Cg* lipid species and instead appeared to recognize apolar *Cg* and *Mtb* lipids. Of note, none of the lipid antigens from *Mtb* or *Cg* that activated dNKT cells stimulated iNKT cells. This pattern of dNKT cell antigen specificity is distinct from both the uniform and pattern recognition receptor-like TCR specificity observed for iNKT cells and the high degree of diversity and antigen specificity observed for MHC-restricted T cells (3) (antigen specificities of NKT cell subsets are summarized in Fig. 7). The α -GalCer antigen and its derivatives, the α -glycan-linked microbial iNKT cell antigens described so far, and the β -GluCer self-antigen are stimulatory for most or all iNKT cells. Although there is some hierarchy in affinity of different mouse iNKT cell TCRs based on TCR β -chain use (22, 23), stimulation with iNKT cell antigens generally results in immediate, innate-like responses of the entire iNKT cell population. iNKT cells, and the recently described semi-invariant V α 10⁺ CD1d-restricted NKT cells, use a unique mode of TCR recognition of lipid/CD1d complexes in which recognition of the lipid antigen is mediated primarily by the semi-invariant TCR α -chain, and the TCR β -chain appears to only modulate affinity (24–26). In contrast, MHC-restricted T cells, as well as human CD1a-, b-, and c-restricted T cells, show a high degree of specificity for unique antigens and stimulation with their cognate antigens results in clonal expansion and adaptive T-cell responses (27). Thus, the ability to discriminate between several lipid antigens as observed here for dNKT cells suggests a different mode for TCR-mediated recognition of lipid/CD1d complexes compared with iNKT cells, which indeed was recently confirmed by structural analysis of a dNKT TCR in complex with CD1d/sulfatide (28, 29).

In humans, CD1b molecules present *Mtb* lipids to CD1b-restricted, microbial lipid-specific T cells that expand following vaccination, and *Mtb* lipid-specific CD1b-restricted T cells elicited by lipid vaccination provided protection in a guinea pig model of tuberculosis (14). In addition, iNKT cells, although not required for optimum immunity to *Mtb* infection in mice, can contribute to the protective immune response during *Mtb* infection and suppress bacterial replication in vitro (30). Here, dNKT cells were activated by *Mtb*-infected APCs, making it tempting to speculate that dNKT cells may also contribute to protective immunity during *Mtb* infection. Although there is no evidence that iNKT cells are capable of generating adaptive, antigen-specific responses, the partially naïve phenotype observed on some dNKT cells, together with their distinct antigen specificities, suggests that they may be capable of generating memory responses to these microbial antigens.

A striking feature of the observed dNKT cell specificity for microbial lipid antigens is the unexpected cross-reactivity for microbial phospholipids and the homologous mammalian phospholipids, because for several iNKT cell antigens, structural

features unique to microbial lipids are critical for antigenicity (21, 31, 32). Both PG and DPG isolated from *Cg* stimulated dNKT cells comparable to their mammalian counterparts, despite apparent structural differences such as the reversal of the 16:0/18:1 fatty acid chains in the sn-1/sn-2 positions. The description here of three DPG/cardioliipin-reactive dNKT cells demonstrates that CD1d-restricted NKT cells that recognize cardioliipin can also use $\alpha\beta$ TCRs, in addition to the cardioliipin-specific $\gamma\delta$ T cells observed in mice (33). Overall, our findings suggest that the cross-reactivity between microbial and self-lipid antigens may be a central feature of dNKT cell function, allowing both presentation of microbial lipids and enhanced availability of stimulatory self-lipids to contribute to dNKT cell activation during infection. Furthermore, it is possible that dNKT cell activation during infection requires TLR- and cytokine-mediated costimulation in addition to the TCR-mediated signals induced by recognition of weak self-lipid or microbial lipid antigens, as has been observed for iNKT cells (34, 35).

In summary, our results show a pattern of antigen specificity for dNKT cells characterized by cross-reactivity between mammalian and microbial phospholipid antigens, and distinct from both the uniform and pattern recognition receptor-like antigen specificity observed for iNKT cells and the high degree of diversity and antigen specificity observed for MHC-restricted T cells. With this example of microbial antigens for dNKT cells, it will be intriguing to determine the role of their recognition in microbial infection and host defense.

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Materials and Methods

dNKT hybridomas (4–6) were cocultured with CD1d-transfected or untransfected RAW cells, or BMDCs from WT or Myd88-deficient mice, in the presence of various concentration of lipids, or following infection of RAW cells with *Mtb* H37Rv. Cytokines were measured in culture supernatants by ELISA. Lipids were extracted from *Mtb* or *Cg* by using organic solvents and isolated by using HPLC and TLC, and structural analysis was performed as described (35). Synthesis of PG will be described elsewhere, and other lipids were from Avanti Polar Lipids. pgsA-deficient *Cg* was generated by targeting NCgl1889 with a construct based on pk19mobsacB and plasmid pEKEx2-1889 served for complementation studies. CD1d binding of lipids and CD1d plate assays used biotinylated CD1d proteins as described (11). For details of experimental conditions and analyses, see *SI Materials and Methods*.

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