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Identification of a Potent Microbial Lipid Antigen for Diverse NKT Cells

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Semi-invariant/type I NKT cells are a well-characterized CD1d-restricted T cell subset. The availability of potent Ags and tetramers for semi-invariant/type I NKT cells allowed this population to be extensively studied and revealed their central roles in infection, autoimmunity, and tumor immunity. In contrast, diverse/type II NKT (dNKT) cells are poorly understood because the lipid Ags that they recognize are largely unknown. We sought to identify dNKT cell lipid Ag(s) by interrogating a panel of dNKT mouse cell hybridomas with lipid extracts from the pathogen Listeria monocytogenes. We identified Listeria phosphatidylglycerol as a microbial Ag that was significantly more potent than a previously characterized dNKT cell Ag, mammalian phosphatidylglycerol. Further, although mammalian phosphatidylglycerol-loaded CD1d tetramers did not stain dNKT cells, the Listeria-derived phosphatidylglycerol-loaded tetramers did. The structure of Listeria phosphatidylglycerol was distinct from mammalian phosphatidylglycerol because it contained shorter, fully-saturated anteiso fatty acid lipid tails. CD1d-binding lipid-displacement studies revealed that the microbial phosphatidylglycerol Ag binds significantly better to CD1d than do counterparts with the same headgroup. These data reveal a highly potent microbial lipid Ag for a subset of dNKT cells and provide an explanation for its increased Ag potency compared with the mammalian counterpart.

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Abbreviations used in this article: AMPP, N-(4-aminomethylphenyl) pyridinium; CD1d-bio, biotinylated mouse CD1d; C/M, chloroform:methanol; DGDC-Sp, DGDG from S. pneurnoniae; DGDG, digalactosyldiacylglycerol; dNKT, semi-invariant/type I NKT; KO, knockout; LC-MS, liquid chromatography–mass spectrometry; GD3, disialoganglioside GD3; iNKT, semi-invariant/type I NKT; MFI, mean fluorescence intensity; MPA, molybdophosphoric acid; NKT, NKT; P30, DGDG from S. pneurnoniae; P30-DK56341, M.B.B. was supported by National Institutes of Health Grants 5K08AI077795 and 1R21AI03616; C.E.A. was supported by Fundacao para a Ciência e Tecnologia International PhD Programme SFHR/BD/74906/2010 from Ministério da Educação e Ciência, Portugal. D.I.G. was supported by the National Health and Medical Research Council of Australia Senior Principal Research Fellowship 1020770. J.R. was supported by the National Health and Medical Research Council of Australia Fellowship; the National Health and Medical Research Council of Australia, and the Australian Research Council. A.P.U. was supported by Australian Research Council Future Fellowship FT140100278.

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loops bind, perched over the A’ pocket, to CD1d, and the CDR3β loop provided the major contact with the bound sulfatide head-group. Whether this is typical of all dNKT TCR–CD1d–Ag interactions remains to be determined, although recent crystallographic studies of a human γδ TCR, and a hybrid δβ TCR, interacting with lipid Ags α-GalCer and sulfatide, presented by CD1d, also showed orthogonal docking over the A’ pocket of CD1d (7–9). The fact that dNKT TCRs use diverse TCRα- and TCRβ-chains, and that the XV19 CD1d–dNKT TCR structural studies revealed that the variable CD3 loops can dominate in lipid Ag recognition, suggests that dNKT cells may possess the capacity to recognize a great range of self and foreign lipid Ags.

One of the key distinguishing features of dNKT cells is that unlike iNKT cells, they do not respond to α-GalCer and, therefore, are not identified by CD1d–α-GalCer tetratrams. With the finding that dNKT cells may be present in humans at higher levels than iNKT cells, there is great interest in identifying physiologically relevant lipid Ags for dNKT cells (6, 10). Many of the identified dNKT cell lipid Ags were identified or confirmed by screening a panel of dNKT cell hybridomas. Using these T–T hybridomas, several endogenous mammalian lipid Ags (e.g., sulfatide, phosphatidyglycerol, lysophosphatidylcholine, lysophosphatidylethanolamine, and diphas- stophagalglycerol) were confirmed as dNKT cell Ags (11–18).

With the notable exceptions of sulfatide-reactive and Gaucher lipid–reactive dNKT cells (12, 19), no other dNKT cell population has been directly identified in vivo because of the failure of tetramer to bind. Instead, the role of dNKT cells was inferred indirectly by comparing mice lacking iNKT cells (Jβ18-knockout [KO] mice) with mice lacking both dNKT and iNKT cells (CD1d-KO mice) (20, 21). Studies with these KO mice demonstrated a protective role for dNKT cells in a variety of pathogenic states, including type 1 diabetes, Con A–induced hepatitis, and murine infection with Schistosoma mansoni or Listeria monocytogenes (1, 10). However, these studies were confounded by the fact that Jβ18-KO mice have additional TCR Jα defects, resulting in a limited TCRα repertoire that has only recently been appreciated (22).

Previously, we identified the first microbial dNKT cell lipid Ags (13). Using dNKT cell hybridomas, we found that phosphatidylglycerol and diphas stophagalglycerol (DPG) derived from Mycobacterium tuberculosis, the related Corynebacterium glutamicum, and mammalian phosphatidyglycerol are Ags for a subset of dNKT cells. These microbe-derived phosphatidyglycerols and DPGs contained the same headgroup as their mammalian counterparts but differed in their fatty acid tail structure. The Corynebacterium–derived phosphatidylglycerol/DPG variants were weak Ags and equivalent to the similarly weak mammalian phosphatidylglycerol/DPG with regard to their ability to activate dNKT cells. We reasoned that microbial lipid Ags for dNKts that are distinctly more active than mammalian phosphatidylglycerol might exist. Therefore, we designed an independent and unbiased search for potential dNKT lipid Ags from other microbes, such as L. monocytogenes.

Listeria is a Gram-positive facultative intracellular bacterium that infects and resides within the cytosol of macrophages, dendritic cells, hepatocytes, and epithelial cells (23–25). This pathogen is a common food contaminant and causes significant mortality in immunocompromised individuals and spontaneous abortions in pregnant women (26). There are three key reasons for investigating Listeria for lipid Ags. First, as an intracellular pathogen the Listeria–derived lipids would be likely to access the intracellular CD1d Ag-presentation system in vivo. Second, data from the comparison of bacterial burdens in Jβ18-KO mice and CD1d-KO mice suggest a role for dNKT cells in clearing this pathogen (27, 28). Third, Listeria has been subjected to lipidomics analysis, whereby Fischer and Leopold (29) identified many unique Listeria lipids that do not have mammalian homologs and, thus, might be recognized as foreign. Furthermore, our analysis of the lipidomics data revealed that a number of these lipids are capable of binding to CD1d, making them potential dNKT lipid Ags.

In this study, we used a panel of iNKT and dNKT hybridomas to screen fractionated Listeria lipids for CD1d-restricted Ags. Interestingly, this unbiased screen revealed reactivity in some of the same chemical classes of lipids identified previously, such as phosphatidyglycerol, but with critically important differences. Listeria–derived phosphatidyglycerol and DPG differed significantly in their fatty acid architecture compared with the mammalian/Corynebacterium variants. Importantly, Listeria–derived phosphatidyglycerol is a strikingly more potent Ag for dNKT cells. By performing Ag CD1d-binding assays and tetramer staining, we provide insights into the structural basis for the high potency of microbial compared with mammalian lipid Ags that share identical lipid headgroups.

Materials and Methods

Growth and lipid extraction of L. monocytogenes

Wild-type L. monocytogenes (strain 10403S; a gift from H. Shen, University of Pennsylvania) was grown in brain-heart infusion broth (BD Biosciences) supplemented with 200 μg/ml streptomycin (Sigma-Aldrich) overnight to stationary phase at 37°C and 225 rpm. The following day, flask-containing prewarmed brain-heart infusion broth (BD Biosciences) supplemented with 200 μg/ml streptomycin were inoculated at ~1:420 v/v and grown until mid-log phase (OD₆₀₀ ≈ 0.4). Once at mid-log phase, bacteria were pelleted by centrifugation, washed with PBS, and lyophi- lized. After up to 48 h of lyophilization, Listeria pellets were processed for extraction of crude polar lipids, as previously described (30). Once iso- lat ed, lipids were weighed, suspended in 2:1 v/v chloroform:methanol (CM), and stored in 15-ml glass tubes at −20°C until used.

Cell lines

The following mouse NKT hybridomas were tested for reactivity against Listeria lipids: 24.9, DN32, 14S.6, 14S.10, 14S.15, 431.A11, TBA7, VII68, VIII24.1.D, and XV19.2 (31–34). The hybridomas not generated in the Brenner laboratory were kindly provided by S. Cardell (Goethebs Universit) and A. Bendelac (University of Chicago). Hybridoma cells were maintained in NKT growth media (RPMI 1640 [Life Technologies] supplemented with 10% v/v FBS [Gemini], 10 mM HEPES [Life Technologies], 2 mM L-glutamine [Life Technologies], 100 μg/ml streptomycin [Life Technologies], and 55 mM 2-ME [Life Technologies]). RAW 264.7 (RAW) and RAW cells stably transfected with mouse CD1d (RAW-CD1d) were maintained in Complete DMEM (DMEM supplemented with 10% v/v FBS [Gemini], 2 mM L-glutamine [Life Technologies], 100 μM penciillin [Life Technologies], 100 μg/ml streptomycin [Life Technologies]); When hybridomas were cocultured with RAW or RAW-CD1d cells or with plate-bound CD1d, cells were incubated overnight in Complete RPMI 1640 [Life Technologies] supplemented with 10% v/v FBS [Gemini], 2 mM L-glutamine [Life Technologies], 100 μM penciillin [Life Technologies], 100 μg/ml streptomycin [Life Technologies]). For tetramer and dextramer experiments, the TCR-deficient T cell hybridoma BW38 (35) was stably transfected with CD3, TCRα, and TCRβ from one of four hybridomas (V88.2 [an iNKT cell hybridoma], TBA7 [TBA7³⁶⁴], 14S.6, or XV19). BW58- and TCR-transfected clones were maintained in DMEM-NKT media (DMEM [Life Technologies] supplemented with 10% v/v FBS [Gemini], 2 mM L-glutamine [Life Technologies], 100 μM penciillin [Life Technologies], 100 μg/ml streptomycin [Life Technologies], and 1% nonessential amino acids [Life Technologies]). All cells were maintained in 37°C incubators at 5% (RPMI media) or 10% (DMEM media) CO₂.

Liquid chromatography–mass spectrometry fractionation of polar Listeria lipids

Preparative HPLC experiments were carried out using a custom Waters (Milford, MA) Autopurification HPLC system comprising a Waters 2767 one-bed injection-collection sample manager, a 2545 quaternary gradient.
module that can pump up to 150 mL/min, a system fluidic organizer coupled to a single-quadrapole Waters 3100 Mass Detector equipped with 2 Spry API ion source, a Waters 2424 evaporative light scattering detector (ELSD), and a Waters 2998 photodiode array detector. In addition, during preparative mode, the system is coupled to two Waters 515 HPLC pumps used for make-up liquid delivery, as well as a 1000:1 splitter that can tolerate a flow rate of 8–30 mL/min. During fractionation, 99.9% of the lipids is sent to a fraction collector for use in future bioassays. A small percentage (0.1%) of the polar lipid is sent to the detectors to identify the lipid-containing fractions that helped with fractionation. The entire system was controlled by MassLynx 4.1 software. Chromatographic analyses and separation of crude polar extracts were performed based on the eluent method on a chemically bonded polyvinyl alcohol-silica stationary phase (36). The total liquid chromatography–mass spectrometry (LC-MS) run was performed by spotting both the TLC-purified lipid and a relevant standard (Escherichia coli). Chromatography–mass spectrometry (LC-MS) was run into 19 fractions using time and an ELSD to designate fractions. Pooled fractions were weighed, resuspended in 2:1 C:M, and stored at −20°C until ready for use.

Analytical and preparative thin layer chromatography

For analytical thin layer chromatography (TLC), lipids were spotted onto aluminum-backed silica TLC plates (EMD Chemicals) and dried under low pressure. Next, lipids were resolved in a solvent system designed for the optimal separation of phospholipids (chloroform:acetic acid:methanol: water at a ratio of 40:25:3:6 v/v/v/v/v). Plates were dried under low pressure, cut (if appropriate), and sprayed with different TLC stains: Dittmer–Lester reagent (phosphate stain), α-naphthyl (sugar stain), molybdophosphoric acid (MPA; a general stain), or ninhydrin (an amino group stain). The plates were then dried under low pressure, resolved in the 40:25:3:6 system, and dried as above. Next, a small segment of the TLC plate was cut off for staining with one of the above TLC stains, which was used to mark the bands of lipid in the unstained section of the plate. The marked silica regions for each lipid band were scraped off the plastic and moved to 15-mL glass tubes, and the lipid was extracted from the silica with three sequential washes of 2:1 C:M. After drying, lipids were weighed, resuspended in 2:1 C:M, and stored at −20°C until use. It is critical to note that the amount of TLC purified lipid in the tube cannot be determined solely by measuring weight, because the measured weight includes some transferred silica. When the identity of the purified lipid was unknown (Fig. 3B), lipids were resuspended in 2:1 C:M based on the total weight of the tube and an estimated expected yield. Because the weights for resuspended lipids were approximated, the amount measured was shown as the fold-dilution of the stock lipid. To determine the relative concentration for identified lipids, concentrations were measured by spotting both the TLC-purified lipid and a relevant standard (Corynebacterium phosphatidylglycerol, DPG, or Streptococcus digalactosyl-diacylglycerol [DDG]) onto an analytical TLC plate at various concentrations to generate a standard curve. The plates were then dried, resolved with the 40:25:3:6 system, redried, stained with MPA, and charred to develop. The marked silica regions for each lipid band were scraped off the plastic and moved to 15-ml glass tubes, and the lipid was extracted from the silica with three sequential washes of 2:1 C:M. After drying, lipids were weighed, resuspended in 2:1 C:M, and stored at −20°C until use. It is critical to note that the amount of TLC purified lipid in the tube cannot be determined solely by measuring weight, because the measured weight includes some transferred silica. When the identity of the purified lipid was unknown (Fig. 3B), lipids were resuspended in 2:1 C:M based on the total weight of the tube and an estimated expected yield. Because the weights for resuspended lipids were approximated, the amount measured was shown as the fold-dilution of the stock lipid. To determine the relative concentration for identified lipids, concentrations were measured by spotting both the TLC-purified lipid and a relevant standard (Corynebacterium phosphatidylglycerol, DPG, or Streptococcus digalactosyl-diacylglycerol [DDG]) onto an analytical TLC plate at various concentrations to generate a standard curve. The plates were then dried, resolved with the 40:25:3:6 system, redried, stained with MPA, and charred to develop. The plates were scanned at 300 dpi, and densitometry was performed with ImageJ software (National Institutes of Health), and quantification was determined by ImageJ

Mass spectrometric identification of lipid structure

Both high-resolution (R = 100,000 at m/z 400) and low-energy collision activated dissociation tandem mass spectrometry (MS/MS) were performed as previously described, with the exceptions that samples were dissolved into methanol instead of 2:1 C:M, the automatic gain control of the ion trap was set to 5 × 10⁴, and the electrospray needle was set to 4.0 kV (13). For structural analysis of fatty acids, TLC-purified lipids were treated via alkaline hydrolysis to liberate fatty acids, which were then isolated and derivatized with β-(4-aminomethylphenyl) pyridinium (AMPP) and subjected to mass spectrometry, as described (37).

Synthesis of lipid standards

Synthesis of mammalian (18:1/16:0) or Listeria (15:0/17:0) phosphatidylglycerols was performed in a stepwise fashion, starting with a glycerol backbone CD1d-loaded CD1d-bio was purified by MonoQ anion-exchange chromatography, immobilized metal-affinity chromatography, and gel filtration. TCR purity was assessed by gel electrophoresis, and predicted mass was confirmed by time-of-flight mass spectrometry. TCR refolding was confirmed by ELISA using an Ab reactive against a conformational epitope for the TCR constant region (clone 12H8; produced in-house) and anti-Vβ8.1/8.2 (clone KJ16-133; eBioscience).

Distalaganglioside GD3 lipid-displacement assay

Distalaganglioside GD3 (GD3; Matreya #1504) was suspended in TBS-Tyl vehicle (mock) and then loaded into ELISAs. GD3-loaded CD1d-bio was purified by MonoQ anion-exchange chromatography, immobilized metal-affinity chromatography, and gel filtration. TheJournal of Immunology 3

Disialoganglioside GD3 (GD3; Matreya #1504) was suspended in TBS-Tyl vehicle (mock) and then loaded into ELISAs. GD3-loaded CD1d-bio was purified by MonoQ anion-exchange chromatography, immobilized metal-affinity chromatography, and gel filtration. TheJournal of Immunology 3

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exchange chromatography. *Listeria* phosphatidylglycerol or *Corynebacterium* phosphatidylglycerol, resuspended at 1 mg/ml in TBS-Ty, was incubated at a 30:1 molar ratio with purified GD3-loaded CD1d-bio. Phosphatidylglycerol-loaded fractions were purified using MonoQ anion-exchange chromatography. These fractions were used in affinity measures with TBA7 by surface plasmon resonance (SPR). Excess lipid and detergent were removed prior to each chromatography run using a PD10 desalting column (Amersham Biosciences).

**SPR analysis**

SPR experiments were performed at 25°C on a Biacore 3000 instrument and conducted in HEPES buffered saline (10 mM HEPES [pH 7.4] 150 mM NaCl); 1% BSA was added to prevent any nonspecific binding. Four thousand response units (RU) CD1d-bio loaded with the lipid Ag *Corynebacterium* phosphatidylglycerol or *Listeria* phosphatidylglycerol–LC-MS fraction E were coupled onto the streptavidin sensor chip. An HLA class I molecule was immobilized on one flow cell for reference subtraction. Biotin was injected to block the free streptavidin sites. Nine serial dilutions of TBA7 from 200 to 0.78 μM were passed through as analyte. BIAevaluation software was used for data analysis.

**Data presentation and statistical analysis**

All IL-2 ELISA graphs, fold-change graphs, and percentage displacement graphs were generated using GraphPad Prism 5.0b. All statistical analyses (one-way ANOVA) were performed with GraphPad Prism 5.0b.

**Results**

We isolated polar *Listeria* lipids from mid-log phase bacteria cultures and cocultured the crude polar lipid mixture with two iNKT cell hybridomas (Fig. 1A, 1B) and eight dNKT cell hybridomas (Fig. 1C–J). To be considered activated, the following requirements were necessary: a dose response between lipid Ag concentration and hybridoma IL-2 production; IL-2 production when cocultured with lipid Ag, and RAW-CD1d is significantly higher than when cocultured with no exogenously added lipid; and little to no response when cocultured with lipid Ag and untransfected RAW cells. By these requirements, both iNKT cell hybridomas (Fig. 1A, 1B) failed to be activated by crude *Listeria* polar lipids, as also noted by other investigators (31–34, 36, 40). In contrast, six of the eight dNKT cell hybridomas were positively activated by *Listeria* polar lipids in a CD1d-dependent manner. Based on these results, we chose two dNKT hybridomas, 14S.6 and TBA7, which gave strong responses to the polar lipid extract for further study to identify the relevant *Listeria* lipid Ags present in the crude extract.

We fractionated the *Listeria* polar lipid extract by preparative HPLC. This preparative LC-MS system involves the use of three isocratic solvent mixtures on a polyvinyl chloride silica column to facilitate separation of lipids based on headgroup structure. Four detectors are used to reveal lipids, including a UV detector, a single quadrupole mass spectrometer, an ELSD, and a photodiode array detector. We used ELSD (measured in light scattering units [LSU]) and retention time as the criteria for separating the total LC-MS run into 19 fractions, which were tested for biological activity. Although fractions encompassing the whole run were tested for activity, the major antigenic fractions correlated with the presence of detectable LSU signals (Fig. 2A, data not shown). The two major LSU peaks appeared at ~10 min and between 38 and 48 min elution time. The peak at 10 min (Peak 1) was identified by TLC to consist of free fatty acids and roughly equal amounts of 1,2- and 1,3-diacylglycerol (data not shown). The stimulatory Peak 1 fraction was weaker than those collected between 38 and 48 min and was not characterized further. The six LC-MS fractions collected between 38 and 48 min activated the diverse hybridomas 14S.6 and TBA7 in a CD1d-dependent manner (Fig. 2B–E).

The fact that all of these fractions are eluting from the LC-MS column around the same time suggested that they may share common polar headgroups. Further, we noted previously that, in our HPLC system, LSU peaks eluting between 38 and 48 min typically indicate the presence of phospholipids. By analyzing

![FIGURE 1.](http://www.jimmunol.org/Downloadedfrom)
migration on TLC plates in a solvent system designed for resolving phospholipids, we found that fractions A–F included the presence of phosphate-containing lipids that migrated to similar heights, suggesting that they all shared similar structures in different ratios (Supplemental Fig. 1). At the concentrations tested, LC-MS fraction C showed a clear dose response across concentrations and was available in sufficient quantities for further analysis (Fig. 2D). After further separation by TLC, we subjected LC-MS fraction C to a variety of stains (Fig. 3A) (13). Dittmer-Lester (phosphate) stains phosphate-containing molecules a blue color on a white background. α-Naphthol (sugar) stains lipids containing carbohydrate groups a dark purple color. MPA (general) is a general lipid stain that is thought to stain fatty acid tails. Finally, LC-MS fraction C also was stained with ninhydrin (amino), which stains amino groups reddish-pink but also can nonspecifically mark some lipids with a brown color.

Based on these TLC stains, we identified 12 lipid bands from LC-MS fraction C and isolated each by preparative TLC. These bands were tested for activity with hybridoma 14S.6 (Fig. 3B). The top six bands (TLC 1–6), which contained all of the phosphate- or sugar-positive lipid bands, activated hybridoma 14S.6, but the bottom six bands did not. MS/MS on the six active TLC bands identified these as lipid species with glycerol backbones, and each lipid was dominated by fatty acid tails of length 17:0/15:0 in the sn1 and sn2 positions, respectively. By MS/MS analysis of the TLC-purified lipids, TLC bands 1–3 consisted of DPG (Fig. 3C), TLC band 4 was identified as phosphatidylglycerol (Fig. 3D), and TLC band 5 was DGDG (Supplemental Fig. 2C). However, further TLC-based analysis of TLC-purified DGDG from Listeria determined that this activity was likely due to a comigrating UV light + molecule. DGDG from either Streptococcus pneumoniae or LC-MS fraction B (that did not contain the comigrating UV + band) was inactive (Supplemental Fig. 2C).
Fig. 2B). TLC band 6 was not visible by any of the four TLC stains, and MS/MS data were inconclusive. We next generated AMPP derivatives of the fatty acid tails for further analysis of the fatty acid structure by gas chromatography-MS (GC-MS) (37). GC-MS on AMPP derivatives from DPG, phosphatidylglycerol, and DGDG revealed that the fatty acids are predominantly anteiso methyl-branched fatty acids (Fig. 3E, 3F), a structure that is not found in mammals (35, 41).

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** TLC purification of LC-MS fraction C identifies six active bands. (A) *Listeria* LC-MS fraction C was spotted at 150 μg/spot on a TLC plate and run in the 40:25:3:6 system. The plate was cut into strips, and each strip was stained with one of four stains: Dittmer-Lester reagent (Phosphate; to stain phosphate groups), α-naphthol (Sugar; to stain sugar groups), MPA (General; a general fatty acid tail stain), and ninhydrin (Amino; to stain amino groups). These stains were used to identify 12 lipid bands for extraction. (B) TLC plate-eluted lipid bands were incubated with 14S.6 cells and RAW (gray) or RAW-CD1d (black) cells in triplicate. Because of the silica plate extraction, weights of TLC bands reflected both silica and lipid weight. Therefore, the lipid bands were resuspended based on total weight, and fold dilutions of the stock lipids are displayed. IL-2 ELISAs were performed on culture supernatants after 16–18 h of coculture. Data are representative of two independent experiments and show mean ± SEM. Collision MS/MS spectra for TLC bands 1–3 were all identified as DPG (C), and TLC band 4 was identified as phosphatidylglycerol (D). Structures of the dominant DPG (E) and phosphatidylglycerol (F) lipids in the TLC bands, with comparison with mammalian (G) and *Corynebacterium* (H) phosphatidylglycerol, demonstrating the different fatty acid tails found in these species.
LC-MS fractions A–F contain phospholipids that resolve on TLC plates with similar mobilities, suggesting that these fractions contain lipids with the same headgroups (Supplemental Fig. 1). Next, we used TLC to isolate putative DPG and phosphatidylglycerol bands from the other LC-MS fractions that contained enough materials for further study (fractions B–E) and confirmed by MS/MS that these were DPG or phosphatidylglycerol and that the major fatty acid tails were anteiso isomers by GC-MS on AMPP derivatives of the fatty acid tails. In total, we isolated DPGs from LC-MS fractions B–E and phosphatidylglycerols from LC-MS fractions C–E. Although the same lipid was found throughout multiple fractions, we identified differences in the fatty acid substituent compositions. These differences included the presence or absence of plasmenyl phosphatidylglycerol (fatty acids with an sn1 ether linker and an sn2 ester linker) (37) and different ratios of phosphatidylglycerol species (e.g., the ratio of 15:0/15:0 to 17:0/17:0) (Fig. 3D, data not shown).

Recently, we reported that phosphatidylglycerol and DPG were dNKt cell Ags derived from mammals or C. glutamicum (13). However, the two species had very similar lipid structures; notably the same dominant fatty acid tails (16:0 and 17:0) were present but opposite in sn1 and sn2 orientation (Fig. 3G, 3H). Importantly, when Corynebacterium phosphatidylglycerol or DPG was compared with mammalian phosphatidylglycerol or DPG, there was no difference in their potency with regard to the activation of dNKt cells (13). Unlike the previously described phosphatidylglycerol and DPG lipids from mammals, Listeria phosphatidylglycerol and DPG have a distinct fatty acid architecture, prompting us to ask whether Listeria phosphatidylglycerol and DPG are more or less stimulatory than the corresponding mammalian (or Corynebacterium) sources. When comparing the ability to activate hybridomas 14S.6 and TBA7, Listeria DPG was an equally potent Ag to Corynebacterium DPG (Supplemental Fig. 2A). However, we found that Listeria-derived phosphatidylglycerols were strikingly more potent Ags than Corynebacterium-derived phosphatidylglycerol, as measured by their ability to activate the dNKt hybridomas at lower lipid concentrations (Fig. 4A). Next, we calculated the Listeria phosphatidylglycerol concentration needed to obtain an equivalent level of IL-2 production as the first Corynebacterium concentration to be clearly above background levels (Table I). When we calculated the fold change in concentration needed to get the same level of activity, we found that the Listeria phosphatidylglycerols were 10–100-fold more potent than Corynebacterium-derived phosphatidylglycerol, which is similar in potency to phosphatidylglycerol from mammals (Fig. 4B, Table I).

To confirm these findings, we synthesized the dominant Listeria phosphatidylglycerol variant (a17:0/a15:0) and compared its ability to activate TBA7 cells with that of synthetic mammalian phosphatidylglycerol (16:0/18:1). These results supported our previous observations that Listeria phosphatidylglycerol was a more potent Ag than mammalian phosphatidylglycerol and had a similar fold difference in activity (~13-fold) to Listeria LC-MS fractions C and D phosphatidylglycerols (Fig. 4C).

To determine whether cellular processing of Listeria phosphatidylglycerol was required for presentation to dNKt cells, we tested activity using an APC-free system. We loaded the most active Listeria phosphatidylglycerol (phosphatidylglycerol–LC-MS fraction E) or the prototypical iNKT cell Ag α-GalCer onto biotinylated CD1d and then bound 0.2 µg of CD1d/well to streptavidin-coated plates. Different NKT hybridomas were incubated with the plate-bound CD1d (Fig. 5A). As expected, α-GalCer–loaded CD1d activated the iNKT DN32 hybridoma but did not activate the dNKt TBA7 hybridoma. Importantly, phosphatidylglycerol–LC-MS fraction E–loaded CD1d activated the dNKt TBA7 hybridoma but not the iNKT DN32 hybridoma. Mock-loaded CD1d did not activate DN32 cells, but it weakly activated TBA7 cells, reflecting the known CD1d autoreactivity seen with many dNKt hybridomas. Next, we determined whether the different antigenic properties of the various phosphatidylglycerols would be reflected in this APC-free system. Indeed, when we loaded Corynebacterium phosphatidylglycerol or the various TLC-purified Listeria phosphatidylglycerols into plate-bound CD1d, we measured a dose-dependent increase in IL-2 production (Fig. 5B, compare left and right panels). The amount of IL-2 produced at 0.25 µg CD1d/well (Fig. 5B, right panel) closely mimicked the fold difference in activity seen in the system using live RAW-CD1d APCs (Fig. 4B, right panel), suggesting that the

**FIGURE 4.** Listeria-derived phosphatidylglycerols are 10–100-fold more antigenic than Corynebacterium or mammalian phosphatidylglycerol. (A) Activity of Listeria phosphatidylglycerol from fractions C–E was compared with that for phosphatidylglycerol–Cg when cocultured in triplicate with 145.6 or TBA7 hybridomas in the presence of RAW-CD1d or RAW cells (data not shown). (B) The fold difference in concentration required for similar activity to Corynebacterium phosphatidylglycerol was calculated. (C) Synthetic Listeria and mammalian phosphatidylglycerols were assayed for activity, as in (A). The fold difference in concentration required for similar activity to synthetic mammalian phosphatidylglycerol was assayed. Data in (A) are representative of three independent experiments, data in (B) are a combination of three experiments, and data in (C) are representative (left panel) or a combination (right panel) of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.05, one-way ANOVA. All graphs are presented as mean ± SEM.
difference in activity is not due to processing of the LC-MS fractions within RAW-C1d1 cells.

These results prompted us to determine whether *Listeria* phosphatidylglycerol was an Ag for the other six dNKT hybridomas. We performed APC-containing (RAW-C1d1 cells) and APC-free (plate-bound C1d1) experiments to determine reactivity to *Listeria* phosphatidylglycerol–LC-MS fraction E (Table III). In the APC-free system, three of the hybridomas (14S.6, 14S.10, and TBA7) were reactive to *Listeria* phosphatidylglycerol. These results match what we (13) found for *Corynebacterium* phosphatidylglycerol. In the APC-containing system, an additional two dNKT hybridomas (VII68 and VIII24.1.D) were activated by *Listeria* phosphatidylglycerol. Because we generally found that the plate-bound C1d1 system is less sensitive for weak Ags than the APC-containing system, this likely reflects a low-affinity reactivity for *Listeria* phosphatidylglycerol by these two hybridomas.

The high activity of *Listeria* phosphatidylglycerol in the C1d1 plate-bound assay compared with other established phosphatidylglycerol sources prompted us to determine whether *Listeria* phosphatidylglycerol-loaded C1d1 tetramers would bind to TBA7 cells. In the C1d1 plate-bound assay, excess detergent and lipid are washed away before adding hybridoma cells to the wells. To minimize excess detergent and lipid in the tetramer preparations, we first optimized the system by modifying our lipid-loading protocol. In addition, we generated hybridoma cell lines that expressed high levels of TCR. The TCR-immortalized T cell line BWS8 was transfected with C3D and the TBA7 CTR (TBA7high) or a typical iNKT cell TCR (Vβ8.2) (35). As expected, C1d1 tetramers loaded with PBS-57 (a synthetic α-GalCer analog) stained Vβ8.2 cells but not TBA7high cells (data not shown).

Further, *Listeria* phosphatidylglycerol-loaded C1d1 tetramers did not bind to Vβ8.2 cells (Fig. 5C), the parent BW58 hybridoma cells (data not shown), or BW58 cells transfected with the phosphatidylglycerol-nonreactive dNKT XV19 hybridoma cell TCR (data not shown). In contrast, we found that tetramers loaded with *Listeria* phosphatidylglycerol–LC-MS fraction E stained TBA7high cells, whereas mock-loaded C1d1 tetramer or tetramers made of C1d1 loaded with the irrelevant lipid DGDG from *S. pneumoniae* (DGDG-Sp) bound only at background levels (Fig. 5C, left panels). Together, these functional and tetramer-staining experiments demonstrate that *Listeria* phosphatidylglycerol-loaded C1d1 is a cognate Ag for the dNKT TBA7 TCR.

*Listeria* phosphatidylglycerol–LC-MS fraction E–loaded tetramers gave a signal that was ∼2-fold higher than vehicle-loaded tetramers (Fig. 5C, right panels). We next attempted to increase the positive–negative signal separation using dextramer technology. Dextramers are dextran backbones containing multiple fluorophore and streptavidin binding sites/molecule (42). Because they contain 12–24 Ag-presenting molecules/dextramer backbone, they are useful for identifying rare primary T cell populations because of their increased TCR avidity and fluorescence compared with tetramers. Indeed, Kasmar et al. (43) successfully used C1d1 dextramers to identify dioxygenumycobactin-restricted T cells ex vivo from human PBMCs. We generated mock-loaded C1d1 dextramers along with *Listeria* phosphatidylglycerol–LC-MS fraction E–, phosphatidylglycerol from *C. glutamicum* (phosphatidylglycerol-Cg), or DGDG-Sp–loaded C1d1 dextramers and used them to stain dNKT TBA7high BW58 cells transfected with the 14S.6 CTR (14S.6high), as well as iNKT Vβ8.2 cells (Fig. 5D, left panel, data not shown). We found that phosphatidylglycerol LC-MS fraction E–loaded C1d1 dextramers specifically bound to TBA7high cells and gave a signal that was ∼8-fold higher than for vehicle-loaded C1d1 dextramers (Fig. 5D, right panel). The phosphatidylglycerol LC-MS fraction E–loaded C1d1 dextramers did not bind to 14S.6high cells (data not shown), suggesting that the 14S.6 CTR affinity for this complex is very low. Finally, we also tested the ability of the less active phosphatidylglycerol from *Corynebacterium* to identify TBA7 CTR–expressing T cells with this newly optimized system. Phosphatidylglycerol-Cg–loaded C1d1 dextramers also consistently stained the TBA7high cells, albeit to a much lower degree than did the *Listeria* phosphatidylglycerol–LC-MS fraction E–loaded dextramers; however, the difference did not reach statistical significance (∼1.5-fold higher MFI than mock loaded).

Previous studies on iNKT cell Ags demonstrated that alterations in the fatty acid tails of lipids can dramatically alter their activity, either by modulating their binding to C1d1 or by indirectly affecting iNKT TCR recognition (44–46). *Listeria* phosphatidylglycerols are dominated by short chain lengths and fully saturated anteiso methyl-branched fatty acids. In contrast, the less antigenic mammalian or *Corynebacterium* phosphatidylglycerol have longer acyl chains, are unsaturated, and lack anteiso branches. These differences in fatty acid tail composition could alter how well the lipid binds into C1d1, alter the orientation of the phosphatidylglycerol headgroup into a more favorable position for TCR binding, or alter C1d1 conformation that subsequently impacts on TCR binding. To test whether the more potent *Listeria* phosphatidylglycerol–LC-MS fraction E loads into C1d1 more efficiently than *Corynebacterium* phosphatidylglycerol, we performed a GD3 lipid-displacement assay. GD3 is a negatively charged lipid with a large sugar-based headgroup that can be displaced from C1d1 by other lipids. We loaded C1d1 with GD3, purified the GD3–loaded C1d1 complexes by MonoQ anion-exchange chromatography, and measured the ability of *Listeria* or *Corynebacterium* to identify dideoxymycobactin-restricted T cells with...
phosphatidylglycerol to displace GD3, which results in earlier elution relative to the CD1d–GD3 complex. We found that Listeria phosphatidylglycerol–LC-MS fraction E displaced ~2-fold more GD3 than did Corynebacterium phosphatidylglycerol under the same conditions (35% versus 19% loaded, Fig. 6B).

We next used SPR to determine whether the TBA7 TCR affinity for Listeria phosphatidylglycerol–LC-MS fraction E differed from that of Corynebacterium phosphatidylglycerol. CD1d was loaded with Listeria phosphatidylglycerol or Corynebacterium phosphatidylglycerol using the GD3-displacement approach described above to ensure optimal loading. CD1d with endogenous lipid Ag was used as a control. These preparations were immobilized to the streptavidin SPR chips via their biotin tags. Purified and soluble TBA7 TCR was then passed over CD1d-Ag, and the binding affinity was measured (in RU). The TCR affinity for Listeria phosphatidylglycerol–LC-MS fraction E–loaded CD1d ($K_D = 71 \mu M$) was similar to CD1d loaded with Corynebacterium phosphatidylglycerol ($K_D = 94 \mu M$) (Fig. 6C, 6D). For comparison, these affinities are lower than the previously published TCR affinities for the dNKT hybridoma XV19 TCR binding to sulfatide + CD1d ($K_D = 24 \mu M$) or the nanomolar iNKT TCR affinity observed for α-GalCer–loaded CD1d ($K_D = 0.07 \mu M$) (6, 38). Accordingly, our data suggest that the increased potency of Listeria phosphatidylglycerol is probably not due to higher-affinity interactions with the TCRs; rather, it may be attributable to improved loading and/or binding to CD1d.
Table III. Reactivity of dNKT hybridomas to Listeria phosphatidylglycerol–LC-MS fraction E.

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>RAW-CD1d APCs</th>
<th>Plate-Bound CD1d</th>
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<tr>
<td>14S.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14S.10</td>
<td>+</td>
<td>+</td>
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<tr>
<td>14S.15</td>
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<td>431.A11</td>
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<td>TBA7</td>
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<td>VII68</td>
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<td>–</td>
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<tr>
<td>VII24.1.D</td>
<td>+</td>
<td>–</td>
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<td>XV19.2</td>
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Reactivity to Listeria phosphatidylglycerol LC-MS fraction E was tested in APC-containing (RAW-CD1d cells) and APC-free (plate-bound CD1d) systems. +, reactive; –, not reactive.

Discussion

There is a growing appreciation that many T cells do not recognize peptides in the context of MHC class I or MHC class II molecules (47). Such non-MHC–restricted T cells, which can recognize lipid Ags (48) or riboflavin metabolites (49), include NKT cells (CD1d (47). Such non-MHC–restricted T cells, which can recognize lipid peptides in the context of MHC class I or MHC class II molecules

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Microbial lipid Ags are ideal targets for dNKT cells. Although mammalian self-lipids may be responsible for dNKT cell selection in the thymus, like self-peptides for MHC-restricted T cells, the most potent Ags recognized in the periphery may be of microbial origin. Because Listeria is an intracellular microbial pathogen, it was an attractive model organism for identifying Ags for dNKT cells (29, 57). By performing an unbiased search for Listeria lipid Ags, we identified the microbial versions of two known dNKT cell phospholipid Ags, phosphatidylglycerol and DPG, as dNKT cell Ags. By measuring the concentration of Listeria phosphatidylglycerol needed to activate 14S.6 and TBA7 hybridoma cells as compared to phosphatidylglycerol-Cg (Fig. 4B), we found that Listeria phosphatidylglycerol is a 10–100-fold more potent Ag than the previously published mammalian or the structurally related Corynebacterium phosphatidylglycerol.

These results prompted us to consider why Listeria phosphatidylglycerol is a more potent Ag than the structurally similar Corynebacterium/mammalian phosphatidylglycerol. Because these two lipids share identical headgroups, it was unsurprising to find that the dNKT TBA7 TCR bound to CD1d loaded with either Listeria or Corynebacterium phosphatidylglycerol with similar affinities. However, we found that Listeria phosphatidylglycerol loaded into CD1d (displacing the charged lipid GD3) ~2-fold more efficiently than did Corynebacterium phosphatidylglycerol. Because TCR activation leads to signaling cascades that can exponentially amplify the original signal, we attribute the higher potency of Listeria phosphatidylglycerol to its increased CD1d loading efficiency over Corynebacterium phosphatidylglycerol. However, we cannot discount the possibility that Listeria phos-
phatidylglycerol is more stably bound within CD1d over time as compared to Corynebacterium phosphatidylglycerol.

There are three differences between the fatty acid tails found in Listeria that could be modulating its ability to load or stay within CD1d more efficiently than mammalian phosphatidylglycerol. These differences include the presence of anteiso methyl groups, lack of a double bond, and shorter tail lengths. One hypothesis is that the anteiso methyl fatty acid branches (found only in some microbes) act as a hook within the CD1d hydrophobic channels and increase the stability of the lipid–CD1d complex. Alternatively, it is possible that other aspects of Listeria phosphatidylglycerol, such as its shorter tails or lack of a double bond, make it easier to load or remain bound within CD1d molecules.

Our previous efforts to generate Corynebacterium or mammalian phosphatidylglycerol CD1d tetramers were not successful. However, identification of the more potent Listeria phosphatidylglycerol variant prompted us to determine whether CD1d tetramers loaded with Listeria phosphatidylglycerol could bind with sufficient avidity to TBA7 cells to stain in flow cytometry. Indeed, Listeria phosphatidylglycerol–LC-MS fraction E–loaded CD1d tetramers specifically bound to TBA7 TCR-transduced cells but not to the same cells transduced with other (or no) TCRs (Fig. 5C, data not shown). By using dextramers that contain ~12 CD1d molecules and multiple fluorophores/molecule, we were able to further increase the signal/noise ratio so that the majority of the Listeria phosphatidylglycerol dextramer-stained cells could be separated from the mock- or irrelevant lipid–dextramer-stained cells. This increase in signal may be critical, because it provides a new reagent that is suitable to identify and interrogate T cells to recognize sulfatide self antigens.

In summary, we identified a new lipid Ag for dNK cells with a distinctively microbial signature: short, fully saturated anteiso lipid tails. Notably, this microbial version of phosphatidylglycerol is much more active than the previously known phosphatidylglycerol Ags from mammals or Corynebacterium, which have structurally related lipid tails. Importantly, by identifying high-potency microbial dNK cell Ags for different dNK cell populations, we can begin to dissect the poorly understood nature of these differences including the presence of anteiso methyl groups, lack of a double bond, and shorter tail lengths. One hypothesis is that the anteiso methyl fatty acid branches (found only in some microbes) act as a hook within the CD1d hydrophobic channels and increase the stability of the lipid–CD1d complex. Alternatively, it is possible that other aspects of Listeria phosphatidylglycerol, such as its shorter tails or lack of a double bond, make it easier to load or remain bound within CD1d molecules.

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Disclosures

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References


