CD1c tetramers detect ex vivo T cell responses to processed phosphomycoketide antigens

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Discovery of CD1 antigen presentation pathways shows how αβ T cells can specifically respond to self- and foreign lipid components of cells. Whereas most CD1 research focuses on CD1d and NKT cells, there are four types of CD1 antigen-presenting molecules, which are each broadly conserved in mammalian evolution (Kasmar et al., 2009). Recent data make clear that CD1a, CD1b, CD1c, and CD1d do not have redundant functions but instead have clearly differing patterns of tissue expression, distinctly shaped antigen binding grooves, diverse pathways of intracellular trafficking, and trigger differing effector functions of responding T cells (Young and Gapin, 2011). CD1c proteins are distinguished from other CD1 proteins by their constitutive expression at high density on the surface of marginal zone or activated B cells (Weller et al., 2004; Roura-Mir et al., 2005a; Allan et al., 2011) and subsets of DCs (Dzionek et al., 2000). Unlike CD1d, which is constitutively expressed on monocyte-derived DCs (MDDCs), CD1c is inducibly expressed on MDDCs after cytokine exposure (Porcelli et al., 1993; Yakimchuk et al., 2011). Thus, CD1c proteins have been proposed to mediate interactions of T cells with B cells and DCs as two major populations of APCs.

Despite extensive knowledge of CD1c protein trafficking in cells, the antigen-presenting function and antigens for the CD1c system are poorly understood. After translation, CD1c is expressed with high density on human dendritic cells (DCs) and B cells, yet its antigen presentation functions are the least well understood among CD1 family members. Using a CD1c-reactive T cell line (DN6) to complete an organism-wide survey of M. tuberculosis lipids, we identified C32 phosphomycoketide (PM) as a previously unknown molecule and a CD1c-presented antigen. CD1c binding and presentation of mycoketide antigens absolutely required the unusual, mycobacteria-specific lipid branching patterns introduced by polyketide synthase 12 (pks12). Unexpectedly, one TCR responded to diversely glycosylated and unglycosylated forms of mycoketide when presented by DCs and B cells. Yet cell-free systems showed that recognition was mediated only by the deglycosylated phosphoantigen. These studies identify antigen processing of a natural bacterial antigen in the human CD1c system, indicating that cells act on glycolipids to generate a highly simplified neoepitope composed of a sugar-free phosphate anion. Using knowledge of this processed antigen, we generated human CD1c tetramers, and demonstrate that CD1c–PM complexes stain T cell receptors (TCRs), providing direct evidence for a ternary interaction among CD1c–lipid–TCR. Furthermore, PM-loaded CD1c tetramers detect fresh human T cells from peripheral blood, demonstrating a polyclonal response to PM antigens in humans ex vivo.
are branched chain phospholipids: synthetic mannosyl phospholipids (MPDs) and mycobacterial mannosyl-β-1-phosphomycoketide (MPM; Moody et al., 2000; Matsunaga et al., 2004). The latter is known to bind within the A pocket of the CD1c groove (Scharf et al., 2010). Patients infected with *M. tuberculosis* have CD1c-dependent polyclonal responses to MPD, providing direct evidence that expansion of phospholipid-reactive T cells is part of the natural history of tuberculosis infection (Moody et al., 2000).

In this study, we sought to identify new antigens in the CD1c system and to develop CD1c tetramers as new tools for biological investigation. We used a CD1c-reactive T cell line (DN6) as a reporter to screen through all types of lipids in *M. tuberculosis*. This organism-wide screen for lipids, combined with genetic and synthetic chemistry approaches, identified a previously unknown molecule, C32 PM, as a CD1c-presented antigen. Using natural and synthetic analogues of mycoketides, we determined that T cell activation by diverse glycolipids is mediated by cellular processing reactions in activated B cells and DCs, which release carbohydrate head groups and reveal a simplified epitope derived from structurally diverse precursors. After identifying the molecular features of PM epitopes needed to contact TCRs,
we generated working CD1c tetramers loaded with PM, which identified polyclonal PM-specific T cells in ex vivo analysis of human blood.

RESULTS

Discovery of PMs in M. tuberculosis

The CD4<sup>+</sup>CD8<sup>-</sup> T cell line DN6 (Beckman et al., 1996) represented a highly useful reporter line to detect new kinds of CD1c-presented antigens because its activation was entirely dependent on, and specific for, CD1c (Fig. 1 A). Furthermore, we reasoned that this clone might detect a natural antigen that is produced during mycobacterial infection because DN6 was specifically activated by lipid extracts of medically important mycobacteria, like <i>M. tuberculosis</i> and <i>Mycobacterium avium</i>, but not soil-dwelling mycobacteria or other bacteria such as <i>Rhodococcus equi</i>, <i>Nocardia farcinia</i> or <i>Escherichia coli</i> (Fig. 1 B).

To isolate the antigens recognized by DN6, we subjected <i>M. tuberculosis</i> to extraction with chloroform and methanol solvents to recover total extractable lipids. We separated these lipids into subclasses using silica columns sequentially eluted with chloroform, acetone, and methanol, which enrich for neutral lipids, glycolipids, and phospholipids, respectively (Fig. 1 C).

After T cell testing showed selective response to the methanol eluate, these polar lipids were further resolved in the normal phase by preparative TLC. Using nondestructive visualization with water spray, 10 bands of lipid-laden silica were scraped, extracted, and screened for stimulatory capacity. The visualized bands were numbered so that higher numbers correspond to higher retention factors (R<sub>f</sub>). We found that DN6 was activated in a dose-dependent activation by bands 3 and 4, with the highest titer noted for band 4 (Fig. 1 C).

To chemically identify the antigen, we further studied band 4 through separation into subfractions using HPLC–mass spectrometry (HPLC-MS). Mass spectrometry revealed an ion at m/z 545.469 that was consistently present in antigenic subfractions of band 4.
This candidate antigen was named Ag545 based on its nominal mass. The accurate mass of Ag545 matched C32H66O4P within 1 ppm, and no other chemical formula matched within the mass accuracy of the QToF (quadrupole time of flight) mass detector. We could not identify any known molecule with this chemical composition in the literature or recently compiled databases of M. tuberculosis lipids (Layre et al., 2011; Sartain et al., 2011). Collision-induced dissociation mass spectrometry (CID-MS) identified a fragment corresponding to phosphate, \( m/z \) 78.959 (expected \( m/z \), 78.9585), with neutral loss of 466.512 (Fig. 1 D). This pattern allowed assignment of Ag545 as a fully saturated C32 alkyphosphate. The C32 length was notable because it is unusual for fatty acids and other common alkane lipids but did match the length of C32 mannosyl β-1 PM (MPM), a previously known CD1c-presented antigen (Matsunaga et al., 2004). Synthesis of antigenic PMs

Based on an apparent structural analogy, we hypothesized that Ag545 was C32 PM, which lacked the mannosyl unit seen in MPM (Fig. 2 A). Because Ag545 was produced only in trace amounts and resistant to extensive collision-induced dissociation (Fig. 1 D), the precise structure of its lipid moiety could not be solved by mass spectrometry. However, based on the assumption that the known mannosyl PM and Ag545 share the same C32 lipid moiety with five repeating branches in the S-configuration (C32 all-β; de Jong et al., 2007), we used stereospecific condensation methods (van Summeren et al., 2006) to synthesize C32 all-S PM. This product matched the retention time and CID-MS pattern as Ag545 from band 4, and it potently stimulated DN6 at nanomolar concentrations (Fig. 1 D and not depicted). These studies identified C32 PM as a previously unknown molecule in M. tuberculosis and as a CD1c-presented antigen. DN6 promiscuously recognizes phospholipids

These data proved that C32 all-S mycoketides are presented by CD1c to T cells, but two aspects of the antigen discovery effort suggested that DN6 responds promiscuously to other mycobacterial phospholipids. First, testing of DN6 to all HPLC subfractions (designated X.Y, where X is the TLC band and Y is the HPLC subfraction) demonstrated a response profile to purified lyso-phospholipids and phospholipids matching to common phospholipids (Layre et al., 2011), identified at least six named anionic phospholipids in fractions 3 and 4 (Fig. 2 C and not depicted). These lipids included lyso-phosphatidylethanolamine or lyso-phosphatidylinositol dimannoside (PIM2), which are similar or identical to known CD1d- or CD1b-presented antigens (de la Salle et al., 2005; Fox et al., 2009; Zeissig et al., 2012). Therefore, we tested DN6 response to purified lyso-phospholipids and phospholipids matching those detected in bands 3 and 4, as well as other structurally related molecules. The lack of response to all tested phospholipids ruled out extreme promiscuity (Fig. 2 C, right).

DN6 might still recognize lipids that were missing from the panel of tested phospholipids or represent some nonmycoketide that could not be detected by LC-MS in M. tuberculosis. Therefore, we tested DN6 response to M. tuberculosis that is selectively deficient in all mycoketides as the result of an engineered deletion in the enzyme that produces mycoketides, pks12, of strain H37Rv. Total lipids from the strains (H37RvΔpks12) or the equivalent deletion in M. tuberculosis strain 1551 or BCG failed to activate DN6 (Fig. 2 D). Yet DN6 did recognize lipids from wild-type and genetically complemented strains at titers expected based on levels of Pks12 complementation. These genetic experiments ruled out cross-recognition of differing lipid anchors, proving that core mycoketide backbone is necessary for the T cell response.

The molecular basis of mycoketide recognition

Next we tested whether the broad response profiles (Fig. 2 B) might result from cross-reactivity of DN6 to differentially modified mycoketides in the biosynthetic pathway (Fig. 2 A). Because structure-function studies of bacterial sources can be confounded by traces of nearly coeluting natural molecules, we synthesized pure, all-synthetic (S) mycoketides with free alcohol, phosphate, and α- or β-1-linked phosphomannose groups (Fig. 3 A). Whereas the CD8-1 T cell line was highly specific for β-mannosylated mycoketides (Moody et al., 2000), DN6 responded promiscuously to unglycosylated and β-mannosylated analogues (Fig. 3 A). The cross-reactivity of DN6 for PM and MPM likely explained the broad response profile of DN6 to mycobacterial lipid subfractions because PM was present in band 4 and MPM in band 3 (Fig. 1 D; and Fig. 2, B and C). Synthetic molecules provided further insights into the pattern of DN6 T cell response, including an absolute requirement for a phosphate moiety. Surprisingly, DN6 showed cross-reactivity for the nonnatural analogue in which mannose is carried in the α-anomeric linkage (Fig. 3 A). These patterns of reactivity, seen with synthetic all-S mycoketide analogues (Fig. 3 A), were further tested with differentially phosphorylated or glycosylated molecules coupled to sterorandom mycoketide or dolichol backbones (Fig. 3, B and C). Among all analogues, we observed a consistent pattern of recognition in that the phosphate was absolutely required, but only DN6 promiscuously recognized glycosylated or unglycosylated analogues (Fig. 3, B and C).
Considering the biological origins of lipids, mycoketides are highly unusual structures that differ from common mammalian straight chain lipids based on their size (C32), length (C27), and five methyl branches in the (S) stereocorefiguration. To directly test the requirement of T cell specificity for the branches that characterize the mycoketide lipid moiety, we tested DN6 response to MPM produced in a form that are shorter in length or in which the (S) methyl branches were missing or converted to (R) stereochemistry (van Summeren et al., 2006; de Jong et al., 2007). The analogues lacking some or all of the methyl branches begin to mimic straight chain lipids. DN6 showed a 100-fold reduction in dose response when one out of five methyl branches were present in the S-configuration. We did not detect any stimulation from analogues with one methyl branch in the R-configuration, or with no methyl branches, or with a shortened lipid (Fig. 3 D). Furthermore, CD1c–lipid complexes were formed and induce an isoelectric focusing (IEF) shift, when all methyl branches were present (Fig. 3 D, inset). Although the shift was small in magnitude, it was reproducible and similar in magnitude to that seen in prior studies of crystallized CD1c–MPM complexes (Scharf et al., 2010). Thus, the S-methyl branches, which are defining for mycoketides, are required for binding to CD1c and activation of T cells.

**CD1c presents two antigens in the mycoketide pathway**

The structural homology of PM and MPM suggested a possible precursor-product relationship (Fig. 2 A). Pks12 (polyketide synthase 12) and Ppm1 (polyprenol monophosphomannose synthase) are two linked genes encoding enzymes with the catalytic domains needed to condense malonyl and methyl malonyl units and to transfer a mannosyl unit, respectively (Gurcha et al., 2002; Matsunaga et al., 2004). Thus, identification of PM clarifies the mechanism of MPM biosynthesis...
Antigen processing of CD1c presented lipids

Based on a recent crystal structure of CD1c with MPM (Scharf et al., 2010), complexes formed with glycolipids of differing α- or β-anomeric linkage would position the mannose at markedly different positions on the outer surface of CD1c, or, alternatively, strain the bond at C1 on mannose. In one well studied example, NKT cells recognize both α- and β-linked hexosyl ceramides, but the linkage influences potency by >100-fold as a result of carbohydrate positioning and C1 bond strain (Brennan et al., 2011; Pellicci et al., 2011). In other examples, the linkage of the proximal carbohydrate is an all-or-nothing determinant of recognition (Kawano et al., 1997; Moody et al., 1997, 2000). Therefore, it would be surprising if mycoketide epitopes formed with α-linked, β-linked, or no carbohydrate were recognized with equal potency (Fig. 3).

We considered the possibility that diverse glycolipid analogues were processed by APCs to create one core epitope so that the same epitope was formed with three different input molecules. APCs trim synthetic or natural glycolipids for presentation by CD1b and CD1d (Prigozy et al., 2001; Zhou et al., 2004; de la Salle et al., 2005). However, whether bacterial glycolipids are frequently processed by CD1c as the site of origin for T cell recognition is unknown.

Cross-reactivity of DN6 to mycoketides with hydrophilic head groups composed of phosphate alone, α-mannosyl, or β-mannosyl units was surprising and remained unexplained. The DN6 line might contain several clonal populations with distinct TCRs that separately recognize each PM analogue, or the T cell line might be activated by TCR-independent means, similar to lipopolysaccharide activation of NKT cells (Brigl et al., 2003). Using DN6 cultures, we amplified TCR-α and -β chain sequences using a diverse TCR primer set based on the international ImMunoGeneTics (IMGT) database and detected several minor bands and two dominant bands corresponding to one TCR-α (TRAV36/DV7) and one -β chain (TRBV6-6). After cloning, we cotransfected these chains into the T lymphoblastoid cell line Hut78 (Fig. 4 A). Whereas untransfected Hut78 with an endogenous TCR showed no reactivity, the DN6 TCR transfec tant was activated by PM, α-MPM, and β-MPM. Therefore, DN6 response was mediated by one αβ TCR that promiscuously recognizes three distinct forms of mycoketide.
Development of CD1c tetramers

Whereas initial efforts to detect polyclonal human T cells with CD1c tetramers loaded with MPM were unsuccessful, detailed knowledge of the processed epitopes recognized by the TCR offered a new approach to CD1c tetramer development. Based on approaches used previously for MHC class I, CD1d, and CD1b (Altman et al., 1996; Matsuda et al., 2000; Karadimitris et al., 2001; Lee et al., 2002; Kasmar et al., 2011), we produced and validated human CD1c tetramers bound to C32 PM. Transmembrane truncated CD1c heavy chains were linked to \( \beta \)-2 microglobulin using leucine zippers and biotinylated using a BirA enzyme. Polyacrylamide gel electrophoresis showed that CD1c–\( \beta \)2m complexes migrated with the expected apparent molecular weight and showed high percentage of biotinylation in avidin pulldown experiments (Fig. 5 A). Also, IEF gel-shift assay of CD1c loaded with PM induced a subtle but visible shift of protein, demonstrating complex formation (Fig. 5 B).

We favored a cognate model in which CD1c–antigen complexes might bind the DN6 TCR. However, alternative models show that certain bacterial lipids activate T cells indirectly through induced cytokine secretion (Brigl et al., 2011) or through bacterially induced endogenous lipid production (De Libero et al., 2005; Brennan et al., 2011). Also, recent studies show that CD1 interacts with cell surface receptors other than TCR, such as Ig-like transcript (ILT; Li et al., 2009, 2012). To distinguish among these models and attempt to detect the ternary complex, DN6 was stained with newly generated CD1c tetramers loaded with C32 PM. This line was comprised of 67% of cells that expressed the clonotypic TRBV6-6+ TCR and 33% of cells with other TCRs, providing a negative control for staining (Fig. 5 C). PM-loaded but not unloaded or rarely altered before TCR recognition was unknown, and antigen processing had not been previously reported for the CD1c system. Conversion of \( \alpha \)- and \( \beta \)-linked MPM to PM product in cells was plausible based on CD1c’s broad trafficking throughout the endosomal network, including lysosomes (Briken et al., 2000; Sugita et al., 2000).

We tested this model by removing all cellular processing factors and compared responses of native T cells to PM, \( \beta \)-MPM, and \( \alpha \)-MPM presented by recombinant CD1c proteins affixed to a plate. In contrast to cells, which present all three antigens, recombinant CD1c presents only the core PM epitope to the native DN6 line (Fig. 4 B) and HUT cells expressing the DN6 TCR (Fig. 4 A). Strong CD8-1 activation at low doses indicated that \( \beta \)-MPM–CD1c complexes were formed under these conditions. Thus, presentation of \( \alpha \)- and \( \beta \)-linked forms requires cellular processing, and the \( \beta \)-mannose unit is neither required nor permissive for activation of DN6. Whereas initial experiments were performed in K562 tumor cells, the same pattern of recognition was seen when presented by myeloid DCs (Fig. 3) and activated human B cells (Fig. 4 C). These studies confirm a recent study reporting that CD1c expression and function on human B cells is promoted by cellular activation (Fig. 4 C; Allan et al., 2011). More generally, these findings indicate that antigen processing occurs efficiently in native human cells that normally express CD1c and strongly support a model in which \( \alpha \)- and \( \beta \)-linked mannose units are removed by APCs so that TCRs contact simple sugar-free phosphate epitope. Binding of TCRs to CD1c had not been previously reported, but development of CD1c tetramers loaded with PM antigens provides a direct test for the key prediction of this model: the deglycosylated epitope might mediate binding of CD1c to a TCR.

Figure 5. C32-PM–loaded CD1c tetramers demonstrate ternary CD1c–TCR interactions. (A) Page gel demonstrates the purity and apparent mass of CD1c: \( \beta \)2M monomers. (B) Native isoelectric focusing gel analysis shows a shift of CD1c loaded complexes at 5 or 20 molar excess C32-PM. (C) C32-PM–loaded CD1c tetramer stains the polyclonal DN6 cell line, which includes a subpopulation of TRBV6-6 TCRs that mediates reactivity to PM. Values within plots indicate percent of all cells within gates and quadrants. (D) C32-PM–loaded CD1c tetramer staining of SupT1 transfected with DN6 TCR.
tetramers brightly stained cells expressing TRBV6-6. Furthermore, lack of staining of TRBV6-6 cells (Fig. 5 C) and the lack of staining of T cells with unloaded tetramers provided evidence that the interaction requires antigen and is specific for TCR structure. Furthermore, we observed PM-dependent staining of the TCR–transfected, but not untransfected, SupT1 cells, proving that the αβ TCR is the sole ligand sufficient for mediating binding of CD1c–PM complexes on the cell surface (Fig. 5 D). We conclude that CD1c binds specifically to the clonotypic DN6 TCR, in a ternary interaction mediated by the PM antigen.

CD1c tetramers detect PM-reactive T cells ex vivo

Production of working tetramers of human CD1c has been a longstanding but unrealized goal in the field. Prior studies showed that MPD added to human DCs causes CD1c-dependent proliferation of T cells in latent tuberculosis patients at higher numbers as compared with uninfected control patients (Moody et al., 2000). Such activation assays provide evidence for CD1c and phospholipid-mediated expansion of T cells during natural tuberculosis infection. CD1c tetramers directly bind to the TCR in ways that rule out potentially confounding results common to all activation assays, including indirect T cell activation by bacterial products and T cell activation by non–T cells. Also, only tetramers allow direct determination of the epitope recognized when unprocessed antigens are added to DCs or other APCs. Therefore loading CD1c tetramers with PM offered an opportunity for direct biophysical detection of fresh CD1c-restricted T cells in humans and mapping of candidate PM epitopes from MPM lipids.

Polyclonal T cells from PBMC of donors with latent M. tuberculosis were stained with PM-loaded CD1c tetramers. PM-loaded but not unloaded CD1c tetramers stain T cells (Fig. 6 A). This result potentially provided proof for CD1c and lipid-reactive cells ex vivo based on biophysical interactions, but it remained formally possible that CD1c was binding to ligands like ILT4 rather than αβ TCRs (Li et al., 2012). Thus, PM-loaded CD1c tetramer stained cells were sorted and expanded and cloned. With this method, we could consistently generate cell lines from all donors tested that stained with C32–PM loaded but not unloaded CD1c tetramer (Fig. 6). Generated lines were initially screened for binding of C32–PM loaded tetramer and then screened for antigen reactivity (Fig. 6 A). All eight newly derived T cell lines were activated by APCs only in the presence of CD1c and antigen, as expected from a TCR-mediated response (Fig. 6). Furthermore, polyclonally expanded lines and T cell lines derived from limiting dilution cloning remained responsive to C32–PM even after repeated expansion in culture (Fig. 6, C and D). DN6 and CD8–1 are CD4–CD8– and CD8+ clones, respectively, and were derived using earlier methods that started with CD4+ T cells to avoid alloreactivity (Porcelli et al., 1989). All CD1c-reactive lines derived here, using an unbiased approach based on CD1c tetramers, are CD4+ (Fig. 6). Finally, newly derived T cell lines captured based on their TCRs binding to C32 PM were activated by both glycosylated and unglycosylated mycoketides, when presented by APCs, consistent with recognition of epitopes processed by deglycosylation (Fig. 6 E). We conclude that C32–PM responsive T cells are present in vivo and that conversion of MPM to yield PM is a generalizable antigen processing recognized by diverse human T cell clones.

DISCUSSION

Here we report the second known natural foreign antigen in the CD1c system as a C32 (all–S) PM. This molecule likely functions as the biochemical precursor of the only other known bacterial antigen for CD1c, mannosyl–β–1–PM (Moody et al., 2000). It is notable that both PM and MPM were both unknown molecules until discovered through unbiased bacterium-wide surveys of many hundreds of lipid types using CD1c-restricted T cells as probes. Based on the global nature of the screens used, the detection of two antigens in the same pathway with the same lipid anchor suggests a specialized role of the CD1c groove in mycoketide capture. Contrasting with the more abundant phospholipids comprised of straight alkyl chains that are common in both mycobacterial and mammalian cells, mycoketides contain five alkyl branches with S configuration, which can be considered a pathogen–specific molecular pattern. A recent crystal structure of MPM bound to CD1c provides a specific model of how the methyl branches in the mycoketide backbone promote binding (Scharf et al., 2010). The C32 mycoketide lipid enters and fully occupies the A’ pocket, which is a toroid cavity buried under the A’ roof. The branched mycoketide moiety is positioned like a spiral staircase such that the linear backbone carbons occupy the smaller inner surface of the toroid and the methyl branches fan out to occupy the larger, outer diameter of the pocket. Here we show that these foreign branches, which are characteristic of mycoketides and absent from common unbranched self-lipids, are the molecular feature needed for binding to CD1c. Furthermore, no lipid produced independently of Pks12 can substitute in generating this CD1c-mediated T cell response. Overall, independent detection of two mycoketides as CD1c-restricted antigens, the hand-in-glove fit of the mycoketide to the A’ pocket of CD1c, and direct evidence for class-defining methyl branches in CD1c binding suggest that mycoketides represent a natural foreign, optimized lipid anchor for CD1c. Although no single antigen would be expected to occupy all CD1c proteins in a cell, these data point to repeating methyl branches as a mechanism for efficient capture of antigens produced at low levels from among the much larger pool of self-lipids.

In cell-free assays, T cell clones do not cross-react between PM and MPM, so the two molecules are separate and valid antigens recognized by distinct TCRs within the CD1c system. Yet activated B cells and MDDCs present MPM to PM-specific TCRs, providing evidence for antigen processing to create a neopeptide formed from a simple phosphate anion. PM and MPM are likely in the middle and end of the MPM biosynthetic pathway, respectively, and only end products of pathways typically accumulate. Supporting this prediction, lipidomic detection of MPM signal occurs at higher intensity than PM, which is just above the limits of detection of sensitive mass
Figure 6. Ex vivo detection of C32-PM–reactive T cells with lipid–loaded CD1c tetramers. (A) T cell–enriched staining of PBMC with C32-PM–loaded CD1c tetramers ex vivo shows frequency of gated population among CD3+ cells. (B) A short-term T cell line derived from tetramer–positive sorting was screened for C32-PM–loaded CD1c tetramer staining and IFN-γ release upon stimulation with C32-PM presented by CD1-expressing APC. (C and D) Polyclonal T cells (C) and T cell lines generated by limiting dilution cloning (D) maintain recognition of C32-PM after repeated expansions when tested at 30 or 80 d after derivation. (E) IFN-γ release by T cell clones stimulated with C32-PM and C32-MPM presented by CD1-expressing APC was measured. In all cases, cell lines were screened for antigen reactivity two or more times, except for donor 1 cell line 22 and donor 3 cell line 30 which failed to propagate long-term after the assays shown here. Data are representative mean ± SD of triplicate measurements.
encounter CD1c. In this scenario, the antigenic function of PM is more physiologically important, considered as a processed product of MPM rather than a de novo product of biosynthesis.

Aside from these biological considerations, detection of a processed epitope provides key practical information for mapping of natural epitopes and detecting ex vivo T cell response. Whereas prior attempts to load CD1c tetramers with MPM and detect T cells ex vivo have been unsuccessful, successful development of CD1c tetramers derives from the insight that PM is a processed product and a natural epitope. Also, a key study that previously detected polyclonal human responses to MPM rather than a de novo product of biosynthesis.

The results are provided from M. tuberculosis CDC1551 and Mycobacterium smegmatis 607 obtained from the Centers for Disease Control and Prevention. M. bovis BCG, Mycobacterium phlei, Mycobacterium fallax, Rhodococcus equi, and Nocardia farcinica were provided from the American Type Culture Collection. M. tb H37Rv Δpks12, M. tb H37Rv Δpks12::pks12, M.tb CDC1551 Δpks12, and M. bovis BCG Δpks12 were generated as previously described (Matsuura et al., 2004). M. tuberculosis H37Rv were maintained on Middlebrook 7H10 plates supplemented with oleic acid–albumin–dextrose complex. For liquid culture, a single colony was inoculated in Middlebrook 7H9 media with 0.2% glycerol supplemented with 10% albumin–dextrose and grown to mid-log phase. M. phlei, M. fallax, Rhodococcus equi, and Nocardia farcinica were grown as previously described (Moody et al., 2002).

**Lipid extraction, chromatography, and synthesis.** Bacteria harvested near mid-log phase were extracted at 5 mg/ml in chloroform/methanol (C/M; 2:1, vol/vol) for 1 h at room temperature followed by centrifugation and removal of supernatants. The pellet was reextracted in C/M (1:1, vol/vol) and C/M (1:2, vol/vol), and all three supernatants were pooled to give total lipids. Total lipids (~50 mg) were used as a silica gel column (25 ml of bed volume; Alltech), equilibrated with chloroform, and washed with 40 ml chloroform, 40 ml acetone, and 50 ml methanol. The methanol eluate was further purified by TLC using silica gel G plates (Analtech Inc.) with a solvent system of chloroform/methanol/water (60:30:6, vol/vol/vol). After visualization with water spray, 10 bands were scraped and extracted from the silica gel with chloroform/methanol (1:2, vol/vol) and stored at −20°C. Synthesis of C32 PM, C30 mannosyl β-PM, C35 MPP, C35 phospholicolchol, all-S α and β anameric C32 mannol PM, and C19:0-C16:0 phosphatidylethanolamine (TBSA) were accomplished previously or with new methods based on those from prior studies (Moody et al., 2000; van Summeren et al., 2006; Ter Horst et al., 2010). Synthetic preparations of the following lipids were commercialized (Avanti Polar Lipids): C18:1/C18:0 phosphatidic acid, C18:3/C18:1 phosphatidylethanolglycerol, C16:0/C16:0 phosphatidylglycerol, C18:0/C18:1 phosphatidylinositol, C18:1 lysophosphatidic acid, C18:0 lysophosphatidylethanolglycerol, C18:0 lysophosphatidylinositol, and C26:0 lysophosphatidylcholine. All lipids were dissolved in chloroform/methanol 1:1 and stored at −20°C. Before use, stock lipids were brought to room temperature and aliquots were dried under gas nitrogen and reconstituted in culture media or PBS for sonication in water bath before use.

**HPLC fractionation of thin layer chromatography extract.** TLC bands 3 and 4 were subjected to separation by HPLC with a Monochrom Diaol column (46 × 250 mm, 3 µm; Varian) and coupled online to a LCQ Advantage ion-trap mass spectrometer (ThermoElectron) equipped with an electrospray ionization (ESI) source. In brief, Mobile phase A consisted of hexane/isopropanol (60:40, vol/vol) containing 0.1% (vol/vol) formic acid, 0.05% (vol/vol) ammonium hydroxide, and 0.05% (vol/vol) triethylamine. A binary gradient was used with flow rate of 0.7 ml/min and column temperature of 25°C starting at 5% mobile phase B, linearly increasing to 15% B in 6 min and held at 15% B for 10 min, linearly increasing to 95% B in 8 min, held at 95% mobile phase B for 6 min, and finally back to 5% B in 2 min. 1-min eluates were collected into glass vials and subfractions were stored at −20°C until use.

**LC-ESI-Q-Tof mass spectrometry analysis of thin layer chromatography extracts.** A universal normal phase HPLC-MS method for rapid separation of mycobacterial lipid classes (Layre et al., 2011) was modified to analyze mycobacterial antigens. In brief, TLC bands 3 and 4 were dried under gas nitrogen and were resuspended at 2 mg/ml in solvent A (hexane/isopropanol 70:30 vol/vol, 0.02% formic acid, 0.01% ammonium hydroxide). Electrospray mass spectrometry (6520 Accurate-Mass Q-Tof; Agilent Technologies) analyses were performed coupled with HPLC (1200 HPLC; Agilent Technologies) using a 3 µm × 150 mm × 2 mm diol column (Varian)
Generation of soluble CD1c and multimerization. Biotinylated CD1c monomers were produced in lentivirus-transduced 293T cells by the National Institutes of Health Tetramer Core Facility (Emory University) and described in detail elsewhere (Kasmar et al., 2011). In brief, human β-2-microglobulin and the extracellular domain of CD1c separated by a 2A-TaV peptide were cloned into the lentiviral expression vector pCMJ4. The chains are followed by a C-terminal acidic or basic leucine zipper which stabilizes the complex. Purified monomers were enzymatically biotinylated at the BSΦφ5 site of the CD1c heavy chain. Monomer purity and composition were confirmed by SDS-PAGE, and biotinylation was confirmed by streptavidin bead pulldown assay. CD1c monomers were loaded with C32-PM or C32-MPM in phosphate-buffered saline, pH 7.4, at a 50-fold molar excess of lipid to protein. Lipid-protein suspension was incubated for 18–24 h at 37°C followed by multimerization with streptavidin-conjugated allophycocyanin or PE (Invitrogen).

Native isoelectric focusing gel shift. CD1c was expressed in Hi5 cells using the baculovirus expression system and purified as described earlier (Scharf et al., 2010). CD1c was treated with endoglycosidase F3 for 2 h at 37°C, purified by MonoQ anion exchange chromatography (GE Healthcare), and used for detergent loading with 1% NP-40. The CD1c aggregates were separated by native PAGE and visualized by silver staining. The biotinylated monomers were loaded into CD1c tetramer preparations, and the samples were run on an 8% native gel (GE Healthcare). A single multiprotein band containing CD1c tetramers was excised, and the sample was digested with trypsin (Promega) in a trypsin buffer (10 mM Hepes, 150 mM NaCl, pH 7.4). The digested samples were analyzed by LC-MS/MS to confirm the presence of CD1c in each band.

αβ TCR construct and expression. The DN6 TCR was identified by PCR screening using human IMGT database primer sets, IPS0000029 for TCR-α chains and IPS0000003 for TCR-β chains. To construct the αβ TCR of DN6, individual TCR-α and TCR-β chains, including leader and constant regions, were cloned from DN6 primary cell line using Phusion high fidelity polymerase (New England Biolabs, Inc.) with primers designed from IMGT reference sequences for TRAV36/DV7 and TRBV6-6. The viral P2A sequence was cloned between TCR chains and a single full-length TCR construct was synthesized for dual expression as previously described (Holst et al., 2006). Primers used were as follows, TRAV36/DV7 5’-GC-CCGACAATTACCATCGAATGGAAGGCTCAACGCTTTATCTACTA-3’ with reverse primer for P2A-TRAC 5’-CTCCACAGTCTCCTGCTT- GCTTTAAACAGAGAACTGTTGGCCTCCCAGGCTGAGGAGCA- GACACCCGA-3’. Primer P2A-TRAB-6 5’-CCTGTTAAAGCAACGCGAAGAAGCTGGAAAGAACCCCCCGTCCCATGACATGCAGCAG- CTCCTGCTG3’-CTC was used with reverse primer TRBC 5’-GCGGTCG- GGCAGGCACCTGACGCTGAGTATCCTTCTCTTCTTTGAC-3’. The products generated were used as template and amplified to a full-length TCR construct using forward primer TRAV36/DRV7 primer with reverse primer TRBC. The partial sequence of the TRAV36/DRV7 with CDR3α junctional region is 5’-CATCCTGGAACATCAGCACCACCCAGACCCGAGACCTCGGGCTCCTCCTGCTTGGCTGTTG- TACTGCTATGAAAGCTACTGGCAGGACTTCGACCCAGGACACCATCCTGATCT- GACTGTCCATCCA…3’ and TRBV6-6 with CDR3β junctional region is 5’…CTCCTCCACAGACTCTGFGAATTCTGCTTGGCTCAGGAG- CATGGACTAGCTTCCTACGAGCAGTACTTCGGGCCGGGCCAG- CAGCTCAGCTGAC…3’. For retroviral expression, the full-length TCR product was directionally cloned into a modified Mo-MLV retroviral vector (pMX) containing a puromycin resistance cassette and the retrovirus was packaged in 293T cells using Amphotec virus (Perkin Elmer). Supernatant-containing virus was used to infect human lymphoblast cell lines SUP-T1 (CRL-1942, ATCC) and HuT78 (TIB-161, ATCC) and selected for puromycin resistance and TCR expression with PM-loaded CD1c tetrants.

Tetramer staining and flow cytometry. DN6 and TCR expressing cell lines were stained with allophycocyanin-labeled or PE-labeled tetrameter at 10 µg/ml in PBS containing 0.5% BSA (FACS buffer) for 45 min at room temperature in the dark and subsequently stained with FITC anti-TCR Vβ.
13.6 (clone IM1330); Beckman Coulter) for an additional 20 min at 4°C. To determine DN6 cell lineageceptor expression, cells were stained with FITC-labeled anti–TCR-αβ (clone WT31), PE-labeled anti-CD8 (clone SK1), and allophycocyanin-labeled anti-CD4 (clone RPA-T4) in FACs buffer for 25 min at 4°C in the dark. Cells were washed in FACs buffer and collected on a FACS Canto II (BD). For cell sorting and ex vivo tetramer staining, T cell-enriched PBMCs were blocked using human AB serum (Gemin) for 10 min at room temperature, washed, and stained with tetramer as above. Subsequently, cells were stained with anti–CD3 (clone SK7), anti–CD56 (clone HCD56), and anti–CD19 (clone HIB19) and sorted for CD1c tetramer+CD3+CD56+CD19+ cells on a FACSaria flow cytometer. Expanded clones were stained as above with tetramer followed by anti–TCR-αβ (clone WT31) or anti–CD3, or anti–CD3 and anti–CD8 antibodies. Collected samples were analyzed using FlowJo flow cytometry analysis software (Tree Star). All antibodies were purchased from BD and eBioscience unless indicated otherwise.

Statistical analysis. Data were analyzed using Prism (GraphPad Software).

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