Like most mammalian species, humans express several structurally distinct CD1 antigen-presenting molecules. The conservation of large CD1 gene families among most mammals suggests that each type of CD1 protein has distinct functions that confer selective advantage. Cellular studies of CD1 proteins increasingly explain how each CD1 protein differs from the others. CD1a, CD1b, CD1c, and CD1d have distinct antigen groove structures, patterns of expression in tissues, intracellular trafficking, and trigger T cells expressing diverse TCRs (Kasmar et al., 2009). CD1d (group 2) diverges most clearly from CD1a, CD1b, and CD1c (group 1) with regard to protein sequence. Also, group 1 and group 2 CD1 proteins show differing transcriptional responses to pathogens, suggesting that they function at different stages of the immune response (Roura-Mir et al., 2005b). Collectively, these cellular studies suggest that group 1 and group 2 CD1 proteins likely have differing roles in immune responses.

The majority of known foreign ligands for group 1 CD1 molecules are mycobacterial in origin, including dideoxymycobactin, mycolic acid, lipoarabinomannan, glucose monomycolate (GMM), glycerol monomycolate, diacylated sulfoglycolipid, phosphatidylinositol mannoside, and mannosyl phosphomycoketide (De Libero and Mori, 2005). Human T cells proliferate or produce interferon-γ in response to several types of mycobacterial lipid antigens presented by group 1 CD1 proteins during latent or active tuberculosis infection, suggesting a function in host response to mycobacteria (Moody et al., 2000b; Roura-Mir et al., 2005b).
In this paper, we show that human CD1b tetramers loaded with a foreign glycolipid bind to group 1 CD1 proteins and CD1b and glycolipid bind to GMMs in vitro. In each of the three scenarios, microbial glycolipids and CD1 proteins are both needed to activate T cells indirectly by upregulating cognate TCRs and selectively track antigen-specific T cells within much larger T cell populations (Altman et al., 1996). Tetramers can identify even rare antigen-specific T cells (Moon et al., 2007) for functional analysis, and CD1d tetramers have allowed single-cell analysis of NKT cells during infection, autoimmune disease, and cancer (Benlagha et al., 2000; Matsuda et al., 2000; Karadimitris et al., 2001; Gumperz et al., 2002). Tetramers take advantage of multimerization to generate high avidity fluorescent staining reagents that bind to individual clonotypic TCRs and selectively track antigen-specific T cells at the population level.

The basic principle of tetramer staining requires that TCRs bind to the antigen-presenting molecule and that this physical interaction is mediated by a groove-bound cognate antigen that physically ligates CD1 to the TCR. For CD1d, lipids like synthetic α-galactosylceramides mediate the trispecific complex of CD1d–antigen–TCR (Borg et al., 2007), so an analogous function of glycolipids in mediating TCR contact with group 1 CD1 proteins is a leading model. However, recent studies have emphasized three alternate mechanisms whereby TCRs bind to CD1 or activate T cells but do not physically ligate CD1 and TCR. Lipopolysaccharide stimulates iNKT cell activation, not as a CD1d-bound lipid antigen but by triggering release of cytokines such as IL-12, which augments CD1d–self-antigen–mediated reactivity (Brigl et al., 2003, 2011). Phosphatidylinositol mannoside might activate T cells indirectly by up-regulating CD1b surface expression (Roura-Mir et al., 2005b), and bacterial lipids induce more stimulatory self-ligands for CD1 proteins (De Libero et al., 2005; Pajot et al., 2007). In each of the three scenarios, microbial glycolipids and CD1 proteins are both needed to activate T cells through indirect mechanisms in which the foreign glycolipid does not physically link CD1 to TCR. Therefore, tetramers represent both a test of cognate antigen recognition by CD1b and a potential tool to physically isolate and characterize a foreign glycolipid-reactive T cell repertoire for the first time.

In this paper, we show that human CD1b tetramers loaded with a mycobacterial glycolipid antigen, GMM, selectively bind to GMM-specific TCRs and directly isolate a natural CD1b and glycolipid-reactive T cell repertoire in humans.

**RESULTS AND DISCUSSION**

**Design of tetramerizable CD1b proteins**

We produced a tetramerizable biotinylated CD1b monomer building on prior designs for MHC and CD1d tetramers (Altman et al., 1996; Gumperz et al., 2002). The extracellular domain of the human CD1b heavy chain was modified with a leucine zipper for binding to β2-microglobulin and a Bir A sequence for biotinylation and complexed with streptavidin-labeled APC (tetramer APC) and tested for staining LDN5 T cells. (d) CD1b tetramers were then loaded with GMMSs of the indicated average chain length (C32, C54, or C80) and tested for staining LDN5 T cells. MFI is mean fluorescence intensity. Data are representative of three or more experiments.

**Figure 1.** CD1b tetramers stain human αβ T cells. (a) Bacterial GMM is formed by glucose linked at the 6-position to a mycolyl unit that contains two chiral centers, which are in the R configuration at positions 2 and 3 (2R, 3R). (b) Tetramerizable CD1b monomers were used in plate-bound antigen presentation experiments to measure IL-2 release by the CD1b-restricted human T cell line LDN5 in response to C32 GMM loaded overnight at 37°C (mean ± SEM). (c) CD1b was loaded with GMMSs that are naturally formed with R configuration at C2 and C3 (R, R) or synthetic GMM prepared with an S configuration at C2 or C3 (2S,3R,S) and complexed to streptavidin-labeled APC (tetramer APC) and tested for staining LDN5 T cells. (d) CD1b tetramers were then loaded with GMMs of the indicated average chain length (C32, C54, or C80) and tested for staining LDN5. MFI is mean fluorescence intensity. Data are representative of three or more experiments.
However, initial attempts to stain were unsuccessful, even after confirming monomer purity, biotinylation, and multimerization of CD1b proteins, as well as successful staining of NKT cells with control CD1d tetramers (Fig. S1 a and Fig. S2). Tetramer staining requires that key aspects of the cellular loading mechanism, which is particularly stringent for CD1b, be replicated in vitro. Therefore, we tested the sufficiency of in vitro conditions for antigen loading. In particular, the absence of cellular loading cofactors like saposin C (Winau et al., 2004) and the lack of essential cellular processing might alter structures in ways that are required for binding. After optimizing the time, pH, and chain length of the antigen, we were able to see high-level T cell activation with a plate bound CD1b monomer loaded with a C32 GMM antigen. This result confirmed that cellular processing and loading cofactors are not absolutely required and proved proper CD1b folding (Fig. 1 b).

**CD1b tetramers bind to T cells**

Using optimized conditions for loading CD1b with C32 GMM (Fig. S1 b), we observed CD1b tetramer staining of LDN5 (Fig. 1 c). Although CD1d tetramers bound to the synthetic superagonist α-galactosylceramide brightly stain CD1d-restricted T cells, self-antigens such as isogloboside 3 or sulfatide result in absent or moderate tetramer staining (Jahng et al., 2004; Zhou et al., 2004; Arrenberg et al., 2010). Therefore, it is notable that GMM, a natural foreign antigen, gives bright staining, such that the mean fluorescence intensity increases 10–100-fold after loading in optimized conditions (Fig. 1 c). To determine whether staining is specific for the structure of the antigen or is a result of nonspecific hydrophobic interactions resulting from the presence of lipids, we exposed CD1b to natural and synthetic antigens that recapitulate certain aspects of the C32 GMM structure. Whereas natural bacterial C32 GMM contains two chiral centers in the R conformation at the C2 and C3 positions of the meromycolate chain GMM (2R, 3R), synthetic C32 GMM diastereomers containing an S configuration at either position GMM (2R, 3S + 2S,3R; Fig. 1 a) are nonantigenic (Moody et al., 2000a). Only C32 GMM (2R, 3R) mediated tetramer staining, indicating that chiral carbons, which determine the orientation of the glucose head group and β-hydroxyl unit relative to the TCR, are required for staining (Fig. 1 c).

In contrast, three preparations of natural bacterial GMMs containing a mean chain length of 32, 54, or 80 carbons and having 2R,3R configuration mediate bright staining (Fig. 1 d). Thus, C48 differences in overall lipid length can be tolerated, leading to high avidity binding. Whereas the length and conformation of the alkane chain hidden within the CD1d groove can significantly influence NKT cell activation (McCarthy et al., 2007), our results strongly suggest that CD1b-restricted TCR binding depends critically on head group positioning but can tolerate very large differences in lipid chain length. These results support and extend prior work suggesting that C80 lipids fill the entire groove, whereas shorter lipids partially fill the groove, allowing smaller spacer lipids to fill in the remaining volume (Gadola et al., 2002; Batuwangala et al., 2004; Garcia-Alles et al., 2006).

**CD1b tetramers bind the TCR-αβ complex**

The cognate model predicts that the surface target of tetramer binding is the heterodimer of rearranged TCR-α and -β chains normally expressed on the LDN5 T cell clone, TRAV 17, and TRBV4-1. However, a physical interaction of TCRs with any group 1 CD1 protein has not been previously observed. In addition to any alternate surface ligands on T cells that are unknown and might bind to CD1b, NK receptors (Carbone et al., 2000) and immunoglobulin-like proteins (ILT; Li et al., 2009) have been implicated in binding CD1 proteins. Therefore, we designed experiments to test the presence and specificity of a proposed interaction between CD1b with the clonotypic αβ TCR. CD1d tetramers made from the same type of construct failed to stain LDN5 but did stain the CD1d-restricted T cell clone J3N.5, implicating CD1 isoform–specific sequences in tetramer staining (Fig. S2). Preincubation with anti-CD1b or anti-TRBV4-1 blocked tetramer staining to background (Fig. 2 a and Fig. S3 a). These
studies were consistent with interaction between CD1b and TCR VB4-1, if binding of monoclonal antibodies directly interfered with contact between TCR-β and the distal surface of CD1b. However, antibodies might have blocked staining in indirect ways involving sequestration of TCRs or CD1b proteins. To definitively test the role of the distal domains of TRBV4-1 and TRAV17 in physical contact with CD1b-GMM complexes, we produced soluble leucine-zippered TCRs comprised of the distal domains of the TCR-α and -β chains from LDN5 (sLDN5) and from CD8-2 (sCD8-2), a TRAV3, TRBV3-1 heterodimer which recognizes CD1a (Fig. 2 b and Fig. S3 b). Preincubation with sCD8-2 TCR did not inhibit tetramer staining, but sLDN5 TCR reduced tetramer staining to background levels (Fig. 2 c). Thus, the clonotypic TCR is necessary for CD1b–GMM binding to cells, proving a cognate TCR interaction with the CD1b–antigen complex, which is TCR specific and necessary for cellular binding.

**CD1b tetramers detect GMM-specific T cells during TB infection**

Development of tetramers for study of patient blood in the setting of an infectious disease requires low background among all types of cells present in PBMCs. To evaluate tetramer specificity, we mixed LDN5 T cells with CD1d-restricted NKT cells and found that GMM-loaded CD1b tetramers selectively stained the TRBV4-1+ clonotypic T cells, with no detectable staining over background of T cells with another TCR (Fig. S3 c). Also, titration of GMM-specific LDN5 T cells into fresh PBMC at known frequencies demonstrated that clonotypic T cells could be sensitively detected at the level of 0.01% of CD3+ cells. A discrete population of brightly staining cells was detected at frequencies near to their actual abundance when titrated into PBMC, so tetramers were not binding to T cells with diverse TCRs (Fig. S3 d). The potential problem of low but detectable background staining on CD3+ cells was minimized by two-color flow cytometry to detect CD3+ tetramer+ cells (Fig. S3 d), setting the stage for clinical studies of human CD1b-restricted T cells.

Several population studies have detected increased interferon-γ responses in tuberculosis patients, indicating that lipid-reactive T cells likely expand during infection (Moody et al., 2000a; Ulrichs et al., 2003; Gilleron et al., 2004; Layre et al., 2009; Montamat-Sicotte et al., 2011). However, group 1 CD1-restricted T cells have never been detected directly ex vivo without stimulation. Tetramer detection is desirable because it rules out false positive results from cytokine production by non-T cells, indirect stimulation of cells by lipid adjuvants, or activation of MHC-restricted cells by contaminating peptide antigens. Also, tetramer-based sorting allows live cell capture for diverse functional and phenotypic studies. Among four subjects with positive intradermal purified protein derivative tests, we observed a similar pattern: a small percentage of blood T cells (∼0.01%) stained brightly such that they were well separated from the pool of nonstaining cells (Fig. 3 a). The absolute frequency of cells was detected at similar rates among patients with latent (patients 1, 2, and 4) and active tuberculosis (patient 3), but staining was not observed in three healthy controls (Fig. S4 c). The detected frequency of T cells from individual patients was similar to one another and highly reproducible among experiments using blood from the same patient to assess different aspects of function and phenotype (Fig. 3, a and b; and Fig. 4, a and b).

To determine whether cells staining with CD1b–GMM complexes functionally recognized CD1b and GMM, we sorted CD3+ cells into tetramer\textsuperscript{high} and tetramer\textsuperscript{low} populations (Fig. 3 b and Fig. S4 b). After recovery, total cells were tested in γ-interferon ELISPOT using K562 cells that do or do not express CD1b (de Jong et al., 2010). Only tetramer\textsuperscript{high} cells produced interferon-γ in response to GMM, and this response required CD1b expression (Fig. 3 b). Thus, CD1b tetramers directly identify populations of foreign glycolipid-reactive T cells in the blood of human tuberculosis patients that constitute a natural sub-repertoire of human αβ T cells. A precursor frequency of 0.01% is similar to that of human NKT cells identified using CD1d tetramers (Gumperz et al., 2002).
Despite limited numbers in the peripheral circulation, lipid-specific T cells have been proposed to act locally near the site as helper cells whose function is magnified by downstream responses of dendritic cells or other T cells (Vincent et al., 2002; Roura-Mir et al., 2005a,b). Sorting of blood-derived cells with CD1b tetramers can address core issues of CD1b-restricted T cell phenotype and function previously addressed in T cell clones which can now be studied ex vivo.

**Distinct features of the CD1b–GMM repertoire**

The first and subsequent studies of group 1 CD1-restricted clones show expression of either γδ or αβ TCRs (Porcelli et al., 1992; Spada et al., 2000) in combination with CD4, CD8, or neither co-receptor. We found that CD1b tetramer\textsuperscript{high} T cells uniformly stain with antibody against invariant components of αβ TCRs in all four patients tested (Fig. 4a). CD4 and CD8 represent key subset markers for NKT cell and MHC-restricted T cells because they strongly influence thymic selection and, thereby, determine effector functions. CD1b-restricted T cell clones can express CD4 or CD8 or neither co-receptor (Porcelli et al., 1992; Moody et al., 1997; Stenger et al., 1998), but any general view of co-receptor expression is limited by the small number of clones studied and the possibility of selective outgrowth in vitro. Given the large number of CD4\textsuperscript{−}CD8\textsuperscript{−} and CD8\textsuperscript{+} clones isolated in early work on CD1b, it was unexpected to observe that CD4 single-positive cells dominate the population of tetramer\textsuperscript{+} T cell populations in all four patients studied (Fig. 4b). The absence of CD4 positivity in early clone-based studies likely resulted from methods that depleted CD4 T cells in cultures to reduce MHC class II alloreactivity during cloning procedures. This was a key intervention that allowed the discovery of CD1-restricted T cells, but unbiased study of the CD1b and GMM repertoire now suggests that the CD4\textsuperscript{+} population dominates.

Identification of the CD1b and GMM reactive repertoire as TCR–αβ\textsuperscript{+}CD4\textsuperscript{+} provides basic information about the CD1b-restricted T cell subset, which raises new questions about potential infection of these cells by HIV as well as a possible role of CD4 in development and effector function of this T cell subset. Furthermore, these results illustrate how any phenotypic question can be approached without confounders relating to in vitro growth or contaminating peptide antigens. Whereas NKT cells can be studied in CD1d-altered mice with human or mouse tetramers, there is no widely used small animal model for CD1b. Therefore, CD1b tetramers open a broad window for detailed study of the immunobiology of these cells. In contrast to highly polymorphic MHC proteins, which require haplotype matching for donors, the low rates of CD1 polymorphism in human populations allow one CD1b sequence in tetramer form to be readily applied to almost any human donor in ways that facilitate population studies. Prior clinical studies indicate that group 1 CD1 T cell responses are frequent in human tuberculosis patients (Moody et al., 2000a; Ulrichs et al., 2003; Gilleron et al., 2004; Layre et al., 2009; Montamat-Sicotte et al., 2011), so CD1 tetramers might be developed as a means of immunodiagnosis. Future studies will take advantage of this technology to determine whether the CD4\textsuperscript{+} T cell populations described in this

**Figure 4.** CD1b-restricted T cell populations express the αβ TCR and CD4. PBMCs from four subjects infected with Mycobacterium tuberculosis were subjected to multicolor FACS analysis. Cells were stained with CD3, violet viability dye, CD14 and CD19–PercP-Cy5.5, loaded CD1b tetramers, and anti–TCR–αβ (a) and CD4 (b).
paper may be expanded in the blood and tissues of tuberculous patients and express effector functions that contribute to control of mycobacterial infection, like interferon-γ, TNF-α, and granulysin (Stenger et al., 1998), or instead have unexpected roles in immunosuppression or immunopathology.

MATERIALS AND METHODS

Generation of soluble CD1b proteins. Soluble biotinylated CD1b monomers were produced in lentivirus-transduced HEK293 T cells by the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA) and tetramerized with fluorescently labeled streptavidin. In brief, human β-2-microglobulin and the extracellular domain of CD1b were cloned into the expression vector pcMCh4 (gift from J. Jacob, Emory University, Atlanta, GA). Lentiviral particles were made in a second generation packaging system (Naldini et al., 1996). The light and heavy chains are expressed under control of the CMV promoter and are separated by the 2A-Tav peptide to generate two separate proteins from a single mRNA. The chains are followed by a C-terminal acidic or basic leucine zipper which stabilizes the complex and is used for affinity purification using the 2H11 monoclonal antibody (E. Reinherz, Harvard, Boston, MA). Purified monomers were enzymatically biotinylated at the BirA site at the C terminus of the heavy chain. Monomer purity and composition were confirmed by PAGE, and biotinylation was confirmed by streptavidin bead pulldown assay. Functional activity was assayed by affixing biotinylated monomers at final concentration of 5 µg/ml onto 96-well streptavidin plates (Thermo Fisher Scientific) in PBS, pH 7.4, for 24 h at 37°C. Lipid antigens were sonicated in PBS for 2 min, added to the wells, and incubated for 24 h at 37°C before washing three times with 200 µl/well sterile PBS. 10^5 LDN5 cells were added in a total volume of 200 µl/well medium per well (RPMe). The plate was incubated for 24 h at 37°C after which culture supernatants were collected for HT2 bioassay.

Generation of soluble clonotypic TCR-αβ complexes. The cDNAs of the α and β chains of TCR (LDN5 and CD8.2) were cloned into the baculovirus transfer vector pAcUW51 (BD). Honey Bee Melittin and envelope glycoprotein gp67 were used as signal peptides to optimize secretion of the TCR from the supernatant was purified using Nickel beads (QIAGEN), and a Strep-Tactin column (IBA), followed by gel filtration chromatography. The pooled protein was concentrated to 1 mg/ml in 20 mM Tris-HCl and 100 mM NaCl, pH 8.0, 20% glycerol, and stored at −80°C in small aliquots.

Loading CD1b monomers with GMM. GMM with differing average chain lengths produced by Rhodococcus equi (C32), Nocardia farcinica (CS4), or Mycobacterium phlei (C80) was isolated as previously described (Moody et al., 2002). Antigen identity and purity were confirmed by biochemical analysis including thin layer chromatography and electrospray ionization mass spectrometry in the positive mode (LXQ Linear Ion Trap Mass Spectrometer; Thermo Fisher Scientific). Loading conditions were guided by results from thin layer chromatography and electrospray ionization mass spectrometry method and the protein was secreted by SF9 insect cells. The secreted chain also has a thrombin cleavage site followed by a basic zipper and Strep-tag II (WSHPQFEK). The TCRs were expressed using the baculovirus cotransfection system (Naldini et al., 1996). The light and heavy chains are expressed under control of the CMV promoter and are separated by the 2A-TaV peptide to generate two separate proteins from a single mRNA. The chains are followed by a C-terminal acidic or basic leucine zipper which stabilizes the complex and is used for affinity purification using the 2H11 monoclonal antibody (E. Reinherz, Harvard, Boston, MA). Purified monomers were enzymatically biotinylated at the BirA site at the C terminus of the heavy chain. Monomer purity and composition were confirmed by PAGE, and biotinylation was confirmed by streptavidin bead pulldown assay. Functional activity was assayed by affixing biotinylated monomers at final concentration of 5 µg/ml onto 96-well streptavidin plates (Thermo Fisher Scientific) in PBS, pH 7.4, for 24 h at 37°C. Lipid antigens were sonicated in PBS for 2 min, added to the wells, and incubated for 24 h at 37°C before washing three times with 200 µl/well sterile PBS. 10^5 LDN5 cells were added in a total volume of 200 µl/well medium per well (RPMe). The plate was incubated for 24 h at 37°C after which culture supernatants were collected for HT2 bioassay.

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CD1b tetramers detect human αβ T cells | Kasnar et al.

Online supplemental material. Fig. S1 shows optimization of tetramer staining of the T cell clone LDN5. Fig. S2 shows a comparison of CD1b and CD1d tetramer staining. Fig. S3 shows tetramers staining clonotypic T cell receptors. Fig. S4 shows FACS gating strategies and tetramer staining of healthy controls. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110665/DC1.

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CD1b tetramer staining of clones. CD1b tetramers were validated by staining the clone LDN5 (Moody et al., 1997). In brief, 2 × 10^5 T cells were treated with human AB serum for 10 min, washed, and then suspended in FACS buffer (PBS with 2% fetal calf serum; Gemini) and stained with 1 μg of fluorescently labeled CD1b tetramer for 60 min at room temperature in the dark. Cells were acquired on a FACS Canto flow cytometer (BD) and analyzed using Flowjo (Tree Star) software with doublet exclusion based on forward and side scatter in the presence or absence of anti-CD1b or recombinant TCRs.

Tetramer staining of human PBMC. After informed consent, 50 ml of blood were collected from healthy controls, asymptomatic tuberculin-positive subjects with no clinical or radiographical evidence of active tuberculosis, and active tuberculosis patients observed by the institutional review boards of the Lemuel Shattuck Hospital (00000786) and Partners Healthcare (2002-P-000661) and the Harvard Committee on Microbiologic Safety (08–184). PBMCs were separated by Ficoll density gradient centrifugation. After thawing, one million PBMCs were treated with human AB serum and stained with 1 μg tetramer for 40 min at room temperature in the dark, after which they were stained with violet fluorescent reactive dye (Invitrogen) to exclude dead cells. Cells were stained with monoclonal antibodies including CD3 (BD), CD14 (BD), and CD19 (ebioscience) for an additional 20 min and then fixed in 2% formaldehyde before FACS analysis. Cells from patient 1 were stained in 12 experiments; cells from patients 2, 3, and 4 were each stained four times. For functional assays, unfixed tetramer-positive cells were sorted using a FACS Aria flow cytometer and tested for antigen specificity using untransfected or CD1b-transfected K562 cells as antigen presenting cells in ELISpot assays (de Jong et al., 2010). Tetramer-positive cells were stained with TCR-αβ FITC (BD) or CD4-PE (BD).

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