CD1b-restricted GEM T cell responses are modulated by Mycobacterium tuberculosis mycolic acid meromycolate chains


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Tuberculosis (TB), caused by Mycobacterium tuberculosis (Mt), remains a major human pandemic. Germline-encoded mycolyl lipid-reactive (GEM) T cells are donor-unrestricted and recognize CD1b-presented mycobacterial mycolates. However, the molecular requirements governing mycolate antigenicity for the GEM T cell receptor (TCR) remain poorly understood. Here, we demonstrate CD1b expression in TB granulomas and reveal a central role for meromycolate chains in influencing GEM-TCR activity. Meromycolate fine structure influences T cell responses in TB-exposed individuals, and meromycolate alterations modulate functional responses by GEM-TCRs. Computational simulations suggest that meromycolate chain dynamics regulate mycolate head group movement, thereby modulating GEM-TCR activity. Our findings have significant implications for the design of future vaccines that target GEM T cells.

Mycobacterium tuberculosis | GEM T cells | CD1b | mycolate lipids | molecular dynamics

Significance

Tuberculosis is a major global pandemic responsible for more deaths than any other infectious disease, yet no effective vaccine exists. Here, we demonstrate CD1b expression within human tuberculosis granulomas, supporting a role for CD1b lipid antigen presentation in host immunity to infection. CD1b presents mycolates, the dominant Mycobacterium tuberculosis (Mt) cell wall lipid class and key virulence factors, to αβ T cells. We reveal that mycolate tail moieties, distal to the head group, are antigenic determinants for the conserved human germline-encoded mycolyl lipid-reactive (GEM) T cell receptors (TCRs). Computational simulations suggest a putative mechanism whereby lipid-ligand dynamics within CD1b regulate GEM-TCR activity. This work provides insights for the development of GEM TCRs.

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Significance

Tuberculosis is a major global pandemic responsible for more deaths than any other infectious disease, yet no effective vaccine exists. Here, we demonstrate CD1b expression within human tuberculosis granulomas, supporting a role for CD1b lipid antigen presentation in host immunity to infection. CD1b presents mycolates, the dominant Mycobacterium tuberculosis (Mt) cell wall lipid class and key virulence factors, to αβ T cells. We reveal that mycolate tail moieties, distal to the head group, are antigenic determinants for the conserved human germline-encoded mycolyl lipid-reactive (GEM) T cell receptors (TCRs). Computational simulations suggest a putative mechanism whereby lipid-ligand dynamics within CD1b regulate GEM-TCR activity. This work provides insights for the development of major histocompatibility complex (MHC)-independent Mt lipid vaccines, including those that target GEM T cells.

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T cell responses directed to Mtb lipids presented by CD1b are important for Mtb containment.

Mycolates are a major lipid component of the Mtb cell wall and are key virulence factors (21). They comprise long-chain \(\beta\)-hydroxy fatty acids, which are composed of a shorter unfunctionalized \(\alpha\)-alkyl chain and a longer meromycolate chain that typically has two functional groups, providing the main source of structural diversity (Fig. S1A). Three major mycolate classes exist in Mtb, including \(\alpha\)-, keto- and methoxy-, based on functional groups within the meromycolate chain, which are proximal or distal to the head group moiety (22) (Fig. S1A). In addition, mycolates occur with different chain lengths and stereoarrangements of functional groups, generating a large spectrum of possible mycolate structures. Mycolates may exist as free mycolic acid (MA), which can be esterified to glycerol (Gro-MM), glucose (GMM), or trehalose (TMM) (Fig. S1 B–D). MA, Gro-MM, and GMM are all CD1b-presented lipid antigens (22–24). When bound to CD1b, the meromycolate chain positions itself within the long \(A\), \(T\), and \(F\) superchannels of CD1b, while the shorter \(\alpha\)-alkyl chain occupies the \(C\) channel, via hydrophobic interactions (25). The hydrophilic head group is exposed above the \(F\) portal, thus contributing directly to the T cell receptor (TCR) interface (25).

Until recently, knowledge of the CD1b-mycolate–specific T cell compartment has been based on a few isolated clones that may not accurately represent the T cell repertoire in vivo (9, 26). More recently, CD1b tetramers have been developed to efficiently capture GMM-specific T cells (9). Emerging data now suggest a pattern of TCR conservation, revealing two T cell compartments that differ in their binding affinity to CD1b. The germline-encoded mycolyl lipid-reactive (GEM) T cells express a conserved TCR and respond to Mtb infection by clonal expansion and secretion of antimycobacterial cytokines (9). GEM-TCRs, which are defined by their TRAV1-2 usage, bind to GMM-loaded CD1b with high affinity. Depending on TCR \(\beta\)-chain usage, GEMs can recognize MA or GMM (9). The second compartment contains the semi-invariant LDN5-like T cells, including LDN5, a T cell clone bearing a TCR that binds CD1b-GMM with moderate affinity (27). Therefore, donor-unrestricted GEM T cells that are activated by MAs presented by nonpolymorphic CD1b molecules are potentially powerful targets for future vaccines or diagnostics that may be effective in the majority of the human population.

A central tenet of CD1b-restricted TCR recognition of mycolates is the fine discrimination of the glycolipid head group moiety (27). However, the major source of mycolate diversity is derived from structural determinants within the meromycolate chain that are distal to the head group moiety (22). This feature has not been systematically investigated in relation to T cell activation. We hypothesized that these structural variations may modulate the activation of CD1b-restricted T cells. We reveal GEM-TCR sensitivity to meromycolate chain functional group structure and stereoarrangement. Molecular simulations of CD1b-MA complexes show marked differences in mycolate behavior, which is related to meromycolate chain interactions with the binding groove of CD1b. Our findings reveal that activation of GEM-TCRs by mycolates is finely tuned by the meromycolate chain structure, which could be exploited for future vaccine or diagnostic approaches.

**Results**

**CD1b Is Expressed in Human Pulmonary TB Granulomas.** CD1b is expressed in lesional tissues that exhibit protective immunity (28, 29), whereas it has been reported that CD1b is down-regulated on the cell surface of CD1\(^+\) APCs infected with Mtb in vitro (30). To investigate CD1b expression in human granulomas, we performed immunohistochemical staining of lung biopsies from five patients with active pulmonary TB. Many of the cells in the granulomas were positive for the macrophage marker CD68, with diffuse positive staining within caseous necrosis (Fig. L4 and Fig. S2). CD1b was expressed within the majority of granulomas stained, with immunoreactive cells situated primarily adjacent to the central caseous core (Fig. 1B and Fig. S2 B, D–F, J, and K). Negative control stains confirmed the absence of nonspecific antibody binding (Fig. 1C and Fig. S2 C, G–I, L, and M). Quantitation of immunoreactive cells in five granuloma areas per biopsy showed a range of CD1b expression (median and interquartile range: 6 ± 10.5 cells per square millimeter). Diffuse foci of CD1b immunoreactivity were also observed within the caseous necrosis (Fig. S3). These results confirm CD1b expression at the site of infection, in line with previous reports demonstrating up-regulation of CD1b in human mycobacterial infection (28, 29) and consistent with a role for CD1b-mediated presentation of Mtb lipids to T cells in the host immune response.

**GEM18-TCR Exhibits Promiscuous Mycolate Head Group Specificity.** Mycolates comprise a structurally diverse species of Mtb cell wall lipids that can activate CD1b-restricted human T cells (9, 26), including GEM T cells (9, 26). However, antigenic determinants of mycobacterial mycolates for CD1b-restricted T cells have not been fully defined. To investigate this, we generated human J.RT3.T3.5 and Nfat-GLuc Jurkat T cells stably expressing the mycolate-specific TCRs, GEM clone 1 (GEM1), GEM clone 18 (GEM18), and LDN5 (9, 26). Jurkat T cells expressing TCR were activated by CD1b in the presence of mycolate, whereas no

![Fig. 1. CD1b expression within human TB granulomas. Human lung biopsies from patients with confirmed TB were stained for the macrophage marker CD68 (A) and CD1b (B). (C) Negative control with secondary antibody (Ab) and avidin biotin–peroxidase complex (ABC) detection only. (A–C, Insets) Large box is a 2.1× magnified version of the small box. (Scale bars: A–C, 50 \(\mu m\).)](image-url)
activation occurred in the absence of the TCR, CD1b, or mycolate (Fig. 2A). To examine the fine specificity of these TCRs to different mycolates, we investigated their reactivity to JR1080, an α-MA, as free MA or when esterified to glycerol, glucose, or trehalose head group moieties (Fig. 2A). GEM1- and LDN5-TCRs were specific for GMM (Fig. 2C–E) and did not respond to MA, Gro-MM, or TMM. In contrast, the GEM18-TCR recognized MA and Gro-MM, as well as GMM to a lesser extent, but did not respond to TMM (Fig. 2E). Similar to a previous report (31), our results demonstrate the promiscuity of GEM18-TCR toward mycolate head group moieties. This suggests that meromycolate chain structure might be an antigenic determinant for GEM18-TCR activity.

Meromycolate Chain Functional Groups Dictate GEM-TCR Activity. We next investigated the role of meromycolate chain structure on GEM-TCR activity using a panel of synthetic mycolates. MA derived from pathogenic bacteria, such as Mtb, generally have distal and proximal functional groups in the long meromycolate chain, defined by X and Y, respectively (Fig. S1A). Functional groups include cyclopropane, methoxy, keto, epoxy, diene, and alkene moieties (Table S1). We first assessed GEM18-TCR activity to a panel of 12 synthetic MAs that all comprise the same short α-alkyl chains of C$_{23}$ or C$_{21}$ but diverse meromycolate chains containing different functional groups at various locations, including the Mtb MAs JR1080, AD129, JRRR124, MH140, JR1046, and JRRR121 (Fig. 3 and Table S1). Initial dose–response studies showed that 10 μg/mL MA was optimal to investigate T cell activation. Stimulation of GEM18 Jurkat T cells with a panel of MAs at 10 μg/mL revealed a distinct hierarchy for GEM18-TCR activation (Fig. 3A and B). A luminescence-based NFAT-GLuc T cell activation assay confirmed this pattern (Fig. S4A). Strong T cell activation was mediated by the diene MA MH157, a MA not expressed by Mtb (32) (Table S1). Of the Mtb mycolates, JR1080 induced the strongest T cell activation, which matched the stereochemistry of the expected major Mtb α-mycolate, based on a common biosynthetic pathway for all three major MA classes (33, 34). This effect was significantly greater than with the other α-MAs tested, such as MMS131 and MMS130, which are not expressed by Mtb (Table S1). AD129, matching the chain lengths and expected stereochemistry of the major keto-MA of Mtb, caused moderate activity, as did JRRR124, matching the expected structure and stereochemistry of the major methoxy-MA.
The keto-MA MH140, matching the corresponding trans-cyclopropane, caused minimal activation, as did the corresponding trans-cyclopropane containing methoxy-MA, JRRR121.

Stereorearrangements of meromycolate chain functional groups are a naturally occurring feature of structural diversity. Therefore, to assess whether the stereochemistry of meromycolate functional groups influenced GEM18-TCR activity, we investigated stereoisomers of JR1080, matching the chain lengths of the most abundant Mtb α-MA (33) (Table S1). This revealed an activation hierarchy dependent on stereochemistry and identified CDL12DU as a more potent antigen of GEM18-TCR than JR1080 at concentrations as low as 0.1 μg/mL (Fig. 3C and Fig. S4B). Next, we investigated GEM18-TCR reactivity against a panel of synthetic Gro-MMs containing diverse meromycolate chains. GEM18 responded in a hierarchical, dose-dependent manner to three of the six Gro-MMs, based on analysis of CD69 up-regulation (Fig. 3D) and luminescence (Fig. S4C). In addition, five Gro-MMs displayed a similar activation pattern as MAs containing the same meromycolate chains (Fig. S4D and Table S1). We further assessed the activation of Jurkat T cells expressing GEM1-, GEM8-, and LDNS-TCRs toward a panel of MAs that comprised similar C23 or C24 short α-alkyl chains but structurally variable meromycolate chains (Fig. S5A and Table S1). We observed differences in GEM18-TCR activation toward these GMMs (Fig. S5B and E). In contrast, minor differences in T cell activation occurred for GEM1-TCR (Fig. S5C and F), and no differences were observed for LDNS-TCR toward these GMMs (Fig. S5D). Taken together, these results demonstrate that the functional group type, position, and relative stereoarrangement within the meromycolate chain have a strong impact on GEM18-TCR activity.

Mtb Mycolates Modulate Functional Human T Cell Responses. We next determined whether meromycolate structural differences affected activation of human peripheral blood T cells from Mtb-exposed individuals. We cocultured MA-loaded autologous CD1b+ monocyte-derived dendritic cells (moDCs) with peripheral blood lymphocytes from 10 patients with latent TB infection. Intracellular cytokine staining was performed for IL-2, IFN-γ, and TNF-α in activated T lymphocytes. Strong T cell activation was observed with the MA JR1080 and the GMM SMP74, while the MAs MMS130 and JRRR121 were weakly stimulatory (Fig. 4A). Significantly more cells produced detectable levels of IL-2, IFN-γ, and TNF-α following stimulation with JR1080 compared with JRRR121, and in the majority of patients, JRRR121 and MMS130 did not activate any T cells.

To overcome limitations associated with low numbers of CD1b-reactive T cells in the periphery ex vivo (9, 35), we transferred the GEM18-TCR into ex vivo-derived T cell populations for high levels of expression to study functional impact (Fig. S6A). T cell function was measured after coculture of GEM18-expressing T cells with CD1b+ T2 lymphoblasts loaded with three strongly stimulatory MAs (CDL12DU, JR1080, and DZ146) and three weakly stimulatory MAs (JRRR121, MMS130, and JR1046). JR1080 exhibited significantly increased cell killing in each case compared with the nonstimulatory ligands (JRRR121: P = 0.0003, MMS130: P = 0.0006, JR1046: P ≤ 0.0001; Fig. 4B). The same was also true of CDL12DU (Fig. S6A: JRRR121: P = 0.0006, MMS130: P = 0.02, JR1046: P = 0.018). We also measured functional cytokine responses, studying proinflammatory and antiinflammatory cytokines known to be critical in antimycobacterial immunity (3). Immunogenic MA induced higher levels of IFN-γ secretion by GEM18-expressing T cells than any of the nonstimulatory mycolates (Fig. 4C). Particularly strong responses were noted for IFN-γ, GM-CSF, IL-2, and TNF-α, which were statistically significant in all cases (Fig. 4D and Fig. S6B).

GEM18-TCR Exhibits Differential Binding to CD1b–MA Complexes. We next investigated binding of GEM18-TCR to CD1b molecules...
Meromycolate Chain Anchoring Modulates MA Antigenicity. Next, we hypothesized that the differential activity of mycolates upon GEM-TCR activation might be due to mechanisms related to lipid behavior within the antigen-binding groove of CD1b. To determine whether structural alterations in regions of the lipid that are distal to the carboxylate head group might be communicated to the surface of the CD1b–ligand complex that interfaces with the TCR, we performed molecular dynamics simulations for CD1b bound to highly stimulatory and weakly stimulatory mycolates. Over the trajectory time course, we examined the position and behavior of the MA head group with different meromycolate chain substitutions. Head group position was measured via the distance moved in reference to the head group of GMM in the existing crystal structure of the CD1b–GMM complex (25). Root-mean-square deviation (rmsd) values were calculated to provide a measure of structural similarity to the putative productive conformation of CD1b-GMM. These simulations showed that JR1080 adopts similar conformations to the head group of CD1b-GMM, whereas the weakly stimulatory JRRR121 adopts markedly different conformations (Fig. 5D). These observations show a substantial increase in overall head group movement in the weakly stimulatory MA JRRR121 (Movies S1 and S2).

Study of the meromycolate chains was then carried out through visualization and comparison of substituent centroids, indicating the geometric center of functional group positions over the trajectory time period. Marked differences in centroid localization and dynamics were apparent between stimulatory and weakly stimulatory MAs. The weakly stimulatory MAs JRRR121 and JR1046 showed much more pronounced localization and dynamics were apparent between stimulatory and weakly stimulatory MAs. The weakly stimulatory MAs JRRR121 and JR1046 showed much more pronounced localization of centroids, in the T′ (distal, red) and A′ channel (proximal, blue) (Fig. 6B and Fig. S7), whereas the strongly stimulatory MAs MH157 and JR1080 showed greater fluidity and weakly stimulatory MAs. The weakly stimulatory MAs JRRR121 and JR1046 showed much more pronounced localization of centroids, in the T′ (distal, red) and A′ channel (proximal, blue) (Fig. 6B and Fig. S7), whereas the strongly stimulatory MAs MH157 and JR1080 showed greater fluidity (Fig. 6C and Fig. S7). Chain fluidity was further investigated to understand differences in this behavior. In instances of strong localization, this was found to be due to interaction of chain substituents with features of the CD1b binding pocket. For example, the JRRR121 proximal and distal chain substituents are strongly localized by their respective interactions with small crevices of the A′ and T′ tunnels, thereby resulting in an “anchoring” mechanism (Movies S3 and S4). This strongly suggests that the different dynamic behavior of lipids within the binding pocket is determined by the position and properties of long-chain substituents.

Discussion

TCRαβ+ CD1b-restricted mycolate-specific GEM lymphocytes are a conserved T cell population in humans that expands upon Mtb infection and exhibits potent antimycobacterial effector...
functions through production of IFN-γ and TNF-α (9, 31, 37, 38). Our demonstration of CD1b expression within human lung TB granulomas provides further evidence for lipid-specific T cell immunity in host defense against TB. CD1b is an attractive target for the development of TB vaccines due to its non-polymorphic nature. However, development of such vaccines requires a precise understanding of the antigenic determinants for CD1b-presented mycolates that are recognized by GEM-TCRs. Using a panel of synthetic pure mycolates, we dissected the role of different structural features in defining recognition and functional responses by GEM-TCRs. Our studies reveal a major and unexpected role for structural determinants in the meromycolate chain, distal to the carbohydrate head group moiety and not expected to bind the TCR based on CD1b-GMM structures, in defining T cell activity.

The concept that deeply buried moieties of CD1-bound lipids can influence T cell activation is supported by several studies. For example, the alkyl chains of Mtb diacylated sulfoglycolipids govern CD1b-mediated T cell activity, including C-methyl substituents, stereochemistry, and alkyl chain position (39). T cell activation is also sensitive to alkyl chain differences in the CD1c antigen mannosyl-β-phosphomycoketide, with length, methyl branching pattern, and stereoarrangements influencing responses (40). Furthermore, the length of the alkyl chains and lipid saturation of the CD1d-antigen α-galactosylceramide are important for controlling CD1d-restricted invariant natural killer T cell activity (41). Consistent with these reports, our data suggest that communication of structural differences in lipid tails to T cells is a central feature of CD1 lipid antigen presentation. Our findings suggest a mechanism for TCR–ligand interaction, which may also be generalizable for ligand recognition by CD1c and CD1d molecules. It may also contribute to the fine-tuning of classical peptide-MHC recognition by TCR (42).

We employed molecular dynamics simulations of MAs to gain a mechanistic understanding for how subtle differences within these lipid structures may impact the potency of the T cell response. These analyses supported the notion that ligand dynamics within the CD1b pocket can be strongly influenced by meromycolate chain substituents. Based on these in silico insights and our experimental data, we propose a model whereby meromycolate chain dynamics within the CD1b groove are directly linked to the ability of the hydrophilic head group to adopt productive conformations for TCR binding. In this model, weakly stimulatory lipids with immobile tails are “trapped” due to the position and nature of their chain substituents, and this trapping consequently restricts the head group from adopting positions that facilitate TCR binding. In contrast, strongly stimulatory lipids have chain substituents that do not “catch” on pocket features and, as such, are more readily accommodated by the binding pocket. This manifests as greater chain mobility, thereby allowing the head group to adopt productive conformations for TCR binding. Thus, ligand dynamics have the potential to fine-tune GEM T cell recognition, and therefore function.

Structural studies of GEM42-TCR in complex with CD1b-GMM recently provided a molecular mechanism for GMM recognition by so-called “typical” GMM-specific GEM-TCRs, such as GEM1, GEM21, and GEM42 (31). Arg107α on the CDR3α loop cooperates with Asp113β on the CDR3β loop, forming a salt bridge that acts as a capstone, stabilizing the α- and β-“tweezers” that grip the glucose head group moiety of GMM (31). This highly rigid and specific mechanism for gripping the glucose moiety likely contributes to the insensitivity of such TCRs toward meromycolate changes. Furthermore, contacts between Arg79 and Thr157 found in the α1 and α2 helices and GMM may stabilize the head group, which may counter any movement due to a lack of backbone anchoring (25). On the other hand, GEM18-TCR differs from typical GMM-recognizing GEM-TCRs in that it possesses a Leu107α residue instead of Arg107α on its CDR3α loop, and Asp113β is absent; therefore, GEM18-TCR lacks the stabilizing tweezers (31). The promiscuity toward different mycolate head groups suggests that GEM18-TCR recognizes a common mycolate epitope that is shared between MA, GMM, and Gro-MM, likely mediated by Gly110α and Phe112α within the CDR3α loop (31). The observed weak GEM18-TCR responses toward GMM
could have resulted through interference from the relatively bulky glucose moiety; however, we could not definitively rule out the processing of GMM to MA after cellular uptake. In addition, our results could not rule out the possibility that MA variants may have altered loading or TCR recognition. Definitive conclusions must await structural determination of GEM18-TCR with CD1b-mycobacterial complexes.

Different strains of Mtb and other mycobacteria express significantly different MA structural profiles, and Mtb is known to considerably change its MA composition in response to different growth conditions and virulence stages (22, 43, 44). It is therefore essential to understand the structure/function relationships of Mtb-derived mycolates using synthetic lipids due to the complex mixtures and difficulty in isolating a single natural molecule. Indeed, an earlier study investigating the response of DNI-TCR hinted at a diverse role for MA structural variants on T cell activation (45). Furthermore, our results are consistent with data from a recent study by Van Rhijn et al. (36) indicating that MA lipid tails are antigenic determinants for T cells. Therefore, an emerging concept is that individual MAs should be considered as distinct lipid antigens that may elicit diverse activation profiles by diverse MA-specific TCRs. It is tempting to speculate that the differential activity of MA on CD1b-restricted TCR may provide a means for Mtb to modulate the host immune response during infection. Consequently, manipulating mycolate structure could be a key strategy to generate optimal antinycobacterial responses for future vaccines. Functional differences between lipids were most pronounced for cytokine release relative to cytotoxicity, likely reflecting the latter being a more downstream effect. Defining the ability of GEMs and other mycolate-specific T cells to detect different meromycolate structures in vivo and characterizing their role in immunity to Mtb are key areas warranting further investigation.

In conclusion, we report a systematic investigation of mycobacterial meromycolate chain structure in regulating CD1b-restricted GEM T cell activity. The fine sensitivity of the conserved GEM-TCR for subtle meromycolate changes and the coevolution of humans and Mtb over the last 70,000 yr suggest an intricate role in protection against mycobacterial infection (46). We provide insights into the molecular antigenic determinants for GEM-TCR activation, and our findings may inform future vaccination strategies that harness the potential of donor-unrestricted T cells to control the ongoing TB pandemic.

Materials and Methods

Immunohistochemistry. Paraffin-embedded Mtb-infected human lung tissue was retrieved from the histology archive at University Hospital Southampton with approval by the Institutional Review Board (Reference 12/NW/0794 SRB04/14). The ethics committee approved immunohistochemical analysis without individual informed consent since it was surplus archived tissue taken as part of routine care. Sections (4 μm thick) were dewaxed and rehydrated, and endogenous peroxidase was blocked. Heat-induced epitope retrieval was performed. Nonspecific staining was blocked, and primary antibodies were applied overnight at 4 °C (1:50 anti-CD1b mouse monoclonal SN13, K5 1B8, Abcam; 1:200 CD68 mouse monoclonal ED1, LifeSpan Biosciences). Negative control sections were incubated with buffer alone. Secondary goat anti-mouse antibody for CD1b, CD68, and the negative control was used at a ratio of 1:800. Sections were developed with avidin biotin-peroxidase complexes (Elite Vectastain ABC kit; Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride (DAB; two-component DAB pack; BioGenex). Slides were counterstained with Mayer’s hematoxylin, dehydrated, cleared, mounted in PERTEX and dried, and then imaged on an Olympus BX51, CC12 DotSlide microscope. Slides were digitalized using an Olympus VS-110 digital slide scanner running Olympus VS-ASW-1100 acquisition software. The number of immunoreactive cells within the granulomas was counted, the granuloma area was measured using ImageJ software (NIH) with the BIOP plug-in, and results were presented as cells per square millimeter.

Cloning.

CD1b construct. MoDCs were lysed with Trizol (Invitrogen), and RNA was reverse transcribed. PCR was performed with the following primers: forward 5′-GCCGTCGACTATGGGATATTCTG-3′ and reverse 5′-GCGCGTCGACTCATGGGATATTCTG-3′. CD1b cDNAs were subsequently amplified and cloned into the pELNS lentivector kindly provided by James Riley, University of Pennsylvania, Philadelphia.

TCR constructs. The publicly available GEM18–TCR-α (TRAV1-2, accession no. JQ778257.1) and GEM18–TCR-β (TRBV6-2, accession no. JQ778257.1) chain sequences (9) were synthesized by GeneArt (Thermo Fisher) and subcloned into the pELNS lentivector. The TCR-β chain (TRBV6, accession no. JQ778264.1) of GEM11-TCR was synthesized and cloned into the GEM18 cassette, replacing the GEM18 TRBV6-2 sequence. Site-directed mutagenesis was subsequently performed on the TCR-β chain to yield a complete GEM1–TCR-α sequence (TRAV1-2, accession no. JQ778263.1), using the following primers: forward 5′-GCCGTCGACTACCCGGGCTGTTACGC-3′ and reverse 5′-AGCCGCGGTACCCGGCAGGCTCAC-3′. GEM18-TCR-β chain sequences were subsequently digested and cloned into the third-generation pELNS lentivector kindly provided by James Riley, University of Pennsylvania, Philadelphia.

Generating transgenic cell lines. Lentiviruses encoding CD1b or TCRs were generated in HEK293T cells after cotransfection of three accessory plasmids: pCMV-VSV-G (1.5 μg), pRSV.REV (3 μg), and pMDLpg.RRE (3 μg), in combination with engineered pELNS lentivector (2.5 μg) (48). Lentiviral particles were harvested 48 h posttransfection. The supernatants were used to infect a/3100 mouse embryonic fibroblast (MEF) cell line expressing the CD1b transgene, with the helper virus NHEVI. Infected MEFs were characterized for TCR expression by flow cytometry, and single-cell preparations were expanded for further experiments.

Immunophenotypic analysis of primary CD1b-restricted TCR activity. The immunophenotypic analysis of primary CD1b-restricted TCR activity was performed at 3 d postinfection using a panel of antibodies against CD4 (1:200 CD4 mouse monoclonal, BD Biosciences), CD68 (1:200, CD68 mouse monoclonal, Biolegend), CD1b (1:200, CD1b mouse monoclonal, Biolegend), PD1 (1:100, PD1 monoclonal, Miltenyi), and CD11c (1:100, CD11c mouse monoclonal, Biolegend). All antibodies were added to the wells at the same concentration and were incubated for 30 min on ice. Cells were washed twice and then stained with mouse IgG1-PE (Biolegend) and mouse IgG1-APC (Biolegend) to block FcR interactions. Stained cells were acquired and analyzed on a FACSCanto (BD Biosciences) flow cytometer using CellQuest software.
were harvested, filtered, and used directly for transduction of T2 lymphoblasts and Jurkat T cell lines. Transduced cells were then cultured with Jurkat T cell lines in a 1:1 ratio in a 96-well plate. After a further 18 h, Jurkat activation was measured by determining CD69 (clone FN50) expression.

MA Preparation and Formulation. MA and their sugar esters were prepared as described previously (32, 49–54). Table S1 provides structural information. Chemically synthesized MAs were dried, resuspended at 1 mg/mL in 9:1 chloroform/methanol, aliquoted, evaporated, and then frozen at –20 °C for future use. When required, the aliquots were resuspended in complete media and sonicated for 30 min at 80 °C before use.

Dendritic Cell Generation. Blood was obtained from asymptomatic donors with latent TB diagnosed by a positive IFN-γ release assay (QuantiFERON-TB Gold In-Tube assay; Cellestis/Qiagen). Written informed consent was obtained from all blood donors (Reference 13 SC 0043). Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hyphaque (GE Healthcare). Monocytes were positively selected by anti-CD14 magnetic microbeads (Miltenyi Biotech) and differentiated into moDCs in complete media (RPMI 1640 supplemented with 1% penicillin/streptomycin, and 10% FCS; all from Lonza), 25 ng/mL GM-CSF, and 20 ng/mL IL-4 (Miltenyi Biotech) for 5 d. CD1b expression was confirmed by flow cytometry.

T Cell Assays. Activation of Jurkat T cells. T2 lymphoblasts were pulsed with lipid for 16 h and then cultured with Jurkat T cell lines in a 1:1 ratio in a 96-well plate. After further 18 h, Jurkat activation was measured by determining CD69 (clone FNS0) up-regulation by flow cytometry. Activation of NFAT-GLuc Jurkat T cells was measured using the Gausia luciferase kit (New England Biolabs) as per the manufacturer’s instructions. GLuc assay solution was added to cell culture supernatant in a 96-well plate (Corning), and luminescence was read (Glo-Max Discover; Promega).

Intracellular cytokine staining. Monocyte-depleted T cell fractions were rapidly thawed and allowed to recover before addition of autologous moDCs pulsed with 5 μg/mL lipid in a ratio of 1:2 in a 96-well plate. The culture was incubated at 37 °C for 6 h in the presence of 2.5 μg/mL anti-CD28, 10 μg/mL Brefeldin A, and 1× Monensin (Biolegend). Cells were then transferred to flow cytometry tubes for intracellular cytokine staining. Positive controls were incubated with phorbol ester (PMA) and ionomycin at 50 nM and 500 nM, respectively. T cell stimulation. GFP-negative TCR transduced T cells were then washed and reconstituted in complete media for 4 h. Cells were then washed and added to lipid-pulsed T2 lymphoblasts at a ratio of 1:2 for 24 h in a total volume of 200 μL in a 96-well plate. After activation, supernatant was collected for cytokine analysis using xMAP assays (R&D Systems), and cell viability was directly assessed using a CytoTox-Glo Cytotoxicity Assay (Promega) according to the manufacturer’s instructions, with luminescence measured by Glo-Max Discover after 15 min. Then, 30 μg/mL digitonin was added to wells to assess total cell death.

Luminex xMAP assays. Concentrations of cytokine were determined using a Bioplex 200 platform (Bio-Rad) according to the manufacturer’s protocol. Cytokines analyzed included the following: IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), IL-17a, TNF-α, IFN-γ, and GM-CSF (R&D Systems).

Soluble TCR and TCR dextramers. Generation of TCR heterodimers was performed as previously described (47). Briefly, the extracellular domains of TCR-α and TCR-β chains were produced in Escherichia coli Rosetta as inclusion bodies after cloning into the bacterial expression vector pGEM7. To produce stably refolded disulfide-linked heterodimers, cytoines were incorporated into the TCR-α and TCR-β chain constant domains by replacing Thr48 and Ser57, respectively. The disulfide-linked GEM18-TCR-β heterodimers were expressed, refolded, and purified as previously described (47). Refolded and purified TCR was assessed by reducing and nonreducing SDS-PAGE gel analysis. Precision Plus Protein Prestained Standard (Bio-Rad) was used as a reference molecular weight marker. GEM18-TCR dextramers were produced using modified TCR-β chains containing a C terminus BirA-tag motif, which was specifically biotinylated. Biotinylated TCR was subsequently purified by size-exclusion chromatography before conjugation to dextran-phycocerythin (PE) (Immudex) to generate fluorescently labeled TCR dextramers.

MA-treated CD1b beads and dextramers. Soluble biotinylated CD1b monomers (Immudex) were treated with methoxy MA similar to a previously published method (36). Briefly, MAs were solubilized in 100 μL of 50 mM citrate buffer (pH 4.5) containing 0.6% CHAPS detergent (Sigma) after sonication in a water bath for 2 h at 40 °C. For beads, solubilized MAs were incubated with CD1b-coated MACS beads (Miltenyi) at 37 °C overnight. Beads were washed in PBS containing 2% FCS before staining with GEM18-TCR dextramer.

Flow Cytometry. The following fluorescent reagents were used: PE-Cy5–CD69–Monensin (Biolegend), anti-CD3-APC–CD14 magnetic microbeads (Miltenyi Biotec) and differentiated into moDCs in complete media (RPMI 1640 supplemented with 1% penicillin/streptomycin, and 10% FCS; all from Lonza), 25 ng/mL GM-CSF, and 20 ng/mL IL-4 (Miltenyi Biotech) for 5 d. CD1b expression was confirmed by flow cytometry.

Molecular Dynamics Simulations. A crystal structure of CD1b in complex with a GMM is available [Protein Data Bank (PDB) ID code 1UQS] (25); however, the low resolution (3.1 Å) prevented its direct use as a simulation starting structure. A 2.6 Å resolution structure (PDB ID code 1GZ0) containing CD1b in complex with a phosphatidylinositol (55) was therefore used to provide the initial geometry of the CD1b and β2-microglobulin chains. Initial ligand structures were generated using flexible alignment of the MA structure with the protein heavy atom restraints to preserve secondary structure elements. The disulfide linkage was represented through the addition of seven Na+ ions. Bond lengths were constrained using the SHAKE algorithm, allowing use of a 2-fs time step. Simulations were conducted at 300 K using a Langevin thermostat with a collision frequency of 3 ps−1. Where relevant below, pressure was regulated using a Monte Carlo barostat, with volume moves attempted every 100 time steps. All systems were initially equilibrated with protein and ligand heavy atom restraints to preserve secondary structure elements. All systems were gradually heated from 100 to 300 K over 0.5 ns. The system volume was then allowed to equilibrate for 2 ns under isothermal–isobaric ensemble dynamics. The system was then cooled over 0.1 ns, and the previous process was repeated with restraints on protein backbone heavy atoms only. Protein backbone restraints were then removed, and the system was equilibrated for a further 2 ns at 300 K.

Statistical Analysis. GraphPad Prism version 7.00 (GraphPad Software, Inc.) was used for statistical analysis, and P values <0.05 were considered statistically significant. The Mann–Whitney U test or one-way ANOVA was used as stated in the figure legends. The heat map was generated using the R software package.


