CD1b-mediated T Cell Recognition of a Glycolipid Antigen Generated from Mycobacterial Lipid and Host Carbohydrate during Infection

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Abstract

T cells recognize microbial glycolipids presented by CD1 proteins, but there is no information regarding the generation of natural glycolipid antigens within infected tissues. Therefore, we determined the molecular basis of CD1b-restricted T cell recognition of mycobacterial glycosylated mycolates, including those produced during tissue infection in vivo. Transfection of the T cell receptor (TCR) α and β chains from a glucose monomycolate (GMM)-specific T cell line reconstituted GMM recognition in TCR-deficient T lymphoblastoma cells. This TCR-mediated response was highly specific for natural mycobacterial glucose-6-*O*-(2*R*, 3*R*) monomycolate, including the precise structure of the glucose moiety, the stereochemistry of the mycolate lipid, and the linkage between the carbohydrate and the lipid. Mycobacterial production of antigenic GMM absolutely required a nonmycobacterial source of glucose that could be supplied by adding glucose to media at concentrations found in mammalian tissues or by infecting tissue in vivo. These results indicate that mycobacteria synthesized antigenic GMM by coupling mycobacterial mycolates to host-derived glucose. Specific T cell recognition of an epitope formed by interaction of host and pathogen biosynthetic pathways provides a mechanism for immune response to those pathogenic mycobacteria that have productively infected tissues, as distinguished from ubiquitous, but innocuous, environmental mycobacteria.

Key words: CD1 • T cell • antigen presentation • mycobacteria • glucose monomycolate

Introduction

In contrast to saprophytic mycobacteria that grow ubiquitously in the environment, pathogenic mycobacteria cause disease by crossing epithelia and growing within tissues. Once mycobacteria successfully invade host tissues, extracellular mycobacteria are generally cleared rapidly by macrophages. Pathogenic mycobacteria persist long term within host tissues, likely within infected phagosomes of host macrophages (1, 2). Intracellular growth and persistence account for the ability of mycobacteria to produce chronic diseases such as leprosy and tuberculosis as well as reactivation tuberculosis in the setting of malnutrition, aging, or acquired immunodeficiency. Cellular immunity to mycobacteria is crucial to the successful host response to infection, and antigen-specific Th1 T cells that activate cell-mediated immunity by IFN-γ production have been experimentally proven to mediate mycobacterial clearance in infected animals (3–6). Most studies of T cell activation by mycobacteria have emphasized the role of MHC class II or class I–peptide antigen presentation systems. However, the discovery of the CD1 antigen presentation system offers an alternative and complementary mechanism by which mycobacterial glycolipids specifically activate T cells (7).

Three human group 1 CD1 isoforms, CD1a, CD1b, and CD1c, present mycobacterial lipids to T cells (8–11). The structures of mycobacterial lipids presented by CD1b in-

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clude free mycolic acids, glucose monomycolate (GMM),[1] and phosphatidylinositol-containing lipoglycans such as lipoarabinomannan, lipomannan, and phosphatidylinositol mannoside (8, 12, 13). CD1c proteins present an unusual fully saturated mycobacterial mannosyl phosphoisoprenoid antigen that is related to eukaryotic dolichyl phosphoglycolipids (14). These mycobacterial lipid antigens are immunologically foreign because they have a mycolic acid, phosphatidylinositol mannoside, or isoprenoid core structure that is unique to mycobacteria and related species, but not found in mammalian cells. Furthermore, several of these lipids are abundant structural components of the protective mycobacterial cell wall and have been shown to influence mycobacterial viability, making them attractive targets for immune recognition in host defense (15, 16). CD1-restricted, mycobacterial lipid–specific T cells possess effector mechanisms that promote clearance of mycobacterial infections, including IFN-γ secretion, cytolysis, and granulysin delivery (7, 17, 18). These observations suggest a straightforward model for CD1 function in host defense whereby pathogenic mycobacteria infect tissues, including $CD1^+$ dendritic cells that present foreign glycolipids to antigen-specific T cells that contribute to bacterial clearance.

Although there is growing evidence that the general mechanism of CD1-mediated glycolipid recognition involves direct interactions of the TCR with a CD1–antigen complex, detailed structural knowledge of the proposed TCR ligands is not yet available, and the extent to which such responses are specific for glycolipid antigen structure is unknown (19, 20). T cell activation may require only that a glycolipid be bound to a CD1 protein, as suggested by studies of murine T cell hybridomas that responded to a series of antigens that differed substantially in the structure of the hydrophilic elements (21). Alternatively, recognition may be highly specific for the molecular structure of the glycolipid, as suggested by a study of native T cell recognition of synthetic analogues of glycosylated mycolates and α-glycosyl ceramides (12, 22, 23).

Furthermore, the tissue expression of antigenic glycolipids is almost completely unexplored, yet the proposed functions of glycolipid-specific T cells require that their antigens be expressed in target tissues in a form recognizable by T cells. For example, CD1d-restricted T cells recognize purified phosphatidylinositols and α-glycosylceramides, yet the former are self-lipids present ubiquitously in cells, whereas the latter are synthetic or marine sponge–derived compounds that are not known to exist at all in mammalian cells (21, 22). Although mycobacteria-specific T cells have been proposed to function by recognizing foreign lipids and glycolipids within infected tissues, there is no information regarding the generation or recognition of T cell epitopes in infected tissues in vivo. T cell recognition of mycobacterial GMM suggests that this glycolipid is a target of the human immune response to mycobacteria (12). However, it is not known whether GMM is even produced by infecting mycobacteria in vivo, and this molecule is produced during in vitro growth only when exogenous glucose is provided in the media (12, 24, 25). Thus, it is not clear whether this CD1b-restricted T cell antigen is produced during infection or whether it is an artifact of in vitro growth conditions.

To address these questions, we undertook a detailed study of T cell specificity for mycobacterial glycosylated mycolates, including those produced during a tissue-based mycobacterial infection. Our results show that T cell recognition of GMM was mediated by the TCR and was highly specific for the precise structure of natural GMM produced by mycobacteria, including the glucose moiety, the linkage of glucose to the mycolate, and the stereochemistry of the mycolate lipid. These results indicate that T cells are capable of extremely precise discrimination of the hydrophilic cap of the antigen that likely functions as a classical TCR epitope by directly contacting TCR variable regions. Importantly, mycobacteria growing extracellularly were not capable of de novo GMM production, but mycobacteria produced antigenic GMM when grown in the presence of exogenous glucose in vitro or within infected mammalian tissues in vivo. Therefore, we propose that during natural infections mycobacteria synthesize GMM by capturing host glucose and esterifying it to mycobacterial mycolates. Specific CD1b-mediated T cell recognition of an antigen produced by the interaction of host and pathogen biosynthetic pathways provides a potential immune mechanism for recognizing and responding only to those mycobacteria that have productively infected host tissues.

Materials and Methods

Glycolipid Antigens. *Mycobacterium leprae* (Colorado State University, Fort Collins, CO) was harvested directly from the liver of infected armadillos as described (26). *Mycobacterium tuberculosis* H37Ra (Colorado State University), *Mycobacterium smegmatis*, *Mycobacterium avium* serovar 4 (H. Remold, Brigham and Women's Hospital, Boston, MA), *Mycobacterium phlei* (American Type Culture Collection), and *Rhodococcus equi* (American Type Culture Collection) were cultured in standard medium (7H9 [Difco]; 0.5 g/liter Tween 80 [Fisher Scientific]) supplemented with 10 g/liter D-glucose, 3-*O*-methyl glucose, fructose, mannose, galactose, L-arabinose, D-arabinose, sorbose, xylose, or glucose (Sigma-Aldrich). Quantitative measurement of *M. avium* GMM was accomplished by cultivation in standard medium that was additionally supplemented with 0.5 g/liter BSA, 0.4 mg/liter catalase (Sigma-Aldrich), and 10 g/liter glycerol followed by 2 d of culture in the absence of glycerol, then by addition of glucose at the indicated concentration and culture for 24 h at an optical density typical of log phase growth (OD_{600} = 0.25–0.35).

Bacteria were washed with PBS, then deionized water, then were lyophilized and extracted for 2 h at 7.5 mg/ml in chloroform/methanol (2:1) to give total extractable lipid. *M. phlei* and *R. equi* GMM were purified using silica columns as described (12). Preparative TLC of crude *M. leprae* lipids was carried out on 200-μm silica-coated glass TLC plates (Scientific Adsorbants Incorporated) developed in chloroform/methanol/water (60:16:2).

[1]*Abbreviations used in this paper:* GMM, glucose monomycolate; G-6-MM, C_{32} glucose-6-*O*-monomycolate; G-3-MM, glucose-3-*O*-monomycolate; MAME, mycolic acid methyl ester; Myc PL, mycolylphospholipid; NMR, nuclear magnetic resonance; R_f, retardation factor.

Silica was scraped in 1-cm intervals and lipids were extracted with three washes of chloroform/methanol (2:1). GMM yields were estimated by silica TLC developed in chloroform/methanol (90:8) and charred with cupric acetate solution at 160°C for 15 min followed by computer-assisted densitometry (Scion Image Software) with quantitative standards.

C_{32} glucose-6-*O*-monomycolate (G-6-MM) was synthesized from 3-*tert*-butyldimethylsilylated mycolic acid and 1,2,3,4-tetra-*O*-*tert*-butyldimethylsilylated glucose as described (12, 27). Glucose-3-*O*-monomycolate (G-3-MM) was synthesized using a similar strategy except that 4,6-*O*-benzylidene-1,2-*O*-isopropylidene derivative of glucose was used to couple via a 3-linkage. Synthetic C_{32} mycolic acid was methylated and resolved in chloroform on preparative silica TLC plates to yield the 2*R*, 3*R* plus 2*S*, 3*S* enantiomeric pair with a retardation factor (R_f) of 0.42, and the 2*R*, 3*S* plus 2*S*, 3*R* enantiomeric pair with an R_f of 0.52, as determined by previous reports and comparison with methyl esters of the natural C_{32} 2*R*, 3*R* mycolate standard from *R. equi* (27). These mycolic acid methyl esters were glucosylated as described and used in T cell assays (27). To confirm that the glucosylation reaction did not racemize the mycolic acids, GMMs were resaponified, methylated, and resolved on analytical TLC as described above.

M. tuberculosis trehalose dimycolate, arabinomycolate, glycerol mycolate, and mycolylphospholipid (Myc PL) were purified as described (12, 28). The *M. avium* hexose phosphoisoprenoid antigen that stimulates the T cell line CD8-1 was partially purified from *M. avium* as described (14). This organic phase was dried under nitrogen, resuspended in chloroform, and loaded on an open silica column that was sequentially eluted with chloroform, acetone, and methanol as described (12). The mixture of methanol-eluting lipids was weighed and used as a source of antigen. The chemical identities of the pure lipid antigens were confirmed using analytical TLC, electrospray ionization mass spectroscopy (Quattro II triple quadrupole mass spectrometer; Micromass), and nuclear magnetic resonance (NMR) (ACE 300; Bruker).

Cells. LDN5, a TCR-$\alpha/\beta^+CD4^-CD8^-$ T cell line, was isolated from a skin biopsy specimen of a human subject with an asymptomatic infection with *M. leprae* diagnosed on the basis of a positive intradermal skin test and a clinical history of contact with a leprosy patient (12). CD8-1, a TCR-$\alpha/\beta^+CD4^-CD8^+$ T cell line, was derived from the peripheral blood of a random donor (10). $CD1^+$ monocyte-derived dendritic cells were prepared from random donor peripheral blood by centrifugation (670 *g* for 15 min) over Ficoll-Hypaque, adherence to plastic tissue culture flasks (Falcon), culture of adherent cells with 300 U/ml granulocyte/monocyte-colony stimulating factor and 400 U/ml IL-4 for 72–96 h, followed by γ-irradiation (5,000 rads). T cells were cultured at a 1:1 ratio with irradiated monocyte-derived dendritic cells in 24-well plates in T cell media (RPMI 1640 supplemented with 10% FCS [Hyclone], L-glutamine, essential amino acids, nonessential amino acids, 2-mercaptoethanol [GIBCO BRL], and 1 nM recombinant human IL-2 [Ajinomoto]). T cell lines were restimulated every 14–28 d with fresh irradiated monocyte-derived dendritic cells and T cell media containing 5 μg/ml of *M. tuberculosis* or *M. phlei* sonicate. C1R lymphoblastoid cells transfected with the vector pSRα-NEO containing the cDNAs encoding human CD1a, CD1b, or CD1c were also used as APCs in cellular assays to determine restriction (11).

TCR Transfections and Flow Cytometry. Transfection methods and sequences of TCR α and β chains from LDN5 (TCRAV3*S*1J9C1, TCRBV7S1D2J2*S*1C2) and CD8-1 (TCRAV-3*S*1J17C1, TCRBV2S1D2S1J2*S*7C2) have been described previously (19, 29). In brief, RNA from 2×10^5–2×10^6 T cells was mixed with carrier DNA from 10^6 EBV-transformed B cells and used to prepare oligo (dT)–primed double stranded cDNA that was incubated with T4 polymerase to form blunt-ended cDNA. This product was circularized with T4 DNA ligase and used as a template for inverse PCR using Cα- or Cβ-specific primers oriented in the opposite directions. The amplified TCR genes were cloned into pBluescript II (Stratagene) and sequenced using the Sequenase v2.0 kit (United States Biochemical). These sequences were used to design Vα- and Vβ-specific primers for the appropriate V genes. RNA isolated from T cells was used to synthesize single stranded cDNA with Superscript II reverse transcriptase (GIBCO BRL) and an oligo (dT) primer. This cDNA was used for PCR amplification followed by digestion with the appropriate endonucleases, gel purification, and ligation into pREP 7 (α chains) or pREP 9 (β chains; both from Invitrogen) expression vectors. These constructs were electrophoretically transfected into TCR-deficient J.RT3-T3.5 (J.RT3) cells which were subsequently maintained in media supplemented with G418 (1 mg/ml) and hygromycin B (0.5 mg/ml) for 2–4 wk. Transfectants were evaluated for surface CD3 expression by staining with 20 μg/ml SPVT3b (anti-CD3ε) at 4°C in PBS/5% FCS at 10^6 cells per 50 μl, followed by 20 μg/ml FITC goat $F(ab')_2$ anti–mouse IgG/IgM (Biosource International). Cells were washed and resuspended in PBS/FCS and analyzed on a FACSort™ flow cytometer (Becton Dickinson). Dead cells were excluded based on forward and side scatter.

T Cell Activation Assays. T cell proliferation was measured by incubating 5×10^4 T cells with antigen and 5×10^4 γ-irradiated (5,000 rads) monocyte-derived dendritic cells in 96-well microtiter plates for 3–4 d followed by addition of 1 μCi [^{3}H]thymidine (New England Nuclear) and an additional 6 h of culture before harvesting and counting β emissions (Beta plate; Wallac) as described (11). IL-2 release was measured by culture of 10^5 J.RT3 cells in the presence of either 10 ng/ml of PMA alone or 10 ng/ml PMA with 5×10^4 monocyte-derived dendritic cells or C1R lymphoblastoid APCs and antigen in 200 μl/well in 96-well microtiter plates. After 24 h, 25 μl of supernatant was transferred to wells containing 75 μl of media and 5×10^3 IL-2–dependent HT-2 cells and cultured for 24 h before adding 1 μCi [^{3}H]thymidine for an additional 6–24 h of culture, followed by harvesting and counting β emissions. Assays were done in triplicate and reported as the mean ± SD except as indicated.

Results

Clonally Variable Segments of the TCR α and β Chains Mediated CD1b-restricted Recognition of GMM. The α/β T cell line LDN5 was derived from the skin biopsy specimen of a human patient infected with *M. leprae* and had been previously shown to recognize mycobacterial GMM presented by $CD1b^+$ APCs (12). To determine whether the TCR mediated GMM recognition, the LDN5 TCR α and β chains were cloned into mammalian expression vectors and cotransfected into J.RT3 T lymphoblastoid cells that expressed low levels of CD3 complexes due to a lack of the endogenous TCR β chain (29, 30). Cotransfection of the TCR α and β genes from LDN5 restored surface expression of CD3 complexes to a high level and conferred upon LDN5αβ/J.RT3 cells the ability to secrete IL-2 in response to GMM (Fig. 1). Recognition of GMM

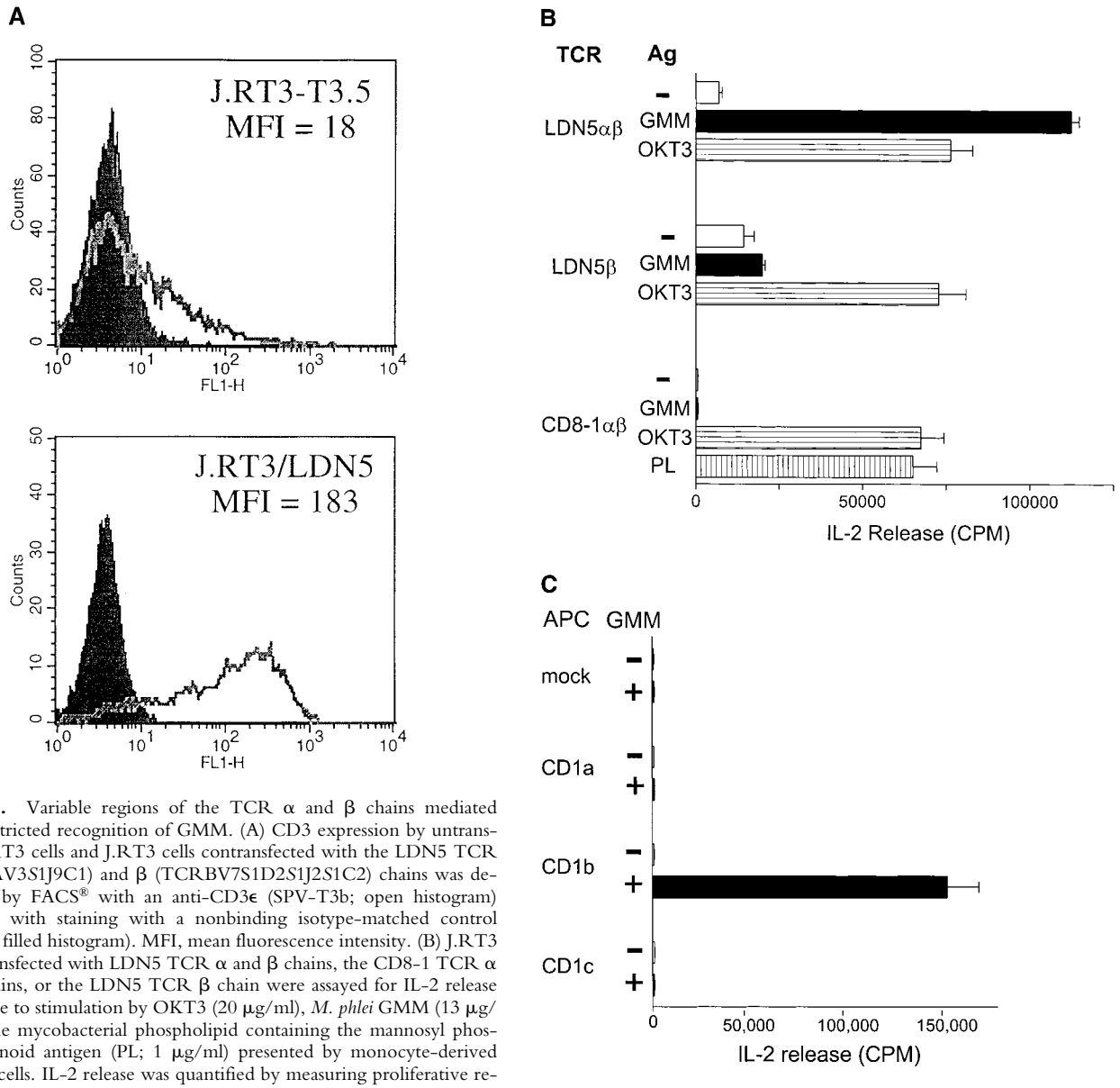

Figure 1. Variable regions of the TCR α and β chains mediated CD1b-restricted recognition of GMM. (A) CD3 expression by untransfected J.RT3 cells and J.RT3 cells contransfected with the LDN5 TCR α (TCRAV3*S*1J9C1) and β (TCRBV7S1D2*S*1J2*S*1C2) chains was determined by FACS® with an anti-CD3ε (SPV-T3b; open histogram) compared with staining with a nonbinding isotype-matched control mAb (P3; filled histogram). MFI, mean fluorescence intensity. (B) J.RT3 cells cotransfected with LDN5 TCR α and β chains, the CD8-1 TCR α and β chains, or the LDN5 TCR β chain were assayed for IL-2 release in response to stimulation by OKT3 (20 μg/ml), *M. phlei* GMM (13 μg/ml), or the mycobacterial phospholipid containing the mannosyl phosphoisoprenoid antigen (PL; 1 μg/ml) presented by monocyte-derived dendritic cells. IL-2 release was quantified by measuring proliferative responses of IL-2–responsive HT-2 cells cultured with J.RT3 supernatants. (C) LDN5αβ/J.RT3 cells were assayed for IL-2 release when stimulated by GMM (5 μg/ml) presented by C1R B lymphoblastoid cells transfected with vector alone (mock) or cDNAs encoding either CD1a, CD1b, or CD1c.

was seen only when B lymphoblastoid APCs were transfected with CD1b but not when mock transfected or transfected with other CD1 isoforms (Fig. 1 C). Thus, recognition of GMM required CD1b expression by the APCs and expression of the TCR α and β chains by the responding T cells.

Since certain lipids can directly activate T cells via invariant components of the CD3 complex–related signaling pathways (31–33), it was important to determine whether TCR transfection conferred GMM recognition simply by reconstituting functional CD3 complexes on J.RT3 cells or also required the TCR variable regions unique to LDN5. Therefore, J.RT3 cells were reconstituted with TCR α and β chain pairs other than those from LDN5. Transfection of TCR α and β chains from the mannosyl phosphoisoprenoid–specific T cell line CD8-1 (CD8-1αβ/J.RT3) did not confer GMM recognition, even though transfection of this TCR-α/β chain pair reconstituted CD3 complexes that mediated IL-2 release in response to OKT3 or the mannosyl phosphoisoprenoid antigen (14; Fig. 1 B). As a further control, the TCR β chain from LDN5 was transfected, allowing pairing with the endogenous JRT.3 TCR α chain (LDN5β/J.RT3). LDN5β/J.RT3 also failed to recognize GMM even though it expressed functional CD3 complexes as measured by OKT3 activation (Fig. 1 B). These results demonstrated that reconstitution of functional CD3 complexes on J.RT3 by TCR α and β chains other than properly paired LDN5 TCR chains was not sufficient to confer GMM recognition, and that variable regions of the TCR unique to LDN5 were required.

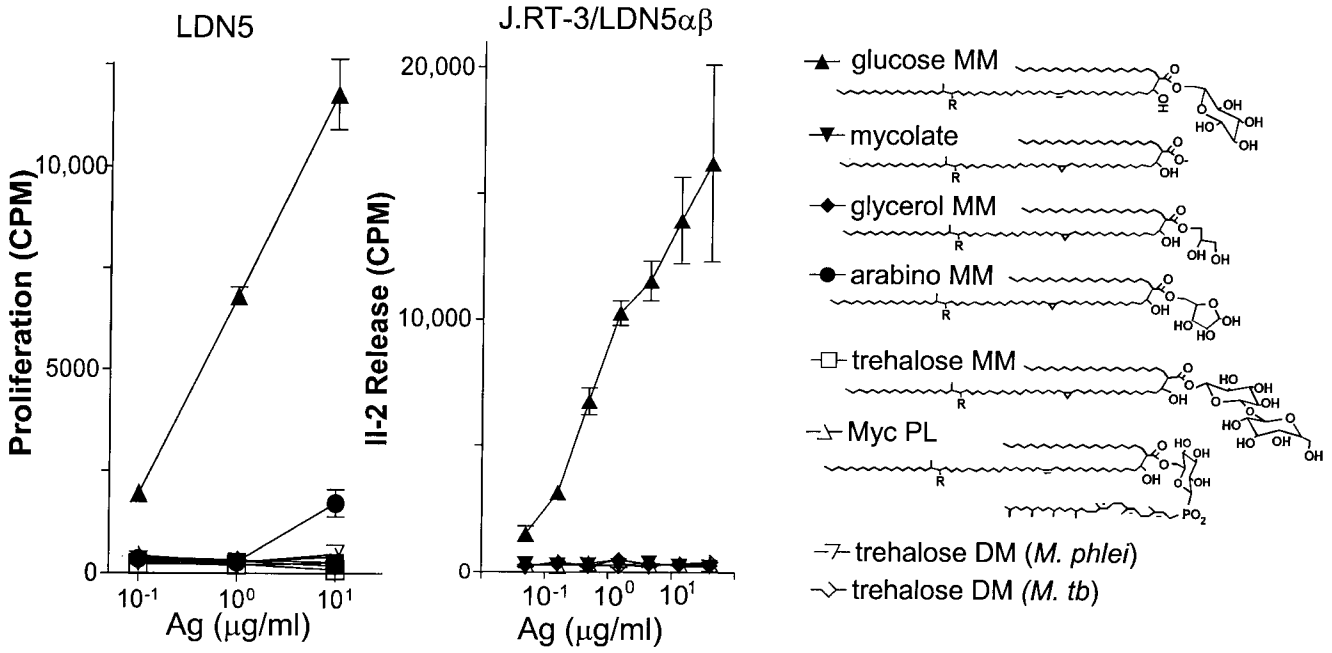

Figure 2. LDN5 and LDN5αβ/J.RT3 cells recognized GMM but not other naturally occurring mycobacterial glycosylated mycolates. Glucose-6-*O*-monomycolate, free mycolate, glycerol mycolate, arabinose-5-*O*-monomycolate, trehalose-6-*O*-monomycolate, Myc PL, and trehalose dimycolate (cord factor) were purified from mycobacteria and cultured with monocyte-derived dendritic cells for presentation to LDN5 and LDN5αβ/J.RT-3. R indicates the meromycolate R group which, if present, may be a keto, wax ester, or methoxy derivative. *M.* tb, *M. tuberculosis*.

The TCR-mediated Antigen Recognition Was Specific for the Carbohydrate Structure of Naturally Occurring Glycosylated Mycolates. Mycobacteria synthesize a variety of natural glycosylated mycolates that are related in structure to GMM. These include free mycolic acids and mycolic acids esterified to carbohydrates including glycerol, arabinose, trehalose, and Myc PL (28, 34–36; Fig. 2). CD1b presents free mycolic acids to several human T cell lines, indicating that the unglycosylated mycolic acid structure alone is sufficient for uptake and presentation by APCs and recognition by certain T cells (8, 12, 37). Since all glycosylated mycolates contain the mycolate moiety, any of these natural glycosylated mycolates might be expected to bind CD1b and be presented to T cells. Therefore, the specificity of LDN5 and LDN5αβ/J.RT3 were evaluated by testing them against a panel of natural glycosylated mycolates produced by mycobacteria. This revealed that LDN5 responded only to GMM, except for slight cross-reactivity for arabinomycolate (Fig. 2). Even antigens such as trehalose monomycolate and trehalose dimycolate that possessed GMM as a substructure failed to stimulate LDN5, indicating that monocyte-derived dendritic cells did not cleave these larger natural glycolipids to generate GMM that could be efficiently presented to T cells (Fig. 2). This pattern of recognition of natural glycosylated mycolates extended the results of a previous study (12), demonstrating that T cell recognition required the glucose moiety, was specific for the structure of the glucose moiety, and was not permissive of a second glycosyl residue at the anomeric carbon of glucose.

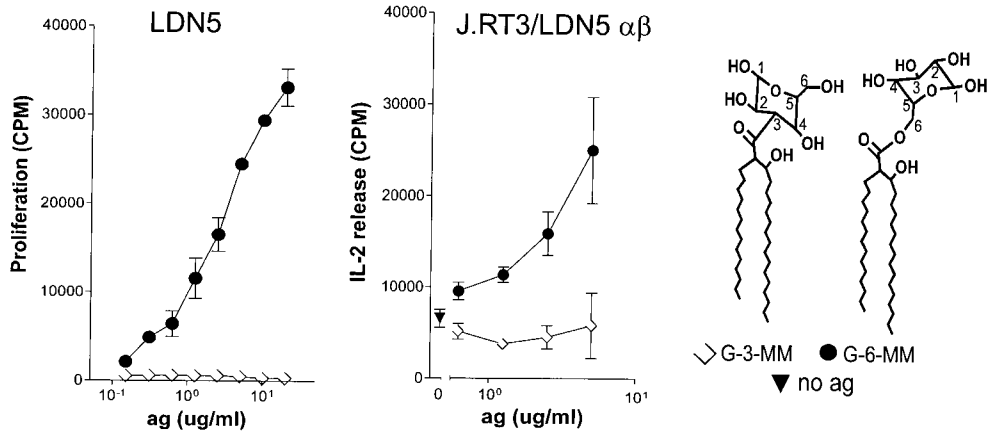

Figure 3. LDN5 and LDN5αβ/J.RT3 recognition of synthetic GMM was specific for the 6-linkage. G-3-MM and G-6-MM were synthesized and the structures were confirmed by electrospray ionization mass spectroscopy and NMR analysis. Antigens (ag) were presented by monocyte-derived dendritic cells and responses were determined by proliferation for LDN5 and by HT-2 assay of IL-2 release for LDN5αβ/J.RT3.

The TCR-mediated Antigen Recognition Was Specific for Linkage and Stereochemistry of the Lipid Portion of Natural GMM. To determine whether the fine specificity of LDN5 was limited to the glucose moiety or also extended to the hydrophilic elements of the mycolate lipid, we produced synthetic analogues of GMM that differed from natural glucose-6-*O*-(2*R*, 3*R*) monomycolate in the linkage or the structure of the lipid. Natural mycobacterial GMM has been previously shown to be composed of D-glucose esterified via the sixth carbon to mycolic acid (38), and NMR analysis of the GMM used in this study confirmed the 6-linkage (data not shown). This naturally occurring 6-linkage was required for LDN5 and LDN5αβ/J.RT3 recognition of GMM, as both lines recognized synthetic G-6-MM but not an isomer linked via the third carbon of glucose, G-3-MM (Fig. 3). This result provided evidence against a nonspecific function of the glucose moiety such as facilitating mycolate solubility, and was most consistent with the generation of an antigenic epitope formed by the glucose and the adjacent hydrophilic elements of the mycolic acid (39).

The mycolate portion of the antigen is produced in vivo by a stereospecific enzymatic condensation of free fatty acids to yield an α-branched, 3-hydroxy fatty acid with an *R* configuration at the two chiral carbons, C_2 and C_3 (40–43). This 2*R*, 3*R* conformation is structurally important for natural mycolates because it allows the hydroxyl group of the mycolate to form a hydrogen bond with the carbonyl function, forming a ring structure that restricts rotation at C_1, C_2, and C_3 of the mycolate (44). In contrast, the chemical synthesis of mycolates yielded this isomer as well as three

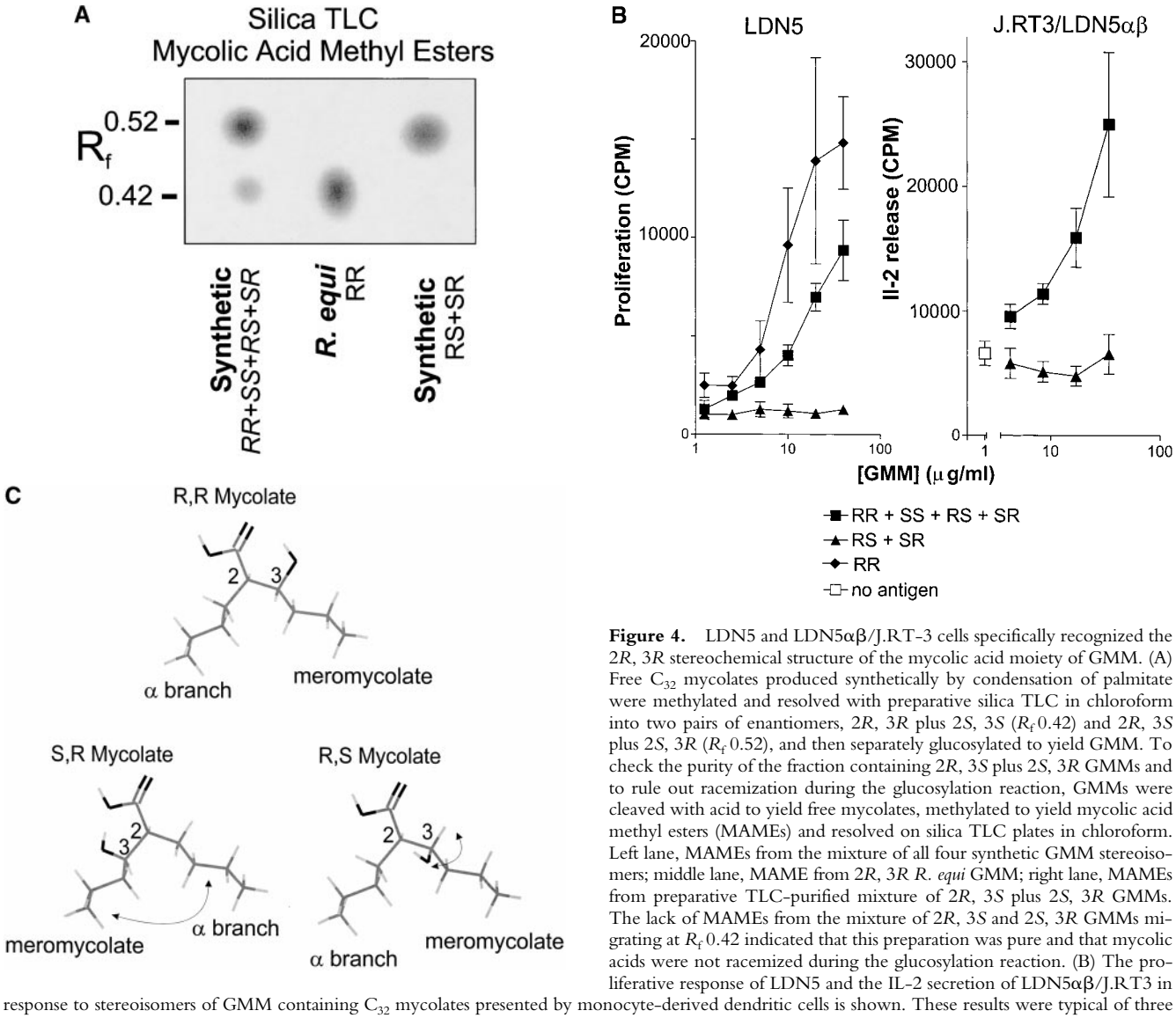

Figure 4. LDN5 and LDN5αβ/J.RT-3 cells specifically recognized the 2*R*, 3*R* stereochemical structure of the mycolic acid moiety of GMM. (A) Free C_{32} mycolates produced synthetically by condensation of palmitate were methylated and resolved with preparative silica TLC in chloroform into two pairs of enantiomers, 2*R*, 3*R* plus 2*S*, 3*S* (R_f 0.42) and 2*R*, 3*S* plus 2*S*, 3*R* (R_f 0.52), and then separately glucosylated to yield GMM. To check the purity of the fraction containing 2*R*, 3*S* plus 2*S*, 3*R* GMMs and to rule out racemization during the glucosylation reaction, GMMs were cleaved with acid to yield free mycolates, methylated to yield mycolic acid methyl esters (MAMEs) and resolved on silica TLC plates in chloroform. Left lane, MAMEs from the mixture of all four synthetic GMM stereoisomers; middle lane, MAME from 2*R*, 3*R R. equi* GMM; right lane, MAMEs from preparative TLC-purified mixture of 2*R*, 3*S* plus 2*S*, 3*R* GMMs. The lack of MAMEs from the mixture of 2*R*, 3*S* and 2*S*, 3*R* GMMs migrating at R_f 0.42 indicated that this preparation was pure and that mycolic acids were not racemized during the glucosylation reaction. (B) The proliferative response of LDN5 and the IL-2 secretion of LDN5αβ/J.RT3 in response to stereoisomers of GMM containing C_{32} mycolates presented by monocyte-derived dendritic cells is shown. These results were typical of three experiments. (C) Structural relationship of natural (2*R*, 3*R*) mycolate composed of C (gray), H (light gray), and O (black) with synthetic analogues that are not known to occur naturally is depicted with truncated meromycolate and α branches. The synthetic 2*S*, 3*R* mycolate form differed from natural 2*R*, 3*R* mycolate in the absolute orientation of the α and meromycolate branches at C_2 as indicated by the arrow. The 2*R*, 3*S* form differed from the natural mycolate in the absolute orientation of the 3-hydroxyl with regard to the meromycolate chain.

stereoisomers that are not known to occur naturally (2*R*, 3*S*; 2*S*, 3*S*; and 2*S*, 3*R*) (Fig. 4, A and C). Therefore, we asked whether LDN5 might also possess specificity for the mycolic acid portion of GMM by assaying for recognition of short chain G-6-MMs that differed only in the stereoconformation at C_2 and C_3 of the lipid. LDN5 recognized natural 2*R*, 3*R* GMM from *R. equi* and also responded at a somewhat higher dose to a mixture of synthetic GMMs containing all four stereoisomers (Fig. 4 B). However, both the native T cell line and the TCR transfectants failed to recognize a mixture of (2*R*, 3*S*) and (2*S*, 3*R*) GMMs, indicating that both the absolute orientation of the lipid branches and the stereoconfiguration of the 3-hydroxyl group found in natural mycobacterial GMM were required for recognition (Fig. 4 C). These results established that the LDN5 TCR–mediated recognition of GMM was highly specific for the hydrophilic portions of the lipid adjacent to the carbohydrate and the linkage of the lipid to the carbohydrate. Moreover, in all cases LDN5 recognized only the synthetic GMMs that recapitulated the hydrophilic structure of the natural from of GMM produced by mycobacteria, glucose-6-*O*-(2*R*, 3*R*) monomycolate.

Mycobacterial Synthesis of Antigenic GMM In Vitro and In Vivo Required an Exogenous Source of Glucose. This precise specificity for the structure of natural mycobacterial GMM suggested that this glycolipid functioned as a target of the human T cell response to mycobacterial infection. However, despite the isolation of GMM from mycobacteria grown in vitro by several groups, it has not been clearly established that infecting mycobacteria growing in vivo produce GMM (24, 25, 38). Furthermore, although mycobacteria grown in glucose-supplemented media synthesized large amounts of GMM, GMM has not generally been recovered from mycobacteria grown in glucose-free media, suggesting that GMM production may even be an artifact of in vitro growth (24, 25).

Since glucose is an abundant metabolite in mammalian cells and tissues and is homeostatically controlled to remain at concentrations near 100 mg/dl (~6 mM), we reasoned that mycobacteria might utilize host glucose for production of GMM within infected tissues in vivo. Therefore, we first sought to determine whether exogenous glucose was necessary for production of antigenic GMM in vitro. *M. phlei* and *M. smegmatis* were cultured in standard media supple-

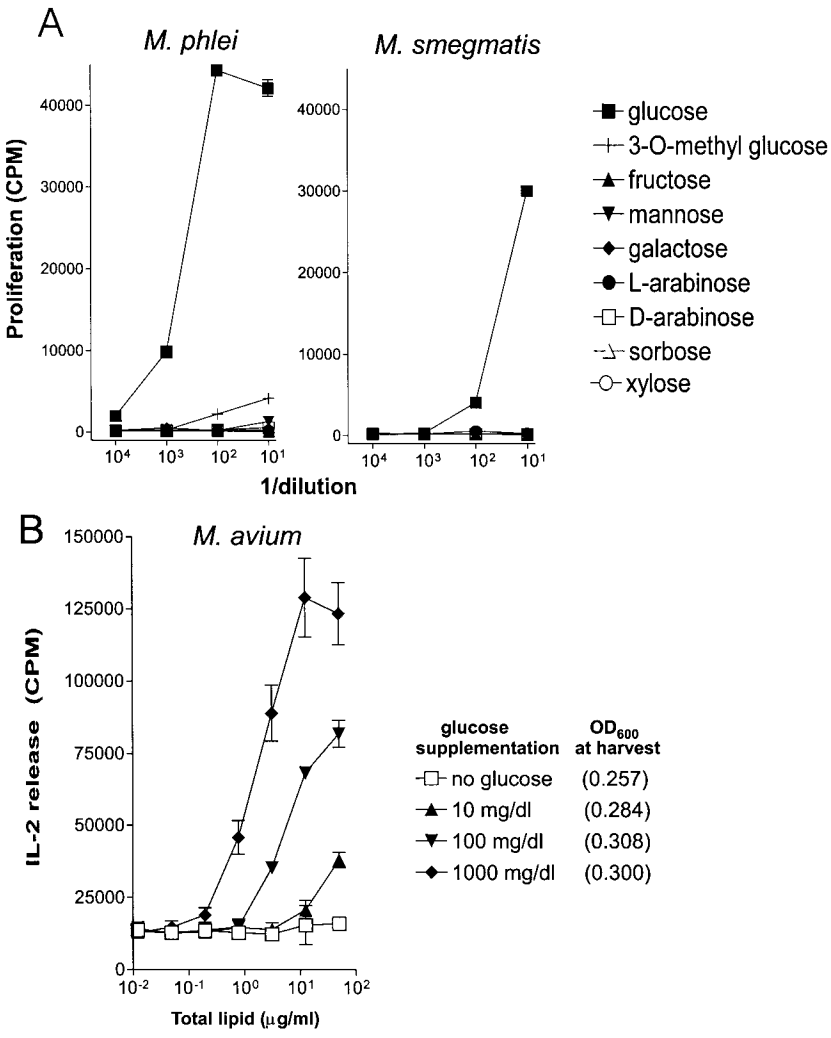

Figure 5. Mycobacterial production of antigenic GMM required exogenous glucose. (A) *M. phlei* and *M. smegmatis* were grown in standard (7H9; GIBCO BRL) medium that was supplemented with 1 g/dl D-glucose or the indicated carbohydrate. Mycobacteria were washed, lyophilized, and then extracted at 7.5 mg/ml with chloroform/methanol to give the total lipid fraction (dilution = 1). LDN5 proliferation in response to total lipid fractions presented by monocyte-derived dendritic cells is shown as the mean of duplicate samples with error bars indicating the range. (B) *M. avium* was cultured in medium supplemented with glucose at the indicated concentration for 24 h. Total extractable lipids were weighed and tested for their ability to stimulate IL-2 secretion by LDN5αβ/J.RT3 cells. The relative yield of mycobacteria after culture for 24 h with each of the indicated glucoses was estimated by the optical density of the culture fluid at 600 nm (OD_{600}). Computer-assisted, densitometric charring of total lipids resolved by TLC indicated that GMM comprised ~1 μg/mg of total lipid from mycobacteria grown at 100 mg/dl (data not shown).

mented either with D-glucose or structurally related sugars that are not present or are present only in trace amounts in mammalian cells. Total lipid extracts from bacteria grown in different conditions were tested for their ability to stimulate the GMM-specific T cell response of LDN5. Both *M. phlei* and *M. smegmatis* grown in glucose-supplemented media produced GMM abundantly as detected by TLC (data not shown), and total lipids from these mycobacteria potently stimulated the LDN5 response (Fig. 5 A). In contrast, mycobacteria grown in standard media supplemented with a carbohydrate other than glucose failed to produce GMM as detected by TLC (data not shown) and did not stimulate LDN5 (Fig. 5 A). This confirmed previous reports that GMM synthesis was dependent on exogenous glucose and demonstrated that this upregulation of GMM production can activate T cells in vitro. Next, we sought to determine the dose dependence of GMM production on exogenous glucose over a range of concentrations including the 100 mg/dl concentration that is typical of mammalian tissues. GMM production by *M. avium* was also dependent on exogenous glucose, and GMM was produced in media containing glucose in the range of concentrations typical of mammalian tissues. GMM comprised ~0.1% of the total extractable lipid when growing at 100 mg/dl of glucose, both as measured by the LDN5 bioassay and by direct charring of TLC plates, making it the single most abundant extractable glycolipid in *M. avium* grown in these conditions (Fig. 5 B). Thus, several species of mycobacteria did not detectably produce GMM de novo, but did produce this antigen in abundance when cultured with exogenous glucose, including glucose concentrations available to mycobacteria during tissue-based growth.

To determine whether mycobacteria would produce GMM during growth within mammalian tissue in vivo, the obligate intracellular parasite, *M. leprae*, was harvested directly from the liver of infected armadillos and tested for stimulation of LDN5. Crude sonicates of *M. leprae* grown in vivo stimulated LDN5 (data not shown), and extraction of the crude sonicate with chloroform/methanol (2:1) revealed that the antigen efficiently partitioned into the lipid-containing phase. Separation of total *M. leprae* lipids by preparative TLC indicated that the antigen was found in glycolipid fractions and comigrated with an authentic GMM standard (Fig. 6). Although the quantities of GMM isolated directly from cells were not adequate for detailed structural characterization, the comigration of the stimulatory glycolipid with a GMM standard and the precise specificity of LDN5 for the structure of GMM provide strong evidence that *M. leprae* growing within tissues in vivo produced antigenic GMM.

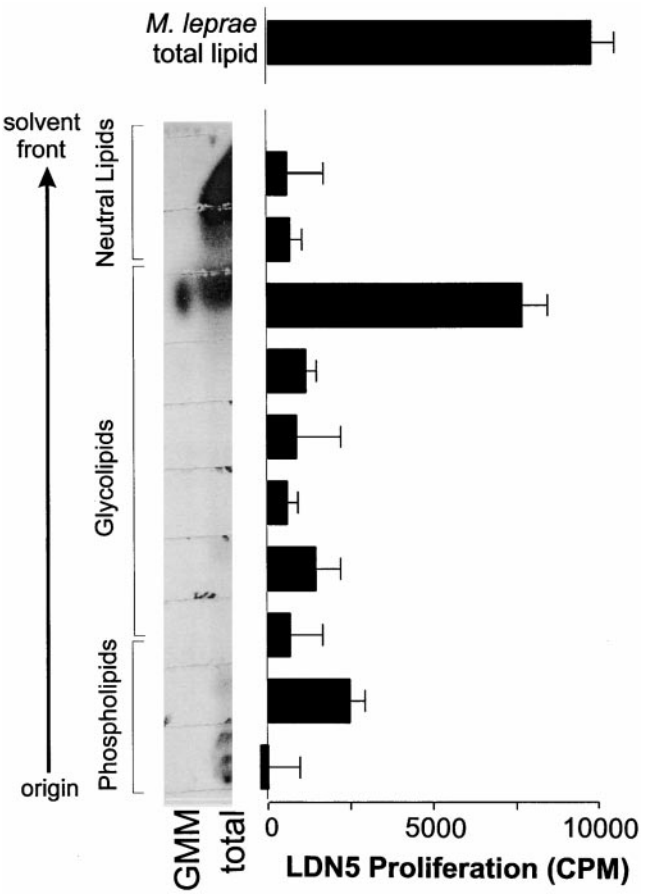

Figure 6. LDN5 recognized a GMM-comigrating lipid from *M. leprae* isolated directly from infected tissue. *M. leprae* was harvested directly from armadillo liver and total lipid was extracted with chloroform/methanol (2:1), loaded on a preparative silica TLC plate, and developed in chloroform/methanol/water (60:16:2) compared with a GMM standard (10 μg) purified from in vitro cultures of *M. phlei*. The TLC plate was scored at 1-cm intervals as indicated, and the lipids were separately extracted from each 1-cm fraction using chloroform/methanol (2:1). Extracts from each fraction were dried under nitrogen, resuspended in T cell medium (1:5 dilution) with monocyte-derived dendritic cells, and tested for stimulation of LDN5. The margin of the preparative plate was reserved and developed by charring with a cupric acetate solution and shown in comparison with the *M. phlei* GMM standard.

Discussion

Self and foreign proteins are composed from essentially the same amino acid pool. Therefore, T cell discrimination of self from foreign proteins is accomplished by reading out differences in primary amino acid sequences by highly specific TCR interactions with antigenic peptides lying within the grooves of MHC class I and class II proteins (45–47). Mammals and microbes differ fundamentally in certain lipid and carbohydrate biosynthetic pathways, so self and foreign glycolipids have certain fundamental differences in structure (19). Thus, T cell discrimination of self from foreign glycolipids might be accomplished with a lesser degree of specificity for antigen structure than seen for peptides, and the few available studies of CD1-restricted T cells have demonstrated varying degrees of antigen specificity (12, 22, 23, 31). In one case, an mCD1d-restricted murine T cell hybridoma recognized phosphatidic acid antigens that differed substantially in their hydrophilic structure, phosphatidyl inositol, phosphatidyl glycerol, and phosphatidyl ethanolamine (21). This study provided a comprehensive evaluation of the molecular determinants of recognition of a naturally occurring bacterial glycolipid antigen, glucose

monomycolate. The results indicated that T cell recognition can be extremely precise for glycolipid antigen structure, particularly the carbohydrate and adjacent elements of the lipid that form the functional unit referred to as the hydrophilic cap (Fig. 7).

We and others have proposed that the molecular mechanism of this specificity occurs by a trimolecular interaction of the TCR with the hydrophilic cap of the antigen as it protrudes from the CD1 groove (12, 19, 20). This study strongly supports this mechanism by directly demonstrating a functional requirement of the clonally variable elements of the LDN5 TCR in mediating a pattern of fine specificity for the hydrophilic elements of the antigen that are proposed to protrude from the CD1 groove. The TCR-mediated response to GMM was specific for all tested elements of the hydrophilic cap, including substitutions of the anomeric carbon of the carbohydrate (Fig. 2), the linkage of the carbohydrate to the mycolate lipid (Fig. 3), the absolute orientation of the mycolate β-hydroxyl (Fig. 4), and the absolute orientation of the two alkyl chains with regard to the carbohydrate (Fig. 4). These data clearly establish that both the carbohydrate and lipid components of GMM are required for recognition by LDN5, most likely by direct contact of TCR variable regions with the double ring structure formed by the linkage of the mycolate to glucose (Fig. 7).

The specificity of LDN5 for only those synthetic GMMs that precisely recapitulate the structure of the hydrophilic cap of natural mycobacterial glucose-6-*O*-(2*R*, 3*R*) monomycolate suggests that this glycolipid is a relevant target of the in vivo human T cell response to mycobacteria. We have reported that phospholipid-specific polyclonal T cell responses occur following mycobacterial infection (14). Recently, we have found similar responses to GMM in PBLs in the majority of subjects infected with *M. tuberculosis*, but not naive controls, indicating that this antigen is a target of the immune response during natural infections (Ulrichs, T, unpublished data). Thus, GMM-specific T cell activation is not just a property of LDN5, but is common among polyclonal lymphocytes of humans infected with *M.*

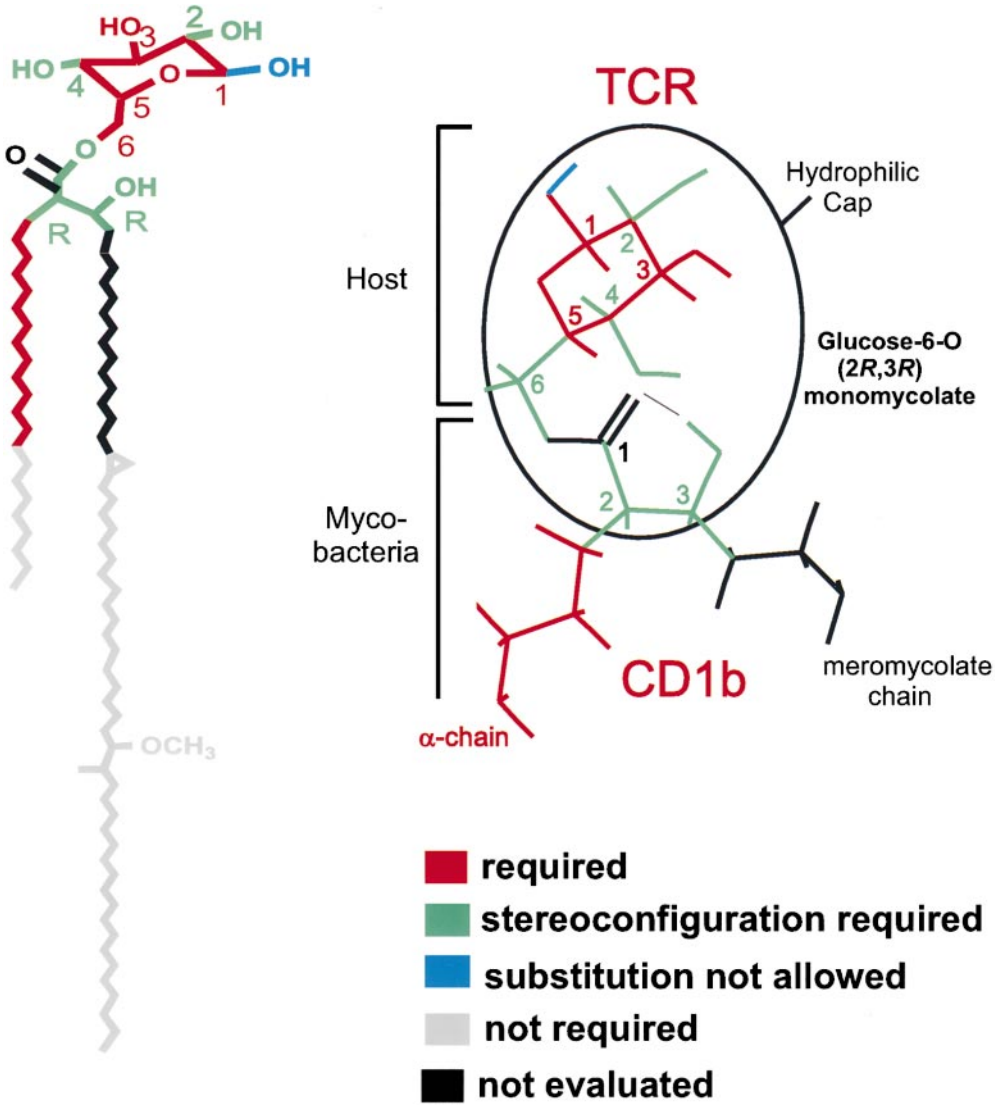

Figure 7. The map of the antigenic determinants of the TCR-mediated response to natural glucose-6-*O*-(2*R*, 3*R*) monomycolate. The hydrophilic cap of GMM is formed from host-derived glucose esterified via the sixth carbon to mycobacterial mycolate. Recognition of GMM required TCR α and β chains with the variable regions corresponding to LDN5, CD1b expression on the APC, and all tested elements of the structure of the hydrophilic cap of natural GMM, including components supplied by the host and by the mycobacteria. Natural or synthetic analogues lacking the indicated chemical element (red) or proper stereoconfiguration (green) of glucose-6-*O*-(2*R*, 3*R*) monomycolate were not recognized by cells bearing the LDN5 TCR α and β chains, but analogues lacking the distal elements of the mycolate alkyl chains (gray) were recognized. Substitution of the anomeric carbon (blue) with α-linked glucose (trehalose mycolate) or α-linked glucose monomycolate (trehalose dimycolate) abolished recognition.

tuberculosis. The biological significance of this reactivity in the context of mycobacterial infection derives from the observation that GMM is, strictly speaking, neither self nor foreign, but instead a combination of a self-carbohydrate and a foreign lipid that are coupled only after successful infection of host tissues by mycobacteria.

Mycobacteria synthesize mycolates using a hybrid fatty acid synthase mechanism that differs from the type I fatty acid synthase mechanism found in eukaryotic cells (16). Thus, the α-branched, β-hydroxy structure of mycolic acids is not found in eukaryotic fatty acids, making mycolic acids inherently foreign in structure to the mammalian immune system. The enzymatic activity leading to coupling of mycolates to glucose has not been identified, but GMM is synthesized by all mycobacterial species studied to date and can be quite abundant in the cell wall, comprising up to 2% of the total extractable lipid (12, 38, 48; and Moody D.B., unpublished data). The results of the current study confirmed that *M. smegmatis*, *M. phlei*, and *M. avium* cannot synthesize GMM de novo, but all three species produce antigenic GMM in abundance when exposed to glucose in vitro. Moreover, the obligate intracellular pathogen *M. leprae* produced GMM while growing within mammalian liver in vivo (Fig. 6). These results indicate that mycobacteria acquire exogenous glucose from the media or host tissues and esterify host glucose to mycobacterial mycolic acids.

Since glucose is not generally found in abundance in mycobacterial growth environments other than infected host tissues, we propose that expression of the GMM antigen in nature is generally restricted to pathogenic mycobacteria that have invaded host tissues and would not be expressed by saprophytic mycobacteria growing in the environment. This issue is particularly relevant for mycobacterial disease, since human exposure to environmental saprophytes is common, but does not generally lead to detectable cell-mediated immunity (49). Specific T cell recognition of a glycolipid antigen derived from the in vivo coupling of host and pathogen factors could allow not only discrimination of self from foreign structures, but also discrimination of bacteria that have invaded tissue from bacteria that have not. Many studies suggested that the host avoids immunopathology by distinguishing foreign antigen that is not a threat to the host (innocuous nonself) from that which is a threat to the host (pathogenic nonself; for a review, see reference 50). Thus, these results not only provide the first information about the expression of a CD1-presented antigen in vivo within tissues, they also suggest a new mechanism for discrimination of pathogenic from innocuous nonself that would allow a vigorous response to pathogenic mycobacteria while avoiding immunopathology from casual exposure to innocuous saprophytes.

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