considerable flexibility in regulating the presentation of lipid antigens, will be critical to improve the rational design of lipid vaccines and adjuvants.

References and Notes
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Figs. 51 to 54
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T Cell Activation by Lipopeptide Antigens

D. Branch Moody,1 David C. Young,1,2 Tan-Yun Cheng,1 Jean-Pierre Rosat,1 Carme Roura-mir,1 Peter B. O’Connor,2 Dirk M. Zajonc,3 Andrew Walz,4 Marvin J. Miller,3 Steven B. Levery,4 Ian A. Wilson,5,6 Catherine E. Costello,2 Michael B. Brenner1

Unlike major histocompatibility proteins, which bind peptides, CD1 proteins display lipid antigens to T cells. Here, we report that CD1a presents a family of previously unknown lipopeptides from Mycobacterium tuberculosis, named didehydroxymycobactins because of their structural relation to mycobactin siderophores. T cell activation was mediated by the β2 T cell receptors and was specific for structure of the acyl and peptidic components of these antigens. These studies identify a means of intracellular pathogen detection and identify lipopeptides as a biochemical class of antigens for T cells, which, like conventional peptides, have a potential for marked specificity.

1Division of Rheumatology, Immunology and Allergy, Brigham and Women’s Hospital and Harvard Medical School, Smith Building Room 514, 1 Jimmy Fund Way, Boston, MA 02115, USA. 2Mass Spectrometry Resource, Boston University School of Medicine, 715 Albany Street, R806, Boston, MA 02115, USA. 3Department of Chemistry and Biochemistry, University of Notre Dame, 251 Nieuwland Science Hall, Notre Dame, IN 46556–5670, USA. 4Department of Chemistry, University of New Hampshire, Durham, NH 03834, USA. 5Department of Molecular Biology and the 6Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

To identify candidate antigens presented by CD1a, TCR α and β chains from the CD1a-restricted and mycobacteria-specific T cell line CD8-2 were cloned and transfected into JRT3-T3.5 T lymphoblastoid cells to generate a reporter cell line, JRT3.CD8-2 (5). Along with untransfected JRT3 cells, this cell line was used to screen for antigens in chromatographic fractions generated from complex mixtures of compounds found in M. tuberculosis cell walls. Antigenic factors capable of JRT3.CD8-2 activation were efficiently extracted from whole mycobacteria by using chloroform: methanol (2:1), suggesting that the antigens were lipids, which were not covalently bound to the arabinoalactan complex of the mycobacterial cell wall (12). Elution of the stimulatory lipids from silica columns in polar solvents, such as methanol, indicated that the antigens displayed characteristics of polar lipids. Purification by high-performance liquid chromatography (HPLC) led to the isolation of a fraction that contained a set of structurally related compounds, which, by mass spectrometry analysis, yielded a prominent [M+H]+ ion at mass/charge ratio (m/z) 838.5. This antigenic compound was initially named 838 on the basis of its nominal mass.

CIR lymphoblastoid cells transfected with human CD1a, but not CD1b, CD1c, or CD1d, were able to present 838 to JRT3.CD8-2 (Fig. 1A). T cell activation was not seen with other known CD1-presented lipid antigens such as mycolic acid, glucose monomycolate, or mannosyl phosphoconjugates (Fig. 1B). Also, 838 did not activate polyclonal T cells or JRT3 transfecteds expressing TCRs that are specific for lipid antigens presented by CD1b or CD1c (Fig. 1B) (13–15). Together, these studies indicated that 838 did not function as a mitogen, but instead activated cells through CD1a-restricted

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 recognition mediated by clonally variable regions of the TCR α and β chains.

Further insights into the molecular composition of 838 were obtained through high-resolution mass measurements. Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) demonstrated the [M+H]+ to be m/z 838.5684, matching the value calculated for C47H76N5O8 (838.5688) (table S1). Tandem mass spectrometry (MS/MS) yielded product ions at m/z 642 and m/z 197, which were each 16 u (the mass of oxygen) smaller than the previously described mycobactin acid and cobactin fragments of mycobactin, a known mycobacterial lipopeptide with iron-scavenging properties (16, 17). Therefore, we named the antigen didehydroxymycobactin (DDM-838). Identification of MS/MS products at m/z 727 and 84 indicated that the hydroxylsines found in mycobactin were substituted by lysines in the proposed structure for DDM (17). The presence of lysine in DDM was confirmed by gas chromatography–mass spectrometry (GC-MS) of acid hydrolysis products. Unexpectedly, GC-MS detected an uncommon amino acid, α-methyl serine, instead of serine and threonine, which are present in most mycobacterial mycobactins (12, 16, 18). The identity of α-methyl serine as a component of DDM was confirmed by the nuclear magnetic resonance (NMR) spectrum of DDM (Fig. 2C), as produced by structure of the CD1a-presented antigens protein (DDM-thr) (17). Mycobacterial mycobactins (and threonine, which are present in most mycobacterial mycobactins, α-methyl serine, instead of serine and threonine, which are present in most mycobacterial mycobactins (12, 16, 18). The identity of α-methyl serine as a component of DDM was confirmed by the nuclear magnetic resonance (NMR) spectrum of DDM (Fig. 2C), as well as by differences in the MS/MS spectra of M. tuberculosis DDM compared with synthetic DDM homologs that contained threonine (DDM-thr) (19). These studies establish the structure of the CD1a-presented antigens produced by M. tuberculosis and show that they conform to a general structure in Fig. 2B, which is composed of salicylic acid, α-methyl serine, acylated lysine, hydroxybutyrate, and cyclized lysine (Fig. 2B).

Didehydroxymycobactin most likely functions as an intermediate in mycobactin synthesis. The mycobactin locus in M. tuberculosis encodes mycobactin synthase genes, MbtA to MbtJ, which function as a nonribosomal peptide synthesis pathway (20). Previously proposed schemes of mycobactin biosynthesis have emphasized a likely role of MbtA, MbtB, MbtC, MbtD, MbtE, and MbtF in activating the salicyl moiety and synthesizing the peptide (Fig. 2D) (19, 21, 22). The final steps in mycobactin synthesis were thought to involve peptide termination by intramolecular attack of the lysine side chain on its carboxy terminus and by acylation and hydroxylation of the two lysine residues (Fig. 2D). Although the precise order in which these terminal steps occur is not known, the discovery of DDM clarifies the order by suggesting that the lysines are incorporated into the peptide and subsequently hydroxylated to yield mycobactins (Fig. 2D). The two hydroxyl moieties, which are present in mycobactin but absent in DDM, form two sites, which mediate high-affinity (~10−26 M) binding to iron (16). Consistent with the predicted roles these hydroxyl groups in iron binding, we found that mycobactin was detected in the iron-bound form solely as [M+Fe(II)]+ at m/z 923.5, whereas DDM was detected solely in the unbound form as a proton adduct of m/z 838.6.

During infection, bacteria must obtain iron from host stores to support a variety of reduction-oxidation reactions necessary for normal bacterial metabolism. Bacteria scavenge iron by producing siderophores, which bind iron with high affinity at or near the bacteria-host interface and deliver iron to the bacterium (22). Mycobacteria produce mycobactin and related siderophores, whose synthesis is triggered by derepression of mycobactin synthase genes during growth in low-iron conditions (24). This process normally occurs during growth in host cells and is known to be necessary for M. tuberculosis survival within human macrophages (25). These considerations suggested that DDM might be synthesized as an intermediate in mycobactin production during intracellular infection. We found that DDM-specific T cell activation occurs efficiently in cells infected with five bacteria per cell of M. tuberculosis, whereas M. tuberculosis grown in complete medium requires the equivalent of 1011 bacteria to produce detectable levels of DDM, and in some cases does not produce DDM at all (Fig. 3A). Because CD1a-restricted T cells are able to kill mycobacteria-infected cells (26), CD1a presentation of DDM may represent an early warning system for intracellular pathogen recognition, whereby bacterial metabolites, which are necessary for adapting to intracellular growth, result in T cell activation.

To analyze more precisely the role of DDM structure in mediating T cell activation, we used HPLC to isolate several natural lipopeptides from M. tuberculosis. In addition to DDM-838 (Fig. 3B, peak E), M. tuberculosis produced a series of structurally related compounds (peaks A to D and F). Although the abundance, as determined by ultraviolet absorbance, correlated well with the measured T cell response for some compounds (Fig. 3B, peaks A, C, D, and...
E), others showed weak (peak F) or no (peak B) ability to activate T cells. Compounds in peaks A, B, E, and F were purified and found to have nominal masses of 810, 812, 838, and 840, respectively. MS/MS demonstrated that DDM-838 bore a C20:1 fatty acid, whereas DDM-840, DDM-812, and DDM-810 contained peptides of equal masses but were substituted with differing fatty acids, C20:0, C18:0, and C18:1, respectively (Figs. 2B and 3C).

A comparison of NMR spectra for DDM-838 and DDM-840 indicated that the unsaturation of the fatty acyl component of DDM-838 was at the C2=C3 position, likely in a cis conformation (Fig. 2C). Whereas DDM-838 gave the most potent T cell response, homologous lipopeptides that had shorter or saturated fatty acids were substantially less stimulatory (Fig. 3C).

The structure of the CD1a-presented antigen, DDM-838. (A and B) The DDM structure was deduced from the MS/MS spectrum of [M+H]⁺ m/z 838. Major complementary ions derived from DDM-838 were present at m/z 624 and m/z 215, which differed by 16 u from mycobactinic acid and cobactin fragments of mycobactin (inset) (16, 19). Simple one-bond fragmentations are indicated by dashed lines and assume hydrogen transfer to nitrogen and oxygen during amide and ester cleavage. Multistep fragmentations explain the ions at m/z 376, m/z 350, m/z 333, m/z 332, and m/z 84. (C) Downfield regions of one-dimensional proton NMR spectra of DMM-838 (upper) and DMM-840 (lower) show resonances that are assigned as aromatic (Hₚ) or other protons (Hₐ-n), as denoted in (B). The olefinic protons Hₚ and Hₐ are present in DDM-838, but not in DDM-840, and are coupled to each other with a J constant of 12.1 Hz. This is consistent with a double bond, which is likely present at C₂₋₃, because the doublet corresponding to Hₚ is directly coupled to only one proton, Hₚ is shifted downfield of its olefinic partner, Hₛ, as expected for a C=CH=C−O system. Further supporting this conclusion, Hₛ protons were also shifted slightly for DDM-838 relative to DDM-840 as a result of the influence of the olefin, which provides evidence for conjugation with the carboxyl group. The identification of α-methyl serine was based in part on the observations that Hₛ protons are only split by each other, suggesting that they are geminal protons in an oxazole ring adjacent to a quaternary carbon.

The acyl chain gave a typical terminal methyl triplet at 0.880 ppm and a large signal centered at 1.257 ppm, which obscures the signal from the methyl protons of the methyl serine associated with the oxazole ring. Two obvious impurity peaks (6.97 and 3.775 ppm, noted with an asterisk). Two-dimensional ¹H-¹H data were also obtained to confirm the presence of methyl serine and to corroborate all implied adjacent proton relations (not shown). (D) This biosynthetic scheme is based on the previously proposed pathway of nonribosomal peptide synthesis (16, 20–22) and on the discovery of DDM as a sideproduct of mycobactin, which does not avidly bind iron. The DDM structure suggests that lysines are incorporated in the peptide, which is terminated by a hydroxylation of the lysine residues prevents T cell recognition (Fig. 3C). Also, lipid fractions containing mycobactinic acid, which corresponds to a truncated lipopeptide lacking the butyric acid–lysine moiety (Fig. 2B, m/z 642 fragment), were recognized weakly or not at all (1/2).

Thus, the T cell response was specific for the structure of the peptide and the length and saturation state of the fatty acyl chain.

These studies identify lipopeptides as a biochemical class of antigens for T cells, which share structural features of MHC-presented peptides and CD1-presented glycolipids. The crystal structure of CD1a shows that the antigen-binding groove is composed of an F’ pocket, which is wide, largely exposed, and broadly contiguous with the predicted TCR contact surface (8). The A’ pocket is largely hydrophobic, with no obvious polar groups that could hydrogen bond with the peptidic portion of DDM in the way that MHC molecules bind to peptides. Also, the A’ pocket is narrow and terminates deep within the CD1a structure, so that it may act as a ruler to select out acyl chains of a particular length. A molecular model shows that the C20:1 fatty acid moiety could fit well within the A’ pocket, positioning the peptidic backbone at the broad junction of the A’ and F’ pockets, so that it would be available for contact with the TCR (Fig. 3D). Although the orientation of the peptidic moiety cannot be predicted precisely, the only polar residues in the binding groove are located at the A’-F’ junction, so it seems plausible that the same residues that are known to bind the sulfoligosaccharide moiety of sulfatide also hydrogen bond with the peptidic portion of DDM (8).
This model, along with the demonstrated role of TCR variable regions in antigen recognition (Figs. 1B and 3B), predict that the polypeptide backbone of the antigen serves as the contact for variable regions of the TCR, as is the case for conventional antigenic peptides presented by MHC proteins (27, 28). Mammalian ribosomes translate DNA-encoded peptide sequences, which are posttranslationally modified by acylation so that their structures resemble mycobacterial DDM but are highly varied in their peptide sequences (29). The autoreactivity of T cells for CD1a proteins suggests that mammalian autoantigens bind to CD1a, and peptides have been shown to regulate the recognition of CD1d presented antigens (4, 11, 30, 31). These observations raise the possibility that CD1 might also function to present structurally diverse lipopeptides encoded in the DNA of bacteria, viruses, or mammalian cells.

References and Notes
Role of LBPA and Alix in Multivesicular Liposome Formation and Endosome Organization

Hirotami Matsuo, Julien Chevallier, Nathalie Mayran, Isabelle Le Blanc, Charles Ferguson, Julien Fauvé, Nathalie Sartori Blanc, Stefan Matile, Jacques Dubochet, Rémy Sadoul, Robert G. Parton, Francis Vilbois, Jean Gruenberg

What are the components that control the assembly of subcellular organelles in eukaryotic cells? Although membranes can clearly be distorted by cytosolic factors, very little is known about the intrinsic mechanisms that control the biogenesis, shape, and organization of organellar membranes. Here, we found that the unconventional phospholipid lysobisphosphatidic acid (LBPA) could induce the formation of multivesicular liposomes. Liposomes prepared at pH 5.5 or pH 7.4, or they were prepared at pH 7.4 and then the internal pH was switched to pH 5.5, were observed in LBPA-containing endosomes in vivo. LBPA has not been detected elsewhere in the cell and is involved in protein and lipid trafficking through late endosomes (3–7).

Membranes and vesicles accumulate within multivesicular or multilamellar endosomes along the degradation pathway leading to lysosomes, and these selectively incorporate some proteins, including down-regulated receptors for growth factors and hormones (1, 2). In late endosomes, LBPA [or bis(monoacylglycerol)phosphate] is abundant in these internal membranes, accounting for ∼15 mole percent of total organelle phospholipids.

Table 1. MVLS. Liposomes were prepared at pH 5.5 or 7.4, or they were prepared at pH 7.4 and then the internal pH was switched to pH 5.5 (10). After labeling with FM 2-10, an aliquot of the assay mixture was exposed to the coveSip (final volume = 3 µl), and the number of MVLS was counted (10). About 300 liposomes from three independent experiments were counted for each condition.

<p>| Liposomes prepared at pH 5.5 or pH 7.4 |</p>
<table>
<thead>
<tr>
<th>n</th>
<th>%</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2′-dioleoyl LBPA</td>
<td>91</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>Control (no LBPA)</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>2,2′-dioleoyl LBPA + aLBPA Ab</td>
<td>4</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>2,2′-dioleoyl LBPA + IC AB</td>
<td>74</td>
<td>81</td>
<td>ND</td>
</tr>
<tr>
<td>3,3′-dioleoyl LBPA</td>
<td>17</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>3,3′-dimyristoyl LBPA</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Trimyristoyl semi-LBPA</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Trioleoyl semi-LBPA</td>
<td>4</td>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Liposomes prepared at pH 7.4 and then switched to pH 5.5

| Liposomes | n | % |
| 2,2′-dioleoyl LBPA | 107 | 100 |
| Control (no LBPA) | 15 | 14 |
| 2,2′-dioleoyl LBPA + aLBPA Ab | 10 | 10 |
| 2,2′-dioleoyl LBPA + IC AB | 64 | 60 |

We synthesized 2,2′-dioleoyl LBPA (8) (Fig. 1, A and B), the major isomer (>90%) in baby hamster kidney (BHK) cells (9), and we prepared large liposomes (10) with a phospholipid composition similar to that of late endosomes (dioleoylphosphatidylinositol: dioleoylphosphatidylethanolamine: phosphatidylinositol:LBPA, 5:2:1:2 mol) (3, 9). When labeled with the fluorescent dye FM2-10, large unilamellar liposomes (diameters of ∼600 to 800 nm) were easily revealed by light microscopy, whether LBPA was present (Fig. 1C) or not present (Table 1). Because the late endosomal lumen is acidic (pH 5.0 to 5.5) (1), we mimicked this situation in vitro by incubating lipids at pH 5.5 during the phase reversal of the liposome assembly process; the external pH was then neutralized to reproduce the pH gradient formed in vivo (10). Liposomes lacking LBPA remained unilamellar (Fig. 1D and Table 1), whereas LBPA liposomes showed 5 to 10 internal vesicles (Fig. 1E and Table 1), thus resembling multivesicular regions of the late endosomes that were observed by electron microscopy (EM) (3, 9, 11, 12). Internal vesicles were also observed within LBPA-containing endosomes.

Materials and Methods

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post date 9 April 2004

REPORTS: “T cell activation by lipopeptide antigens” by D. B. Moody et al. (23 Jan. 2004, p. 527). In Fig. 2C, the text labels for the NMR peaks were inadvertently shifted to the right. The correct figure appears here.