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Intersection of Group I CD1 Molecules and Mycobacteria in Different Intracellular Compartments of Dendritic Cells

Ulrich E. Schaible, Kristine Hagens, Karsten Fischer, Helen L. Collins, and Stefan H. E. Kaufmann

Human CD1a, CD1b, and CD1c molecules can present mycobacterial glycolipids to T cells. Because phagosomes containing viable mycobacteria represent early endosomal compartments, we studied where mycobacterial glycolipids intersect with CD1 molecules in infected APC. CD1b and CD1c, but not CD1a, localized to late endosomes/lysosomes. CD1a and CD1c were predominantly expressed on the cell surface and in mycobacterial phagosomes of the early endosomal stage. In contrast, CD1b was present in a subset of mycobacterial phagosomes representing mature phagolysosomes. Released mycobacterial glycolipids including lipoarabinomannan and phosphatidylinositol mannosides were transported from the phagosome into late endosomes/lysosomes and to uninfected bystander cells. The macrophage mannose receptor, which has been implicated in glycolipid uptake by APC for CD1b-mediated presentation, was absent from mycobacterial phagosomes and may therefore not be involved in trafficking of glycolipids between phagosomes and late endosomes/lysosomes. In conclusion, all three CD1 molecules have access to mycobacteria and glycolipids thereof, but at different intracellular sites. This allows sampling by CD1a, CD1b, and CD1c of mycobacterial glycolipids from different intracellular sites of the infected cell, which has important implications for processing and presentation of such Ags during mycobacterial infections.

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3 Abbreviations used in this paper: DC, dendritic cells; LAM, lipoarabinomannan; PIM, phosphatidylinositol mannosides; Mφ, macrophages; IFN-γ, interferon-γ; LAMP-1, lysosome-associated membrane protein-1; PFA, paraformaldehyde.

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Materials and Methods

Antibodies

Murine mAbs against CD1a (10H3.9.3) and CD1b (4A7.6.5) were provided by Dr. Daniel Olive (Marseilles, France) (17), and additional mAb against CD1b and CD1c were supplied by Dr. Walter Knapp (University of...
Table I. Distribution of human CD1 molecules and the MMR in relation to intracellular tracers and marker molecules as revealed by confocal microscopy

<table>
<thead>
<tr>
<th>Marker/Tracer</th>
<th>Compartment</th>
<th>CD1a</th>
<th>CD1b</th>
<th>CD1c</th>
<th>MMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF-FITC (1-h pulse)</td>
<td>EE</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>ManBSA (2-h pulse)</td>
<td>EE-LE</td>
<td>−</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>ManBSA-FITC (2-h pulse/30-min chase)</td>
<td>LE</td>
<td>−</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Ova-FITC/dex-TR (16-h pulse/2-h chase)</td>
<td>L</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>EE-L</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>CiM6PR</td>
<td>LE</td>
<td>−</td>
<td>±</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>LE-L</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>LBPA</td>
<td>LE</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>MHC class II (intracellular)</td>
<td>LE-L</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>MHC class II (surface)</td>
<td>PM</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>MHC class I (surface)</td>
<td>PM</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Golgi matrix protein</td>
<td>Golgi</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>BCG (ova-Cy3)</td>
<td>EE</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>BCG (ova-Cy3)</td>
<td>LE/L</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

* Human IL-4/GM-CSF-derived APC were either labeled with TF-FITC, manBSA-FITC, or Ova-FITC to label early endosomes (EE), late endosomes (LE) or lysosomes (L), respectively, or left untreated. APC were fixed in PFA and stained with mAb to CD1a, CD1b, or CD1c and Cy3-labeled secondary Abs. Double labeling was performed with Abs against the following marker molecules: LAMP-1, CiM6PR, cathepsin D, LBPA, MHC class II, or MHC class I. Cells were analyzed by confocal laser scanning microscopy as described in Fig. 1. Scoring: −, no colocalization; ±, partial colocalization; +, strong colocalization. PM, plasma membrane.

Vienna, Vienna, Austria (18). The CD1a-specific mAb OKT6 was purchased from American Type Culture Collection (Manassas, VA), and additional mAb to CD1b and CD1c were obtained from Serotech (Oxford, U.K.). The murine mAb to human CD14 (M5E2), CD64, and MMR were obtained from PharMingen (Hamburg, Germany). Those to human MHC class II (L243); human HLA-A, -B, and -C (MHC class I; W6/32); and human transferrin receptor (TfR; L5.1) were obtained from American Type Culture Collection. Those against human lysosome-associated membrane protein-1 (LAMP-1) (HA3) were a gift from Dr. John August (provided through Developmental Hybridoma Bank, University of Iowa, Ames, IA). The murine mAb against PIM (3D6) (19) and LAM (CS35) (20) were provided by Dr. Mario Ehlers (University of Cape Town, Cape Town, South Africa) and Dr. John Belisle (University of Colorado, Fort Collins, CO), respectively. The mAb to lysobiphosphatic acid (LBPA; 2C6) was a gift from Drs. Toshihide Kobayashi and Jean Gruenberg, (University of Geneva, Geneva, Switzerland) (21). Rabbit polyclonal Abs to human cathepsins B and D were purchased from Calbiochem (Bad Soden, Germany), and the affinity-purified rabbit polyclonal Abs to the cation-independent mannose-6-phosphate receptor (ciM6PR) was provided by Dr. Albert Haas (University of Wurzburg, Wurzburg, Germany). Species-specific secondary Abs labeled with peroxidase, Texas Red, FITC, Cy2, Cy3, or Cy5 were purchased from Dianova (Hamburg, Germany) and absorbed against Mycobacterium bovis BCG lysates before use. Cells were fixed in 4% paraformaldehyde and embedded in moviol.

Bacteria and labeling

*M. bovis* BCG (Copenhagen) or *M. tuberculosis* (H37Ra) were grown in 7H10 Middlebrook medium supplemented with oleic acid albumin dextrose complex (OADC; Difco, Detroit, MI), harvested during logarithmic growth phase (1–2 × 10^9/ml), and washed three times in PBS. *M. bovis* BCG carrying the green fluorescent protein (GFP) gene under the 60-kDa heat shock protein promoter were provided by Dr. Camille Loicht (Unit 447, Institut Nationale de la Sante de la Recherche Mdicale, Institut Pasteur de Lille, Lille, France).

For labeling with N-hydroxysuccinimidyl ester carboxyfluorescin (NHS-FITC; Boehringer Mannheim, Mannheim, Germany), bacteria were resuspended in PBS containing 1 mg/ml of the dye and shaken for 1 h at room temperature. To label mycobacteria with hydrazide compounds, bacteria were agitated in acetate buffer (pH 5.5) containing 15 mM sodium periodate for 15 min. The reaction was stopped by adding 15 mM sodium bisulfite for 5 min, and the sugar moieties on the bacterial surface were labeled with Alexa568-hydrazide (50 mM; Molecular Probes, Leiden, The Netherlands) at room temperature for 3 h. Labeled bacteria were washed five times in PBS and then fixed in medium before fixation. Bacteria retained ~95% viability after labeling. Confocal microscopy using this dye was performed on live DC and Mφ cultured on coverslips. The cells were kept in Ringer solution at 37°C for the time of observation.

Cell culture

PBMC from healthy donors (Bloodbank, Charite, Berlin) were purified from buffy coats using consecutive Ficoll and Percoll gradients. Cells were resuspended in RPMI (10% FCS) and either directly plated to produce Mφ or further cultured for 2 or 6 days in the presence of human rGM-CSF (100 ng/ml) and rIL-4 (100 ng/ml), R&D Systems, Wiesbaden, Germany) in petri dishes, on multichamber slides (Nunc, Roskilde, Denmark), or on coverslips to produce DC (37°C, 7% CO2). Where appropriate, cells were infected with mycobacteria at a multiplicity of infection of 10:1 for 2 h. Cells were washed and further incubated for the time periods indicated. Cells cultured for 6 days in GM-CSF/IL-4 were positive for MHC class I, MHC class II, CD1a, CD1b, and CD1c, but were negative for CD14 and CD64 (Fc-y). Cells showing typical DC-like morphology were clustered around adherent cells (data not shown). Although the latter cells are more Mφ-like, they nevertheless expressed all three group I CD1 molecules. In the text, all GM-CSF/IL-4-treated cells are termed DC.

Intracellular tracers

Texas Red-labeled dextran (dex-TR) was purchased from Molecular Probes (Leiden, The Netherlands), and FITC-labeled mannosylated BSA (ova-Cy3) was obtained from Sigma (Munich, Germany). FITC- or Cy3-labeled OVA or transferrin (Tf) were labeled with either NHS-FITC (Boehringer Mannheim) or Cy3 (Dianova) in carbonate buffer. Tagged proteins were purified using PD10 columns (Pharmacia, Freiburg, Germany). Competition experiments using unlabeled Tf confirmed that the labeled compound retained its receptor-specific binding capacity (12). Tracers were applied either in cell culture medium (dex-TR, OVA-FITC, OVA-Cy3) or Ringer solution (manBSA-FITC, Tf-FITC). Viable cells were fluorescently labeled using 2.5 ng/ml of the vital stain carboxyfluorescein diacetate-NHS (CFDA; Molecular Probes) for 5 min at room temperature in PBS and washed three times in PBS. Cells maintained fluorescence for up to 4 days. Tosyl-activated magnetic beads (Dynal, Hamburg, Germany) were labeled with human IgG (Sigma) and incubated with APC in a ratio of 5:1 for 2 h. For fluorescence microscopy either a conventional Leica DMIRB or a Leica TCS-NT confocal scanner (Leica, Bensheim, Germany) was used. To control the confocal data for false colocalization, independent sequential scans were performed and compared/overlayed thereafter. Confocal data were further processed using the Leica TCS-NT software and Adobe Photoshop.

Western blotting and TLC

To characterize the mycobacterial material that has been labeled with Alexa568-hydrazide, labeled mycobacteria were submitted to SDS-PAGE and Western blotting or to TLC. For SDS-PAGE, 10^4 mycobacteria/lane were separated on 4–20% polyacrylamide gradient gels containing 4 M urea. Fluorescence labeling was visualized by UV light before Western

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FIGURE 1. Differential intracellular localization of CD1 molecules in uninfected DC. A–C, Human GM-CSF/IL-4-derived DC were labeled with Tf-FITC, manBSA-FITC, OVA-FITC, or OVA-Cy3 to mark early endosomes, late endosomes, or lysosomes, respectively. DC were fixed in paraformaldehyde (PFA) and stained with Abs to CD1a (A), CD1b (B), or CD1c (C) and Cy3- or Cy2-labeled secondary Abs. DC were double stained with rabbit polyclonal Abs to either ciM6PR or cathepsin D. Cells were analyzed by confocal laser scanning microscopy, and data are presented as false color overlay images. Colocalization is indicated by yellow. The pictures shown are representative examples of at least five experiments performed with DC from different donors. Magnification, ×2500.
blotting onto nitrocellulose. Western blots were probed with mAb to LAM and PIM and species-specific secondary Abs labeled with peroxidase (HRP). Blots were developed using an enhanced chemiluminescence procedure (New England Nuclear, Boston, MA). For TLC, 10⁷ mycobacteria were extracted twice for 24 h in chloroform/methanol/water (16/6/1), and the extract was loaded onto high performance TLC silica plates. Plates were developed in the first dimension in chloroform/methanol (80/20; alkaline) and in the second dimension in propanol/water/acetic acid (80/10/10; acidic) and were visualized using UV light. For immunostaining, TLC plates were fixed in Plexigum (Röhm, Darmstadt, Germany), blocked in 10% BSA/0.05% Tween 20 in PBS, stained with mAb and HRP-labeled secondary Abs, and developed by enhanced chemiluminescence.

Results
Differential intracellular trafficking of CD1a, CD1b, and CD1c in uninfected DC

Uninfected DC expressed CD1a, CD1b, and CD1c at 2 and 6 days after cytokine stimulation, although the percentage of positive cells varied among donors (data not shown). The distribution of the three CD1 molecules in uninfected and infected DC as well as the tracers/markers used in this study, with respect to their specificity for intracellular compartments, are summarized in Table I. CD1a was exclusively found on the plasma membrane of DC and did not colocalize with tracers/markers for intracellular vesicles (Fig. 1A and Table I). This distribution is reminiscent of the cellular location of MHC class I molecules (15). In contrast, CD1b was less intensively expressed on the plasma membrane, but was strongly expressed in intracellular compartments. These CD1b-positive compartments colocalized with late endosome/lysosome-specific tracers/markers, but not with those specific for early endosomes (Fig. 1B and Table I). CD1b also partially overlapped with man-BSA-FITC internalized for 2 h but not chased, which labels the continuum between early and late endosomes (Table I). As previously shown, CD1b distribution partially colocalized with the late endosomal/lysosomal proteins cathepsin D, LAMP-1, and MHC class II (14). We have further extended these observations by showing partial overlap of CD1b with ciM6PR (Fig. 1D) and LBPA (Table I), which are markers for late endosomes (15, 21, 22).

Compared with CD1b, CD1c was more highly expressed on the plasma membrane (Fig. 1C). Furthermore, CD1c was additionally found in small vesicles, which in part colocalized with late endosomal/lysosomal tracers/markers such as man-BSA-FITC, OVA-Cy3, cathepsin D, LAMP-1, MHC class II, LBPA, and ciM6PR (Fig. 1, C and D, and Table I). CD1c was also found in a small percentage of Tf-FITC-labeled early endosomes (Fig. 1C and Table I). None of the three group I CD1 molecules colocalized with a Golgi-specific marker (Table I). This was probably due to their slow turnover/long half-life and consequently the small amount of newly synthesized CD1 protein (1). These experiments were repeated with three different mAb to either CD1a or CD1b and with two different mAb to CD1c and revealed similar results (data not shown). Additionally, we also used dex-TR at different incubation times to trace early endosomes (5-min pulse), late endosomes (30-min pulse), or lysosomes (2-h pulse, 1-h chase). These experiments gave similar results with respect to colocalization with the three CD1 molecules (data not shown). Taken together, the plasma membrane restricted localization of CD1a differed from additional late endosomal/lysosomal localization of CD1b and CD1c.

Intracellular trafficking of CD1a, CD1b, and CD1c in mycobacteria-infected DC

Infection of DC with M. bovis BCG or M. tuberculosis for 2–48 h did not alter the distribution of any of the group I CD1 molecules. However, the overall amount of surface-expressed CD1a, CD1b, and CD1c was slightly diminished 1–2 days after infection as previously described (data not shown) (23). All three CD1 molecules
were present in phagosomes containing mycobacteria (Fig. 2A). In DC, mycobacteria resided in phagosomes that either did not fuse (early endosomal phagosomes; 66% for BCG phagosomes) or fused with late endosomes/lysosomes (phagolysosomes; 44%) that were labeled with OVA-FITC or OVA-Cy3 (16-h pulse, 2-h chase). Interestingly, CD1a was exclusively present in the early endosomal subset of the mycobacterial phagosomes negative for OVA labeling (Fig. 2BA). In contrast, CD1b was only found in the OVA-positive late endosomal/lysosomal subset (Fig. 2BB). As previously reported, phagosomes with late endosomal/lysosomal characteristics most probably represent mature phagolysosomes containing nonviable mycobacteria (24). Similar to CD1a, CD1c was found in mycobacterial phagosomes with characteristics for early endosomes (Fig. 2BC). These results were further corroborated by the finding that CD1b associated strongly with IgG-bead phagosomes, CD1c associated faintly, and CD1a not at all (Fig. 2C). Thus, although all three CD1 molecules intersect with intracellular mycobacteria they do so in phagosomes at different stages of maturation: early endosomal for CD1a and CD1c vs late endosomal/lysosomal for CD1b.

Export of mycobacterial glycolipids from the phagosome

As previously shown by immunoelectron microscopy, LAM is exported from the mycobacterial phagosome and delivered into lysosomes (25). To analyze the overall trafficking of mycobacterial glycolipids from the phagosomes into the host cell vesicular system, terminal sugar moieties of cell wall components of M. bovis BCG, either wild type or expressing GFP, were labeled using Alexa568-hydrazide. The labeled material was analyzed by SDS-PAGE/Western blot as well as TLC as shown in Fig. 3. The two main fluorescent species observed by SDS-PAGE were a smear of ~30–40 kDa and a band of ~12 kDa running close to the dye front (Fig. 3A). Western blotting using specific mAb revealed that these bands corresponded to LAM (a smear between 30 and 40 kDa) (20) and PIM (~12 kDa) (19), respectively (Fig. 3A). By TLC, we were able to separate six Alexa568-hydrazide-labeled compounds of mycobacterial origin (Fig. 3B, arrows, spots 1–6). Furthermore, TLC analysis revealed that Alexa568-labeled mycobacteria were devoid of any uncoupled dye (Fig. 3B vs C). The compounds separated by TLC are currently under investigation, but are distinct from LAM (data not shown). Immunostaining of the TLC revealed that three of these spots stain with this mAb probably reflects PIM with various amounts of mannose. When analyzed 24 h p.i. in isolated phagosomes and nonphagosomal vesicles, spots 3–6 were still associated with the bacteria, whereas spots 1 and 2 were only found in other vesicles (data not shown).

Alexa568-labeled mycobacteria were used to infect human DC and macrophages. Two hours p.i., mycobacteria were internalized by the cells with no detectable label outside of the phagosomes.
This picture changed at 10 h p.i. and was even more pronounced 24 and 48 h p.i. At these time points the Alexa568-labeled glycolipids were widely distributed through the intracellular network, including tubular as well as large perinuclear lysosomal structures (Fig. 4A). This transfer of labeled glycolipids was not observed when mycobacteria were heat killed before labeling with Alexa568-hydrazide (data not shown). Following additional labeling of infected cells with intracellular tracers, we found that exported glycolipids colocalized predominantly with the late endosomal/lysosomal tracers, OVA-FITC and manBSA-FITC, but not with the early endosomal tracer Tf-FITC (Fig. 4B). Similar results were obtained in DC and Mφ (data not shown). Fluorescence labeling of mycobacterial surface proteins with NHS-FITC did not lead to this vast export of labeled material from the phagosome, suggesting that glycoproteins are only a small part of the exported material labeled with Alexa568-hydrazide (data not shown).

We obtained evidence that glycolipids were transferred to DC, which themselves did not contain mycobacteria. To verify this finding, we cocultured human Mφ infected with Alexa568-labeled \textit{M. bovis} BCG and uninfected DC labeled with CFDA. By 24 h most of the bystander DC contained significant amounts of mycobacterially derived label (Fig. 4C). Longer than 48-h coculture resulted in bystander DC becoming infected (data not shown).
These data reveal that mycobacterial glycolipids are exported from phagosomes containing live mycobacteria into late endosomes/lysosomes and to uninfected APC in their vicinity.

**MMR expression in early endosomal compartments**

The MMR has been proposed as a putative receptor and transport molecule to deliver mycobacterial glycolipids to CD1b-bearing vesicles (14). In our experiments the intracellular distribution of the MMR in DC was mainly restricted to small peripheral vesicles and colocalized with the early endosome-specific tracer Tf-FITC and strongly with manBSA-FITC, which labels the continuum between early and late endosomes (2-h pulse, no chase; Fig. 5). Localization of the MMR did not overlap with late endosome/lysosome-specific tracers (Fig. 5) or markers (cathepsin D, ciM6PR, MHC class II; Table I). The intracellular distribution of the MMR was not altered upon infection of the DC with mycobacteria, and the MMR was not observed in mycobacterial phagosomes (Fig. 5). These data suggest that the MMR does not participate in intracellular glycolipid transfer from the phagosome but, rather, is involved in the uptake of free mycobacterial glycolipids by uninfected DC (14).

**Discussion**

Mycobacteria persist dormantly within mononuclear phagocytes. Most immunocompetent individuals infected with tubercle bacilli do not develop disease, which indicates the important role of the specific immune response in restricting mycobacterial growth (26). The protective immune response comprises both CD4 and CD8 T cells and perhaps unconventional T cell populations such as γδ T cells (27). Recently, T cells recognizing glycolipids in conjunction with CD1 have been added to the antmycobacterial armory (9). Of the three group I CD1 molecules, only CD1b has been studied in detail with respect to its intracellular localization (14). These experiments were performed with purified LAM, which leaves open how, or even if, mycobacteria intersect with CD1 molecules during natural infection. The data presented here show that all three group I CD1 molecules intersect with mycobacterial phagosomes, although at different stages; whereas CD1a and CD1c gain access to phagosomes arrested at the early endosomal stage, CD1b trafficks to phagolysosomes. Furthermore, CD1b and CD1c molecules intersect with late endosomes/lysosomes carrying exported glycolipids.

The intracellular distribution of CD1b is similar to that of MHC class II, i.e., it has been localized together with HLA-DR and the invariant chain to late endosomal/lysosomal multilamellar vesicles reminiscent of the MIIC (14, 15). The MIIC also contains CD63, cathepsin D, and LAMP-1 and accesses the fluid phase marker dex-TR (14). In addition to these late endosomal/lysosomal markers, we show that CD1b associates with vesicles accessing manBSA. This tracer is taken up via the MMR and is released from its receptor in an early compartment to be delivered to late endosomes/lysosomes. Furthermore, we identified CD1b in vesicles carrying LBPA (21) and the ciM6PR (22), markers for late endosome.

In contrast to CD1b, CD1a colocalized neither with markers for early endosomes nor with those for late endosomes/lysosomes. Rather, CD1a was exclusively expressed on the plasma membrane, similar to MHC class I molecules (15). This observation is consistent with previous findings that the cytoplasmic tail of CD1a does not contain the endosomal targeting sequence YXXZ (Y = tyrosine, X = any amino acid, Z = bulky hydrophobic residue) present in other CD1 molecules (1, 28). Mutations introduced into the endosomal targeting motif of CD1b prevent these molecules from trafficking to the MIIC and inhibit presentation of mycobacterial glycolipids such as mycolic acids to T cells (29).
Presentation of glycolipid Ags by CD1b depends on the integrity of acidified compartments as a prerequisite for processing of Ags and their loading into the Ag binding groove of the CD1 molecules (27, 28). Similar to processing/presentation of MHC class II-restricted protein Ags, chloroquine inhibits CD1b-mediated T cell stimulation (5, 28, 29). Moreover, the CD1b molecule accommodates its ligands more efficiently when loading is performed at low pH, which probably facilitates widening of the groove (30). It can also be speculated that glycolipids are processed by host cell-derived lysosomal enzymes, which have lower pH optima. Preliminary data show that mycobacterial glycolipids are modified in infected APC (K. Fischer et al., unpublished observations). The data presented here, in combination with the presence of the YXXZ motif in the cytoplasmic tail of CD1c, suggests that CD1c is also trafficked through late endosomes/lysosomes and may pick up glycolipid Ags in an acidified environment similar to CD1b, although direct evidence is not yet available. The intracellular distribution of CD1c was not completely identical with that of CD1b. Compared with CD1b, CD1c was expressed at higher levels on the plasma membrane and less intensely in intracellular vesicles. CD1c only partially overlapped with late endosomes/lysosomes and was additionally present in mycobacterial phagosomes arrested at the early endosomal stage. In addition, CD1c was also found in a few Tf-FITC-labeled early endosomes. Compared with CD1b, CD1c is less efficiently trafficked into phagolysosomes containing beads. This indicates that CD1c can also pick up glycolipid ligands in earlier compartments, such as the early endosomal mycobacterial phagosomal similar to CD1a. Although we observed a slight decrease in the overall expression of all three CD1 molecules in mycobacteria-infected cells, as has been described by others (23), their intracellular distribution was not significantly altered during infection. The only apparent difference was the presence in mycobacterial phagosomes of CD1a, which otherwise was plasma membrane associated. The fact that we do not see a substantial decrease in CD1 expression in infected APC as described by Stenger et al. (23) could be due to experimental differences such as mycobacterial strains and different infection rates. The presence of CD1a and CD1c in the mycobacterial phagosome is not surprising in light of earlier reports showing 1) that surface-derived MHC class I and II molecules are present in the mycobacterial phagosome (31), and 2) that there is a continuous exchange of material between the phagosome and the plasma membrane (32). The significant amount of CD1a and CD1c on the cell surface compared with CD1b may facilitate simultaneous uptake or continuous import of these molecules into mycobacterial phagosomes arrested in the early endosomal stage. Although CD1a was present in early mycobacterial phagosomes that accumulated Tf, it was not detected in early endosomes of the transferrin pathway. This could be due to the following explanations. Either endosomes of the early recycling pathway engulf distinct receptors such as the TfR and exclude other surface molecules such as CD1a, or CD1a is recycled at an earlier endosomal stage than Tf.

Mycobacterial survival is facilitated in an early endosomal compartment characterized by an almost neutral pH due to a paucity in the vacuolar H+ATPase (10). The mycobacterial phagosome has access to the Tf/TfR pathway and also carries the early endosomal marker rab5 (11–13). It is, therefore, an intriguing question how mycobacterial glycolipids are trafficked from the phagosome into CD1-bearing compartments. Our data reveal that mycobacterial glycolipids are exported from the phagosome into late endosomes/lysosomes. A previous immunoelectron microscopy study has described LAM in lysosomes of infected murine macrophages (25). We show that LAM, PIM (19, 20), and three other as yet unidentified glycolipids, fluorescently labeled via their terminal sugar moieties, were released from the mycobacterial surface and transported out of the phagosome into late endosomes/lysosomes. These glycolipids reached compartments carrying CD1b and CD1c. Both LAM and PIM have been described as T cell Ags presented by CD1b and CD1c (3, 5, 7). Trafficking of fluorescent glycolipids has only been observed with live mycobacteria, which usually reside in early endosomal compartments. The early stage of the phagosome probably facilitated export of material into the downstream lysosomal pathway.

The observation that labeled glycolipids were transferred to uninfected bystander cells may have important implications for CD1-mediated immune responses. Although CD1-positive DC have been demonstrated in leprosy lesions and can harbor mycobacteria, Mφ represent the major habitat for mycobacteria in vivo (24, 33, 34). Macrophages, on the other hand, do not express CD1 in appreciable amounts. Furthermore, data by others revealed that CD1b expression is down-regulated upon infection with M. tuberculosis (23). Therefore, transfer of Ags from infected to noninfected APC would allow induction of an immune response even when the Ag-presenting capacity of the infected APC is impaired. The data presented here suggest that mycobacterial glycolipids can be transferred from infected Mφ to CD1-expressing DC to facilitate presentation to T cells. Preliminary data suggest that the transfer to bystander cells is mediated by extracellular vesicles, which are released into the culture medium. These vesicles possibly resemble exosomes (35) and blebs probably generated by apoptosis (U. E. Schaible, V. Brinkmann, and K. Fischer, unpublished observations), which is a well described outcome of mycobacterial infection in APC (36, 37). This transfer was not inhibited by Abs against the MMR (data not shown) and may therefore require additional receptors. At present, we cannot exclude the possibility that Alexa568-hydrazide labeling may alter the trafficking behavior of cell wall glycolipids. However, the observation that uptake by macrophages of Alexa568-hydrazide-labeled purified LAM can be competed by nonlabeled LAM would argue against this idea (data not shown).

It is of interest that in DC we found relatively high numbers of mycobacteria in phagolysosomes. This could be due to the cell type investigated, i.e., human IL-4/GM-CSF-induced DC, and could account for the fact that in these cells CD1b can interact with the late endosomal/lysosomal subset of phagosomes probably containing nonviable mycobacteria (24). The presence of CD1b, but not CD1a, in mycobacterial phagosomes of the late endosomal/lysosomal stage is consistent with the presence of CD1b in phagolysosomes containing IgG-coupled beads. These data together with a recent report showing that phagolysosomes represent competent compartments to process protein Ags for MHC class II presentation to T cells (38) suggest that CD1b can pick up and present glycolipids from dying mycobacteria in phagolysosomes.

Intracellular trafficking and intercellular transfer of glycolipids probably involve receptor molecules of the PRR family, such as the MMR. The MMR participates in delivery of exogenously added LAM to CD1b-bearing vesicles, thereby facilitating presentation of LAM to CD1b-restricted T cells (14). However, the MMR is predominantly present in early endosomes. As shown here, the MMR resides in some Tf- or manBSA-carrying vesicles. The latter ones are probably compartments where mannosylated material is separated from the MMR and trafficks further down the lysosomal pathway (39). These manBSA-labeled vesicles can also carry CD1b and CD1c and may represent the meeting point for MMR-delivered material and these molecules. To date, the MMR has not been localized to mycobacterial phagosomes, and therefore may not be involved in direct transfer of glycolipids from the phagosome into late endosomes/lysosomes. Because the MMR is
predominantly expressed in early endosomes and on the plasma membrane, it may, rather, participate in the engulfment of free glycolipids by bystander cells, as shown for purified LAM (14).

This raises the question of whether other PRR are involved in glycolipid transfer from phagosomes to lysosomes and bystander cells. CD14, which binds LAM directly (40), is present in mycobacterial phagosomes as well as in late endosomes/lysosomes (U. E. Schaible et al., manuscript in preparation). Hence, CD14 is a candidate for glycolipid trafficking in mycobacteria-infected Mφ. Furthermore, CD14 has been shown to be involved in the uptake of apoptotic blebs by human Mφ (41). Moreover, we cannot exclude participation of other PRR or the possibility that glycolipids such as LAM, which can directly integrate into the host cell membrane, are trafficked independently of specific receptors, similar to glycosyl phosphatidyl inositol-anchored cellular proteins (42).

Purified mycobacterial glycolipids are presented by group I CD1 molecules to specialized T cells (43). This finding has been taken as evidence that this T cell population plays an important role in protection against tuberculosis. Here we identified distinct intracellular compartments where mycobacteria, mycobacterial glycolipids, and the three group I CD1 molecules interact during mycobacterial infection; this suggests that CD1a, CD1b, and CD1c lipid, and the three group I CD1 molecules intersect during mycobacterial infection. This suggests that CD1a, CD1b, and CD1c are trafficked independently of specific receptors, similar to such as LAM, which can directly participate in the engulfment of free glycolipid Ags for a novel antituberculosis vaccine.

Note added in proof.

It should be mentioned that two recent publications are of note. First, Roark et al. (44) describe trafficking of mycobacterial lipids from phagosomes to lysosomes and noninfected bystander cells via exosomes. Second, Sugita et al. (45) published data that CD1a is present in recycling endosomes.

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References


