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**Mycobacterium tuberculosis** Regulates CD1 Antigen Presentation Pathways through TLR-2

*Carme Roura-Mir,*2,2* Lisheng Wang,2,3* Tan-Yun Cheng,* Isamu Matsunaga,* Christopher C. Dascher,* Stanford L. Peng,4* Matthew J. Fenton,5* Carsten Kirschning,‡ and D. Branch Moody*6*

*Mycobacterium tuberculosis* remains a major pathogen of worldwide importance, which releases lipid Ags that are presented to human T cells during the course of tuberculosis infections. Here we report that cellular infection with live *M. tuberculosis* or exposure to mycobacterial cell wall products converted CD1+ myeloid precursors into competent APCs that expressed group 1 CD1 proteins (CD1a, CD1b, and CD1c). The appearance of group 1 CD1 proteins at the surface of infected or activated cells occurred via transcriptional regulation, and new CD1 protein synthesis and was accompanied by down-regulation of CD1d transcripts and protein. Isolation of CD1-inducing factors from *M. tuberculosis* using normal phase chromatography, as well as the use of purified natural and synthetic compounds, showed that this process involved polar lipids that signaled through TLR-2, and we found that TLR-2 was necessary for the up-regulation of CD1 protein expression. Thus, mycobacterial cell wall lipids provide two distinct signals for the activation of lipid-reactive T cells: lipid Ags that activate T cell receptors and lipid adjuvants that activate APCs through TLR-2. These dual activation signals may represent a system for selectively promoting the presentation of exogenous foreign lipids by those myeloid APCs, which come into direct contact with pathogens. The Journal of Immunology, 2005, 175: 1758–1766.

**The Journal of Immunology**
of the cytokines that promote the early phases of differentiation to DCs, GM-CSF, and IL-4 (1, 27). Related to this, studies of MHC class II-mediated Ag processing have shown that the transition of myeloid precursors into immature DCs in response to GM-CSF and IL-4 is associated with increasing rates of macrophagocytosis and related mechanisms of bulk flow Ag uptake (28). This raises the possibility that monocytes in the peripheral blood generally lack the ability to present lipids on group 1 CD1 proteins, until they traffic to local sites of inflammation or infection, where as yet undefined stimuli lead to CD1 expression.

In these studies, we considered the possibility that microbial pathogens might modulate CD1 expression and Ag-presenting function in human myeloid cells. We initially focused on *M. tuberculosis* because this prevalent and deadly pathogen produces many types of lipid Ags that are presented by the CD1 system, including mycolic acids, GMM, LAM, mannosyl phosphocytoketides, acylated sulfofetahalos, and DDM lipopeptides (3, 4, 5–9, 29). Furthermore, prior studies had shown that human myeloid cells that migrate to the sites of cutaneous infection by *Mycobacterium leprae* or pulmonary infection by *M. tuberculosis* infection express CD1a, CD1b, or CD1c (30–32).

To test the hypothesis that *M. tuberculosis* itself might regulate CD1 function, we developed systems to study the mechanism of CD1 expression and Ag-presenting function in human monocytes infected with *M. tuberculosis*. We found that *M. tuberculosis* or its cell wall lipids strongly induced the expression of all three group 1 CD1 isoforms (CD1a, CD1b, and CD1c), but not the only member of human group 2 CD1 proteins, CD1d. CD1 induction was accompanied by increases in the rate of lipid Ag uptake and was sufficient to convert monocytes into fully competent APCs. Isolation of the mycobacterial factors that controlled this process showed that the stimuli were polar lipids shed from the mycobacterial cell wall, which signal through TLR-2. Furthermore, we found that TLR-2 was necessary for the induction of CD1 by mycobacteria. These studies define a new immunological function for TLR-2 by showing that primary activation of this innate pattern recognition receptor controls the subsequent functions of group 1 CD1 proteins. Furthermore, these studies suggest a model in which microbial cell walls provide two signals that lead to CD1-mediated T cell activation: Ags that bind to CD1 and adjuvants that trigger CD1 expression through activation of TLR-2.

**Materials and Methods**

**Cells and flow cytometry**

Human monocytes were isolated from peripheral blood using Ficoll and adherence to tissue-culture flasks and were cultured in RPMI 1640 medium with 10% FBS (33). In selected experiments, monocytes were further depleted of residual lymphocytes using anti-CD20 (L26), anti-CD3 (TC58), anti-CD56 (B159), and goat anti-mouse Ig-coated magnetic beads (Dynal) such that lymphocytes constituted <2% of total cells as determined by flow cytometric staining with non-cross-reactive lymphocyte markers. These additional purification steps to remove lymphocytes did not substantially influence CD1 induction on myeloid cells. Cell surface protein expression was measured using murine mAb against human CD1a (OKT6), CD1b (BCD1b.3 or 4A7), CD1c (F10/21A3.1), CD1d (CD1d4.2), CD80 (L307.4), CD83 (HB15e), CD86 (FUN-1), CD11c (B-ly6), and CD14 (M5E2) from BD Biosciences; CD64 (10.1; Caltag Laboratories); CD20 (eB-h209; eBioscience); HLA-DR (L243; American Type Culture Collection), and IgG1 (F(ab')2) or mouse IgG1 PE.

**Real-time PCR analysis**

Total RNA from monocytes was isolated using the RNeasy kit (Qiagen) and was retrotranscribed using Superscript II H-Reverse Transcriptase (Invitrogen Life Technologies). The relative mRNA for CD1 expression was analyzed by real-time PCR (RT-PCR) using a Taqman universal master mix reagent kit in the ABI PRISM 7700 System (Applied Biosystems). CD1 mRNA levels were determined by the comparative Ct method relative to the expression of the housekeeping gene GAPDH. Primers are: CD1a (5'-TGTATGGTCTTCCAGGTGA-3' forward, 5'-AGATGCGATCAGATCAT-3' reverse), CD1b (5'-CCATTTCCAACGTG-3' forward, 5'-GAAAAGGGTGTCGGCC-3' reverse), CD1c (5'-TTGGTGCAATGACGAGCCA-3' forward, 5'-GGTGTGACGAAAGGAAAA-3' reverse) and CD1d (5'-AGATGCCCGCA-3' forward, 5'-TATTGGCGAAAGGACGATCT-3' reverse). The fluorogenic probes are: CD1a (5'-FAM-AGACGGGGCCTCAAGGACCTCTCCTCCTCCATAMRA-3'), CD1b (5'-FAM-CCTCGTGTTCAAGGAATGTCCCTTTTATGCTAMRA-3'), CD1c (5'-FAM-CCCAGGAAACGCTCTCCTTCCATGTCTAMRA-3'), and CD1d (5'-FAM-AGGCTTTCCTCCGCTGCTAMRA-3'). The PCR cycling parameters were 40 cycles of 95°C denaturation and 60°C annealing and extension.

**Cellular assays**

IL-2 release by the human CD1b-restricted T cell line LDN5 was measured after 1 day in culture at a 1:1 ratio with lipid-treated monocytes (6). Activation of JRT-3 cells transfected with the TCR α- and β-chains from the human CD1a-restricted T cell line CD8-2 (34, 35) was measured by flow cytometry of cells treated with the calcium-sensitive fluorophores Fluo-4 and Fura-red (23). Glycolipid uptake was measured by incubating monocytes at 100,000 cells per well in 96-well plates with *M. tuberculosis* lipids (10 μg/ml) or medium alone for 3 days followed by addition of 1′C-labeled GMM as described (23). Macropinocytosis was measured by culturing 10³ monocytes/ml with *M. tuberculosis* lipids (10 μg/ml) for 3 days and washing before further culture with FITC-dextran (1 mg/ml; m.w. 40,000 Da; Sigma-Aldrich) for 1 h. Cells were washed and then subjected to flow cytometric analysis.

**Live infection**

*M. tuberculosis* strain H37Ra or strain H37Rv (Colorado State University, Fort Collins, CO) or *M. tuberculosis* H37Ra or H37Rv expressing GFP (36) was grown in 7H9 medium with albumin-dextrose-catalase supplement, glycerol, and Tween 80. Mid-log-phase cultures were washed with medium and filtered through a 5-μm filter. Aliquots were thawed and used to infect monocytes at 2 or 10 bacteria per cell as determined by counting in Petroff-Hauser chambers or from colony forming unit determinations on Middlebrook 7H11 plates. After extensive washing to remove extracellular bacteria, cells were cultured 1–3 days in medium, stained with anti-CD1 mAb, fixed with 2% paraformaldehyde, and analyzed by flow cytometry. For confocal microscopy, cells stained with anti-CD1 Abs were examined as cytoplasm preparations on glass slides using a Zeiss Axiovert S100 microscope equipped with a Bio-Rad Mrc 1024 confocal imaging system.

**Microbial extracts and glycolipids**

Total lipid extracts of *M. tuberculosis* H37Ra, *M. tuberculosis* H37Rv (Colorado State University), *Mycobacterium avium* (H. Remold, Harvard University, Cambridge, MA), *Mycobacterium phlei* (L. Tielens, Universiteit Utrecht, Utrecht, The Netherlands, and D. van der Kleij, Leiden University Medical Center, Leiden, The Netherlands) were prepared by extracting lyophilized organisms at 5 mg/ml in chloroform/methanol (2:1) for 2 h. *M. tuberculosis* H37Rv was further fractionated by drying under nitrogen, resuspending in chloroform, and eluting from an open silica column (6). In selected experiments, *M. tuberculosis* polar lipids were further purified by one-dimensional TLC using 200-μm silica plates with 10 mg of polar lipids, resolved in chloroform/methanol (2:1) and eluted from open silica column after 1 day in culture at a 1:1 ratio with lipid-treated monocytes (6).

**TLR analysis**

Monocytes were incubated in the presence or absence of different concentrations of poly(I:C) (Sigma-Aldrich), PAM3 (EMC Microcollections) or Cpg oligonucleotides (Integrated DNA Technologies). For inhibition of the TLR-2-dependent activity, monocytes were incubated with an anti-TLR2 Ab (T2.5) (37) or isotype control Ab for 1 h before adding the *M. tuberculosis* lipids (10 μg/ml). Cells were extensively washed after...
2 h, resuspended in fresh medium containing 20 μg/ml of the TLR-2 Ab or control Ab, and further cultured for 3 days. CD1 expression was measured by flow cytometry using fluorescein-labeled CD1a (CB-T6), CD1b (SN13/K5-1B8), CD1c (M241), or isotype control Abs (Ansell). TLR-2 agonism was tested using Chinese hamster ovary (CHO) cells cotransfected with TLR-2 and a CD25 expression vector under the control of NF-κB as described (38).

Results
M. tuberculosis induces group 1 CD1 expression
To test the possibility that mycobacteria could modulate CD1 expression, we infected human monocytes with M. tuberculosis and measured CD1 expression by flow cytometry over a period of several days. Whereas fresh monocytes and control cells treated with medium for 3 days failed to detectably stain with mAbs specific for group 1 CD1 proteins, a large number of cells in infected cultures stained brightly with Abs against CD1a, CD1b, and CD1c. Each of these group 1 CD1 proteins was induced to high levels by day 2 of infection, and expression levels were typically maximal at day 3 (Fig. 1A).

To investigate the cellular mechanisms by which M. tuberculosis induced CD1 cell surface expression, we first sought to determine whether CD1 staining was found on cells that were infected with bacteria, uninfected bystander cells, or both. This was of interest because the subset of cells with productive intracellular infection had the greatest contact with bacteria and might have received the highest doses of CD1-inducing products. In contrast, prior studies of mycobacteria-infected cells conducted with mixed agonist and antagonist signals suggested that live mycobacteria can inhibit CD1 expression. In particular, previous studies had found that when monocytes were first treated with GM-CSF and IL-4 so that they expressed high levels of CD1 before mycobacterial infection, group 1 CD1 protein expression was either unaffected (39), moderately down-regulated (40), or down-regulated to undetectable levels after infection (41). The question of whether mycobacteria can induce CD1 expression in the first instance had not been previously addressed, and the data shown in Fig. 1A indicated that whatever inhibitory signals might be provided by M. tuberculosis, these were not sufficient to block the initial induction of CD1a, CD1b, and CD1c on fresh monocytes. Nevertheless, because not all the monocytes in the culture up-regulated CD1, it remained possible that cells with live intracellular bacteria might have been prevented from expressing CD1 and that CD1 might have been present predominantly on noninfected bystander cells.

To separately measure CD1 expression on cells with and without bacteria, we infected monocytes with M. tuberculosis expressing GFP (M. tb-GFP) (36). Two-color immunofluorescence microscopy of monocytes showed bright staining for CD1a on cells with and without overlying fluorescent bacteria 3 days after infection (Fig. 1B). Confocal microscopy showed bright staining for CD1a, CD1b, and CD1c on infected cells as well as colocalization of CD1b within compartments that contained bacteria (Fig. 1C and data not shown). In addition, flow cytometric analysis of the entire population of infected cells showed clear evidence for CD1a, CD1b, and CD1c expression on a large subpopulation of both GFP<sup>hi</sup> and GFP<sup>lo</sup> cells (Fig. 1D). These experiments provided strong evidence that group 1 CD1 proteins are up-regulated over a period of 2–3 days on infected and bystander cells alike.

CD1 induction is mediated by mycobacterial lipids
The appearance of group 1 CD1 proteins on uninfected bystander cells suggested that the mechanism of CD1 induction involved secretion or cell-to-cell transfer of stimulatory factors that were derived from the mycobacterium, the monocytes, or the interaction of the mycobacterium with the monocytes. Further studies showed

FIGURE 1. Mycobacterial infection of monocytes induces group 1 CD1 proteins on the surface of infected and bystander cells. A, Fresh human monocytes were infected with M. tuberculosis H37Ra (10 bacteria/cell) for 4 h, washed extensively, and cultured before flow cytometric detection of cell surface CD1. The percentage of cells staining with mAb against CD1a (OKT6), CD1b (BCD1b3.1), or CD1c (F10/21A3.1) above background levels seen with an isotype-matched mAb (P3) is shown. B, Monocytes were infected with M. tb-GFP, washed, and cultured for 3 days before staining with anti-CD1a and anti-mouse Ig PE (red). Representative phase contrast and overlay immunofluorescence micrographs show four cells, three of which express CD1a and one of which is infected. C, The upper panel is a representative confocal micrograph of a human monocyte 2 days after M. tb-GFP infection and is stained with anti-CD1b (red) with colocalization shown in yellow. The lower panel is a composite image of the Z plane corresponding to the superimposed line in the upper panel. D, Two-color flow cytometric analysis of monocytes after 2 days of culture in medium or infection with M. tb-GFP and staining with anti-CD1a, anti-CD1b (4A7.6.5), anti-CD1c, or control (P3) Ab and FITC-labeled goat anti-mouse F(ab')<sub>2</sub>. These experiments are representative of >10 experiments performed.
that killed mycobacteria and filtered supernatants of bacteria-conditioned culture medium could substitute for live *M. tuberculosis* in inducing CD1 expression (data not shown). Therefore, this process did not require growth of live bacteria within cells and could be mediated by products physically shed from the cell wall. To purify those factors, intact *M. tuberculosis* organisms were extracted with chloroform:methanol to yield fractions enriched in neutral lipids, glycolipids, and polar lipids, respectively (6). Fractions were dried, sonicated into medium at 10 μg/ml (A and C) or the indicated concentration (B), cultured with monocytes for 3 days or the indicated time, and stained as described in Fig. 1.

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**FIGURE 2.** Polar lipids from *M. tuberculosis* induce cell surface expression of group 1 CD1 proteins by human myeloid cells. Whole *M. tuberculosis* strain H37Rv was lyophilized and extracted with chloroform:methanol (2:1) to obtain soluble *M. tuberculosis* lipid and a pellet composed of CWSs. The *M. tuberculosis* lipid fraction was loaded onto a silica chromatographic column that was eluted sequentially with chloroform, acetone, and methanol to yield fractions enriched in neutral lipids, glycolipids, and polar lipids, respectively (6). Fractions were dried, sonicated into medium at 10 μg/ml (A and C) or the indicated concentration (B), cultured with monocytes for 3 days or the indicated time, and stained as described in Fig. 1.

**FIGURE 3.** Differentiation markers of monocytes treated with *M. tuberculosis* lipids. *A*. After 3 days of culture with total *M. tuberculosis* lipids (10 μg/ml), cells were stained with mAb specific for CD80 (L307.4), CD86 (B7209), CD11c (B-ly6), and HLA-DR (L243) on the CD1a+ cells. These results are representative of more than three experiments.

**CD1 induction involves new protein synthesis**

Flow cytometric analysis of cells treated with *M. tuberculosis* lipids showed that CD1 expression was not up-regulated, in contrast with the results seen for CD1a, CD1b, and CD1c (Fig. 3A). Compared with medium-treated cells, B7 costimulatory molecules (CD80, CD86) were up-regulated in parallel with group 1 CD1 proteins. Similar to previous reports of an inability of *M. tuberculosis* to promote the complete maturation of DCs (41), cells did not express CD83 (Fig. 3A). To further characterize the phenotype of the subset of monocytes that differentiated into CD1-expressing cells, we conducted two-color flow cytometry of cells for markers of myeloid cell differentiation on cells that fell into a gate defined by high CD1a expression. CD1a+ cells were found to down-regulate CD14 and CD64 in response to *M. tuberculosis* lipid treatment, so they did not have a surface phenotype corresponding to activated macrophages (Fig. 3B). Instead, CD1a+ cells uniformly up-regulated CD11c to high levels and showed modest increases in DC-specific ICAM-3-grabbing nonintegrin (CD209) expression and high levels of MHC class II. Thus, mycobacteria lipid-treated cells that came to express group 1 CD1 proteins also expressed many markers that are also found on immature myeloid DCs.

Next, we sought to determine the cellular mechanism for the appearance of group 1 CD1 proteins at the surface of cells. Although it was known that group 1 CD1 protein expression on myeloid cells is dependent on activation (1), the precise cellular mechanisms by which CD1 proteins come to be expressed on the surface had not been previously investigated. For MHC Ag-presenting molecules, peptide ligands can stabilize Ag complexes and thereby increase steady state levels of cell surface expression. The up-regulation of MHC class II during myeloid DC maturation also prominently involves redirected transport of these complexes from late endosomes and lysosomes to the cell surface (42). Therefore, we investigated these, as well as transcription, as candidate mechanisms underlying the appearance of group 1 CD1 proteins on the cell surface.

*M. tuberculosis* lipids extracts that induced group 1 CD1 expression contained lipid ligands of CD1, but these did not account for increased CD1 expression at the cell surface, as purified Ags presented by CD1a (DDM), CD1b (mycolic acid, GM1 ganglioside, and GMM), or CD1c (mannosyl phosphomycoketides) did not result in detectable levels of CD1 protein at the cell surface, even after 3 days of exposure to high Ag concentrations (Fig. 4A).

This study (Fig. 1A) and prior studies have generally found that monocytes do not have any detectable group 1 CD1 proteins at the...
increase in group 1 CD1 mRNA, we found that the absolute levels of CD1d mRNA in fresh monocytes declined on the first day of lipid treatment and then partially recovered by day 3 (Fig. 4B). The reduction of CD1d expression correlated with the reduction of cell surface CD1d protein expression under the same conditions (Fig. 3A). Therefore, we conclude that levels of group 1 and group 2 CD1 protein expression are oppositely regulated by exposure to mycobacterial cell wall products.

M. tuberculosis lipid stimulates lipid Ag uptake and processing

Previous studies had shown that CD1 Ag presentation required not only cell surface expression of CD1 but also an efficient transport of exogenous lipid Ags into endosomes, where CD1-lipid complexes are formed (1, 4, 23, 25, 43). Therefore, we sought to determine whether M. tuberculosis lipids, in addition to inducing CD1 translation, could also promote Ag uptake and other cellular functions related to the processing of lipid Ags in the endosomal network. We found that untreated monocytes did not rapidly internalize fluorescent dextran particles that serve as a marker for macrophagocytosis. However, monocytes incubated with M. tuberculosis total lipids for 3 days efficiently internalized both FITC-dextran and radiolabeled C32 GMM, a CD1b-presented lipid Ag (Fig. 5, A and B).

To quantitatively measure CD1 gene transcription after exposure to M. tuberculosis lipid, we designed RT-PCR primers to amplify isotype-specific sequences of the otherwise homologous human CD1a, CD1b, CD1c, and CD1d genes. Primers for each CD1 isoform failed to cross-prime any other CD1 isoform and amplify isotype-specific sequences of the otherwise homologous human CD1a, CD1b, CD1c, and CD1d genes. Primers for each CD1 isoform failed to cross-prime any other CD1 isoform and showed similar sensitivity and amplification efficiency (data not shown). Compared with untreated or medium-treated monocytes, monocytes cultured with M. tuberculosis lipid were consistently found to have 100- to 3000-fold increases in mRNA encoding CD1a, CD1b, and CD1c. Comparison of group 1 CD1 mRNA and protein production showed that they occurred with similar kinetics and that the mRNA slightly preceded the appearance of CD1 protein at the cell surface (Figs. 1A and 4B). In contrast with the increase in group 1 CD1 mRNA, we found that the absolute levels of CD1d mRNA in fresh monocytes declined on the first day of lipid treatment and then partially recovered by day 3 (Fig. 4B). The reduction of CD1d expression correlated with the reduction of cell surface CD1d protein expression under the same conditions (Fig. 3A). Therefore, we conclude that levels of group 1 and group 2 CD1 protein expression are oppositely regulated by exposure to mycobacterial cell wall products.

FIGURE 4. CD1 up-regulation by M. tuberculosis lipids involves new protein synthesis. A, Monocyte expression of CD1 was measured by flow cytometry after coculture for 3 days with lipids (10 μg/ml except for MPI) that was used at a 10-fold excess of the concentration that stimulates T cells) that were known from previous studies to be presented by CD1a (DDM), CD1b (GMM, mycolic acid, GM1 ganglioside), or CD1c (manosyl phosphoisooprenoid (MPI)). B, After treatment of monocytes with M. tuberculosis lipids (10 μg/ml) for the indicated time, total RNA was extracted, retrotranscribed, and analyzed by RT-PCR using complementary oligonucleotide fluorescent probes (Taqman reagent). CD1 mRNA levels were determined by the comparative Ct method relative to the expression of the housekeeping gene GAPDH. These results are representative of those seen in four experiments completed in triplicate and are expressed as the mean ± SD.

FIGURE 5. Mycobacterial lipids have separate adjuvant and antigenic effects on CD1-restricted T cells. A and B, Monocytes were cultured for 3 days in medium alone (dashed line) or M. tuberculosis lipid (solid line; 10 μg/ml) and then were incubated with FITC-labeled dextran for 1 h, washed, and analyzed by flow cytometry. Alternatively, monocytes were incubated with [3H]labeled C32 GMM for 6 h, washed, and subjected to scintillation counting to measure internalization. C, Monocytes were treated with medium or M. tuberculosis lipids (10 μg/ml), washed, and incubated for 24 h with C32 GMM (2 μM) and the CD1b-restricted T cell line LDN5. IL-2 in supernatants was measured by [3H]thymidine incorporation by the IL-2-dependent T cell line HT-2. D, Monocytes were treated in C and then cultured with DDM, CD1a-presented lipopeptide. These cells were washed to remove Ag and were mixed with J.RT-3/CD8.2 cells and subjected to flow cytometric analysis for calcium flux (7).
To determine whether *M. tuberculosis* lipids could convert monocytes into cells that have all the features necessary to present exogenous lipid Ags to T cells, monocytes were treated with *M. tuberculosis* lipids for 3 days and were tested for presentation of lipid Ags that were known to require endosomal processing. The mixture of *M. tuberculosis* lipids might have activated T cells by supplying cognate lipid Ags that bind CD1 and contact the TCR, by stimulating CD1 Ag processing pathways or via both effects. It was possible to distinguish between these potential mechanisms by using T cells, which specifically recognize lipid Ags that are made by *M. tuberculosis* in vivo, but are not produced at detectable levels under the nonpermissive conditions of in vitro growth used to prepare these *M. tuberculosis* lipid extracts. These Ags were GMM, a CD1b-presented Ag that is processed in late endosomal compartments (23), and DDM, a CD1a-presented Ag that requires internalization into early endosomal compartments before being recognized (5, 25). Monocytes that were pretreated with *M. tuberculosis* lipid for 3 days and then pulsed with GMM or DDM Ags were found to activate Ag-specific T cells, but untreated monocytes pulsed with Ag did not (Fig. 5, C and D). The failure of untreated monocytes to present GMM or DDM suggested that these two lipid Ags did not have CD1-inducing properties that could substitute for the mixture of all cell wall lipids, and this was confirmed in separate experiments in which these and other known CD1-presented Ags failed to induce CD1 when applied at high concentrations for 3 days (Fig. 4A). Thus, certain lipids within the

**FIGURE 6.** Monocyte response to human, mycobacterial, bacterial, protozoan, and fungal lipids. Total lipid extracts of human C1R B lymphoblastoid cells (human) or the indicated microbe were prepared by extraction of intact organisms with chloroform:methanol (2:1). Total lipid extracts were dried, sonicated into medium at 10 μg/ml, cocultured with human monocytes for 3 days, and subjected to flow cytometry. These results are typical of more than three experiments.

**FIGURE 7.** TLR-2 mediates the induction of CD1 expression on monocytes. A, CHO cells (3E10) transfected with human TLR-2 and a reporter plasmid controlling CD25 expression (CHO-TLR-2) were treated overnight with medium, Pam3Cys (100 ng/ml), or polar *M. tuberculosis* lipids isolated as in Fig. 2A and further purified by one-dimensional silica TLC (1.5 μg/ml). Results are shown as the mean ratio of duplicate samples of CD25 expression by CHO-TLR-2 compared with mock-transfected CHO cells cultured in the same way. B, Monocytes were treated for 3 days with GM-CSF (300 U/ml) and IL-4 (200 U/ml) or *M. tuberculosis* total lipids (10 μg/ml), *M. tuberculosis* dimannosyl phosphatidylinositol mannoside (PIM; 5 μg/ml), Mycobacterium smegmatis araLAM (5 μg/ml), or *M. tuberculosis* mannos-capped LAM (Man LAM; 5 μg/ml). C, Monocytes were cultured for 3 days with medium alone or with different concentrations of synthetic Pam3Cys (100 and 10 ng/ml) and were analyzed as in A. D, Monocytes were treated for 3 days in medium, in the presence of *M. tuberculosis* total lipids (10 μg/ml) or with unmethylated CpG oligonucleotides or poly(I:C) at the indicated concentrations. E, Monocytes were cultured for 3 days with *M. tuberculosis* lipids (10 μg/ml) in the absence or presence of a TLR-2 neutralizing Ab (T2.5) or an isotype control Ab (30 μg/ml). Data in this figure are representative of at least three experiments.
mycobacterial cell wall function as Ags that bind to CD1, and others function as adjuvants that initiate CD1 expression.

**CD1 induction is mediated by microbial agonists of TLR-2**

To identify the lipid stimulants of CD1, we first screened lipid extracts from a variety of microbial pathogens and human cells. Lipids from human cells, protozoa (*S. mansoni, L. donovani*), and fungi (*C. albicans, A. niger*) did not induce group 1 CD1 expression, and a Gram-negative bacterium (*Salmonella typhimurium*) induced only trace levels of CD1. Lipid mixtures from *S. aureus* and all four tested species of mycobacteria (*M. tuberculosis H37Ra, M. tuberculosis H37Rv, M. phlei*, and *M. avium*) stimulated high levels of CD1a, CD1b, and CD1c expression (Fig. 6). None of the lipid preparations up-regulated CD1d expression. This initial screen provided evidence that CD1-inducing factors were not likely to be abundant lipids that compose the membranes of human or other eukaryotic cells. Instead, any potent stimulatory factors were conserved among a more limited range of mycobacterial and bacterial species.

Furthermore, several features of these patterns of reactivity implicated TLR-2 in mediating the response. The staphylococcal and mycobacterial species that induced CD1 represented two types of pathogens that were known to produce TLR-2 agonists (44–46), and the monocytes under study expressed TLR-2 (data not shown). In addition, we found that CD1-inducing lipid extracts from *M. tuberculosis* also had TLR-2 agonistic properties, as CHO cells expressing CD25 under an NF-κB reporter construct were stimulated to express CD25 by mycobacterial lipids only when the cells were cotransfected with TLR-2 (CHO/TLR-2) (Fig. 7A). These observations led us to further purify the stimulatory factors from *M. tuberculosis* total lipids using open silica columns, and we found that the CD1-inducing factors were detected exclusively in polar lipid fractions that are highly enriched for phospholipids (Fig. 2A). *M. tuberculosis* produces two phospholipids that were known to agonize TLR-2: phosphatidylidylinositol mannoside (PIM) and LAM with arabinosylated termini (araLAM) (47, 48). Highly purified preparations of araLAM and PIM induced group 1 CD1 expression (Fig. 7B), but CD1 was not up-regulated in response to mannosylated LAM, a phospholipid that is structurally related to araLAM.

These results provided strong correlative evidence suggesting that TLR-2 mediates CD1 induction, so we sought to more directly test this hypothesis with selective agonists and antagonists of TLR function. A synthetic agonist of TLR-2, 3-bis(palmitoyloxy)-(2-RS)-propyl-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys(4)-OH(5), which mimicked the end-terminal lipopeptides of *Borrelia burgdorferi*, potently induced group 1 CD1 proteins (Fig. 7C) (49). CD1 induction was not seen in response to unmethylated CpG oligonucleotides or polyinosin-polycytidyl acid (poly[I:C]), which agonize TLR-9 and TLR-3, respectively (Fig. 7D). As with intact Gram-negative bacteria, purified LPS, a TLR-4 agonist, only slightly induced CD1 when applied at 10 ng/ml and higher concentrations (>100 ng/ml) did not detectably induce CD1 (data not shown). Last, CD1 induction by *M. tuberculosis* lipids was significantly inhibited in the presence of a neutralizing anti-TLR-2 Ab, demonstrating that TLR-2 activation is necessary for CD1 induction by mycobacterial lipids (Fig. 7E). Thus, these cellular studies indicate that CD1a, CD1b, and CD1c represent an inducible Ag processing system, whose function is regulated by microbial products and an innate pattern recognition receptor, TLR-2.

**Discussion**

TLR-2 plays a central role in mediating interactions between myeloid cells and mycobacterial and staphylococcal pathogens, and our findings identify a new function for TLR-2 as an initiator of group 1 CD1 Ag presentation pathways (38, 44–46, 50, 51). All natural and synthetic TLR-2 agonists tested were sufficient to stimulate CD1 expression, and inhibition of TLR-2 function with blocking Abs provided evidence that signaling through TLR-2 was necessary to initiate the differentiation of human monocytes in response to mycobacterial lipids (Fig. 7). Because *M. tuberculosis* lipids also promoted lipid Ag uptake and were sufficient to allow monocytes to mediate T cell activation by Ags that require endosomal processing (Fig. 5), this appears to be a coordinated pathway of cellular differentiation that converts monocytes into fully competent lipid APCs. In terms of their cell surface phenotype (CD14lowCD11bhigh, intermediate DC-specific ICAM-3-grabbing nonintegrin), high Ag uptake capacity, and their ability to secrete IL-12 (data not shown), these mycobacterial lipid-treated cells have acquired the phenotype of DCs, which have not reached full maturity such that they express CD83 (27). These results are in agreement with prior studies showing that *M. tuberculosis* does not fully mature DCs such that they express CD83 and are able to strongly prime MHC class II-restricted cells (52). However, any potential maturation arrest induced by mycobacteria is not sufficient to block the expression or function of group 1 CD1 proteins.

Both infected and lipid-activated cells showed increases in cell surface expression of group 1 CD1 and MHC class II protein, but our data show that the cellular mechanisms of up-regulation are distinct. MHC class II normally increases from intermediate to high levels via a mechanism that involves redistribution of peptide-loaded MHC class II complexes from lysosomes to the cell surface over a period of 5 or more days (53, 54). In contrast, group 1 CD1 proteins increased from undetectable to high levels within 2 days after the contact with mycobacterial products (Fig. 1). The latter situation represents a more rapid, all or nothing mode of induction that is mediated predominantly or wholly by increases in transcription (Fig. 4). The acquisition of peptide and lipid Ag processing pathways by maturing DCs may represent interrelated processes. For example, the appearance of group 1 CD1 proteins relatively early in the maturation process is consistent with their proposed function in activating CD1 autoreactive T cells that secrete cytokines to promote the terminal phases of DC maturation (55). In addition, the ability of *M. tuberculosis* lipids to up-regulate bulk flow mechanisms for acquisition of large antigenic particles likely serves the dual purpose of bringing both lipids and proteins into endosomes for processing by DCs (Fig. 5, A and B) (44).

The lack of expression of group 1 CD1 proteins on blood-derived monocytes, along with new evidence that TLR-2 selectively up-regulates group 1 CD1 but not group 2 CD1 expression, contributes to emerging ideas about the separate roles of group 1 and group 2 CD1 proteins in innate responses. It has been convincingly argued that the constitutive expression of CD1d on myeloid cells and B cells, as well as its rapid presentation of endogenous Ags to invariant NK T cells in vivo, points to a function in innate immunity (56). However, human CD1a, CD1b, and CD1c do not as clearly fit into this paradigm because their expression on DCs is delayed, and these isoforms present a more diverse array of self and foreign Ags to T cells with diverse TCRs (35). The cellular signaling mechanisms by which TLR-2 agonism leads to CD1 expression likely involved NF-κB, but have not yet been comprehensively investigated. The 24- to 48-h delay in the appearance of high levels of group 1 CD1 transcripts and proteins after exposure to *M. tuberculosis* suggests the possibility that this process may involve cytokine secretion.

Our results provide evidence that group 1 CD1 expression is controlled by an innate pattern recognition receptor for bacterial products, and they show how group 1 CD1-restricted T cells can be
most efficiently stimulated after APCs are preactivated by local contact with microbial cell wall products (Figs. 5 and 7). The ability of microbial products and innate receptors to activate CD1 Ag processing can be compared with other systems in which antecedent activation of the innate system promotes subsequent adaptive responses, such as the role of CD19 ligation in B cells or the role of TLR-mediated priming of MHC-restricted T cells (57, 58). Viewed in this way, the regulated and inducible expression of CD1a, CD1b, and CD1c on maturing DCs can be contrasted with that of CD1d-restricted NK T cells, as the former likely represents a delayed pathogen recognition system rather than an innate response. Furthermore, the ability of a single microbial stimulus to oppositely regulate group 1 and 2 protein expression points to distinct roles for the two groups of CD1 proteins in immune responses.

This kind of system suggests a model in which mycobacterial cell walls are complex mixtures of lipids that have two separate functions in simultaneously inducing CD1 Ag processing pathways and providing lipids that enter cellular Ag processing pathways. We found that most mycobacterial lipids previously reported to function as cognate Ags for lipid-reactive TCRs, including mannosyl phosphorylketides, mycotic acid, GMM, and DDM, did not induce CD1 expression (Fig. 4A) (19, 59–61). Therefore, individual lipid components of the mycobacterial cell wall promote CD1-mediated T cell activation by two distinct mechanisms: cognate recognition of Ags bound to CD1 proteins or as adjuvants that mediate induction of CD1 expression through TLR-2 signaling. PIM and LAM may represent special cases in which a single lipid can activate T cells indirectly through TLRs and also directly through TCRs. However, further studies of LAM will be needed to determine whether the dominant mechanism of T activation involves synthesis of new CD1 proteins or presentation of LAM bound within the CD1b groove (4, 18). These in vitro studies of human monocytes now suggest that the simultaneous delivery of both types of activating signals by intact bacteria or sloughed lipids could promote the efficient uptake and presentation of foreign lipids by those myeloid precursors of DCs that encounter pathogens at sites of infection in vivo.

Last, the discovery of microbial adjuvants for CD1 expression raises the possibility that natural or synthetic microbial lipids could be applied to monocytes to engineer DC-based vaccines with lipid Ag presentation capabilities. Most existing adjuvant schemes take advantage of LPS, CD40L, TNF-α, B7-1/2 costimulatory molecules, and MHC class II-mediated Ag presentation capabilities. Most existing adjuvant schemes take advantage of LPS, CD40L, TNF-α, B7-1/2 costimulatory molecules, and MHC class II-mediated Ag presentation capabilities. The authors have no financial conflict of interest.

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