

Role of lipid trimming and CD1 groove size in cellular antigen presentation

Tan-Yun Cheng¹, Miguel Relloso¹, Ildiko Van Rhijn², David C Young¹, Gurdyal S Besra³, Volker Briken⁴, Dirk M Zajonc⁵, Ian A Wilson^{6,7}, Steven Porcelli⁴ and D Branch Moody^{1,*}

¹Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA, ²Department of Infection and Immunity, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands, ³School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, UK, ⁴Department of Immunology and Microbiology, Albert Einstein College of Medicine, Bronx, NY, USA, ⁵Division of Cellular Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA, ⁶Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA, USA and ⁷Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA, USA

Cellular CD1 proteins bind lipids that differ in length (C_{12–80}), including antigens that exceed the capacity of the CD1 groove. This could be accomplished by trimming lipids to a uniform length before loading or by inserting each lipid so that it penetrates the groove to a varying extent. New assays to detect antigen fragments generated within human dendritic cells showed that bacterial antigens remained intact, even after delivery to lysosomes, where control lipids were cleaved. Further, recombinant CD1b proteins could bind and present C₈₀ lipid antigens using a mechanism that did not involve cellular enzymes or lipid cleavage, but was regulated by pH in the physiologic range. We conclude that endosomal acidification acts directly, rather than through enzymatic trimming, to insert lipids into CD1b. Lipids are loaded in an intact form, so that they likely protrude through a portal near the bottom of the groove, which represents an escape hatch for long lipids from mycobacterial pathogens.

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Introduction

Cellular CD1 proteins mediate T-cell activation in response to a variety of lipid, glycolipid and lipopeptide antigens, leading to the emerging view that the CD1 antigen presentation system allows T cells to broadly survey the lipid content of target cells. The mechanism for display of lipid antigens to

T-cell receptors (TCR) is illustrated by crystal structures of CD1-β2-microglobulin in complex with phosphatidylinositol, ganglioside GM2, sulfatide, glucose monomycolate (GMM), lipopeptide, phosphatidylcholine and α-galactosyl ceramide (Gadola *et al*, 2002; Zajonc *et al*, 2003, 2005a, b; Batuwangala *et al*, 2004; Giabbai *et al*, 2005; Koch *et al*, 2005). The phosphate, sulfate, carbohydrate or peptide moieties lie on the outer, α-helical surface of CD1, where they can directly contact TCRs. The lipid moieties traverse a small portal positioned between the α1- and α2-helices and are inserted deeply within the α1–α2 superdomain of CD1 proteins, where they form extensive hydrophobic interactions with the non-polar residues that line the CD1 groove (Zeng *et al*, 1997; Gadola *et al*, 2002). These crystal structures clarify general mechanism of seating lipids within the groove, but there is little understanding of basic questions relating to how CD1 proteins capture antigens with structurally diverse lipid anchors or prioritize among the many types of lipid antigens available within cells.

The lipid anchors in CD1-presented antigens can be composed of mycolic acids (MA) (Beckman *et al*, 1994; Moody *et al*, 1997), diacylglycerols (Sieling *et al*, 1995; Joyce *et al*, 1998; Rauch *et al*, 2003; Agea *et al*, 2005), ceramides (Kawano *et al*, 1997; Shamshiev *et al*, 1999, 2002; Kinjo *et al*, 2005; Mattner *et al*, 2005), polyisoprenols (Moody *et al*, 2000b), polyketides (Matsunaga *et al*, 2004), phthioceranoates (Gilleron *et al*, 2004) or fatty acyl chains (Moody *et al*, 2004). These lipids can have either one or two alkyl chains that vary greatly in their overall length. In fact, exogenous lipid antigens presented by CD1-expressing APCs have lipid moieties that are much larger or smaller than the known volume of the antigen binding grooves found in CD1a (1300 Å³), CD1b (2200 Å³) or CD1d proteins (1650 Å³) (Zeng *et al*, 1997; Gadola *et al*, 2002; Zajonc *et al*, 2003). The apparent mismatch of the CD1 groove volume with lipid length is greatest for CD1b proteins, which present diacylglycerols, sphingolipids, mycolates and polyacylated carbohydrates, ranging in length from C₁₂ to C₈₀ (Moody *et al*, 1997; Shamshiev *et al*, 1999; Gilleron *et al*, 2004). The CD1b groove is composed of four contiguous pockets named A', C', F' and T', which are accessible by two portals located above the F' pocket and at the bottom of the C' pocket (Gadola *et al*, 2002). The crystal structure of CD1b in complex with a GMM antigen shows that a C₅₆ lipid occupies nearly the entire groove (Batuwangala *et al*, 2004); so the interior of the CD1b groove could optimally accommodate antigens with an overall lipid length of C_{60–64}. However, *in vivo* studies of naturally occurring mycobacterial lipids show that cellular CD1b proteins mediate T-cell recognition of antigens exceeding this volume, such as C₈₀ MA and C₈₀ GMM (Ulrichs *et al*, 2003). In addition, acylated sulfotrehaloses may also exceed this limit depending on the number of acyl chains inserted into the groove (Gilleron *et al*, 2004).

CD1 proteins could present lipids of diverse length by one of two general strategies. Antigens might be trimmed by cells

*Corresponding author. Division of Rheumatology Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Smith Building 514, 1 Jimmy Fund Way, Boston, MA 2115, USA. Tel.: +1 617 525 1037; Fax: +1 617 525 1010; E-mail: bmoody@rics.bwh.harvard.edu

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before loading into the CD1 groove. Alternatively, each type of antigen might bind so that the hydrophilic cap sits at the main entrance to the groove, but the lipid moiety present in each type of antigen penetrates the groove to a varying extent. Both hypotheses have some indirect support from studies of lipid antigen presentation in intact cells. In support of the trimming hypothesis, it is known that many CD1-presented antigens must be taken up into late endosomes or lysosomes before their recognition by T cells, which offers an opportunity for exposure to hydrolytically active enzymes before loading antigens into the groove (Porcelli *et al*, 1992; Sieling *et al*, 1995; Chiu *et al*, 1999; Moody *et al*, 2002; Gilleron *et al*, 2004). However, the actual molecular events that render these antigens into a recognizable form are not known and might be accounted for by any of several explanations.

Low pH directly promotes the association of recombinant CD1b proteins with lipid antigens *in vitro* (Ernst *et al*, 1998), suggesting that low pH generated by vesicular ATPases might relax the α -helical structures that protect the binding groove and thereby directly promote antigen insertion. Secondly, loading of antigens onto CD1b and CD1d is increased by saposin and apolipoprotein lipid transfer proteins, which normally localize to late endosomes and lysosomes (Kang and Cresswell, 2004; Winau *et al*, 2004; Zhou *et al*, 2004a; van den Elzen *et al*, 2005). Last, lysosomal hydrolases, such as α -galactosidase A and β -hexosaminidase, chemically modify the carbohydrate moieties of antigens in ways that create epitopes for TCRs (Prigozy *et al*, 2001; Zhou *et al*, 2004b). Although there have been no prior studies of potential modifications of the lipid moieties of antigens, these deglycosylation reactions raise the possibility that other lysosomal enzymes might chemically modify the lipid moieties so that they more closely match the volume of CD1 grooves.

As an alternative to trimming, each lipid antigen may be inserted in an intact form and occupy the CD1 groove to a varying extent, depending on the length and number of its alkyl chains. Antigens with lipid moieties that are significantly smaller than the groove volume, such as gangliosides, sulfatides and shorter forms of GMM (C_{12} GMM, C_{32} GMM), might bind together with smaller space-holding lipids, which occupy the remainder of the groove (Gadola *et al*, 2002). Longer chain antigens might occupy the groove more completely. In the absence of trimming, the largest C_{80} lipids would fully occupy its volume and then exit from the hydrophobic interior to the outer, solvent-exposed face of CD1b, possibly through a small portal located at the bottom of the C' pocket (Gadola *et al*, 2002).

To distinguish among these possibilities, we developed assays to determine whether dendritic cells (DCs) alter the length of long-chain bacterial lipids. Parallel analyses of antigen uptake, generation of antigenic complexes and detection of antigen breakdown products showed that lipid antigen cleavage products could not be detected, even at time periods that greatly exceed those necessary to load long-chain antigens onto CD1b. Further, it was possible to bypass all endosomal factors and form antigenic complexes with long-chain lipids in cell-free conditions, as long as the loading reactions were carried out under a narrow pH range that corresponds to that of late endosomes. We conclude that cellular presentation of long-chain lipids involves acid-mediated loading of intact antigens using a mechanism in

which large lipids can enter the $\alpha 1$ - $\alpha 2$ superdomain, traverse the antigen binding groove and then likely re-emerge with their termini positioned below the groove on the outer surface of CD1.

Results

Endosomal and non-endosomal antigen loading occur over distinct time frames

Before designing assays for measuring cell-mediated alterations in antigen structure, we first sought to determine the time frame of functional antigen processing by monocyte-derived DCs. We treated DCs with MA and GMM antigens with short (C_{32}), intermediate (C_{54}) or long (C_{80}) lipids and measured the elapsed time before these antigens activated CD1b-restricted T-cell lines. DCs were found to present C_{32} GMM within 2 min of exposure. In contrast, all of the longer chain GMM and MA antigens were first recognized after more than 30 min of incubation with DCs (Figure 1B and data not shown). In separate studies of proliferation carried out under conditions of steady-state antigen processing for 72 h, C_{80} GMM (0.012 μ M) and C_{54} GMM (0.56 μ M) were found to half-maximally activate T cells at low concentrations compared to those for C_{32} GMM (3.6 μ M). Therefore, the delayed recognition of long-chain GMM antigens was not accounted for by an intrinsically lower potency.

Instead, this paradoxical delay in recognition of the most potent antigens suggested that the T-cell recognition of longer chain antigens was dependent upon processing within endosomes. This conclusion is supported by prior studies showing that C_{80} and C_{54} GMM required endosomal processing by CD1b-transfected lymphoblastoid cells, whereas C_{32} GMM did not (Moody *et al*, 2002). Thus, the delayed presentation by DCs was seen only for those antigens that were known to require endosomal processing as defined by other criteria. Also, 2 min is not generally sufficient to allow antigens and surface proteins to recycle between the surface and late endosomes. Instead, the 30–60 min delay before T-cell recognition correlates with the time necessary for CD1 and other proteins to recycle to endosomes and back to the surface (Jayawardena-Wolf *et al*, 2001; Briken *et al*, 2002; Moody *et al*, 2002). Thus, kinetic studies of CD1b-mediated antigen presentation clarify the frames necessary for non-endosomal (0–5 min) and late endosomal (>30 min) antigen processing events in DCs.

Mycolyl lipids are transported to CD1b⁺, MHC II⁺, LAMP-1⁺ endosomes

To more directly measure the uptake and subcellular localization of mycolate antigens into DCs, we prepared labeled antigens by coupling a small fluorophore, BODIPY, to free MA (BODIPY-MA) in a process that alters mycolate structure by adding a small label that is comparable in molecular weight to a hexose sugar. Also, we prepared biosynthetically radiolabeled antigens of native structure from mycobacteria grown in the presence of 14 C]acetate (Figure 1A). To measure the uptake of GMM antigens, DCs were cultured with radiolabeled C_{32} GMM for up to 24 h, washed to remove extracellular antigens and treated with organic solvents to extract lipids. Consistent with prior studies, uptake of GMM by immature DCs was typically in the range of 50 ng/10⁶ cells/24 h (Moody *et al*, 2002). To more precisely determine

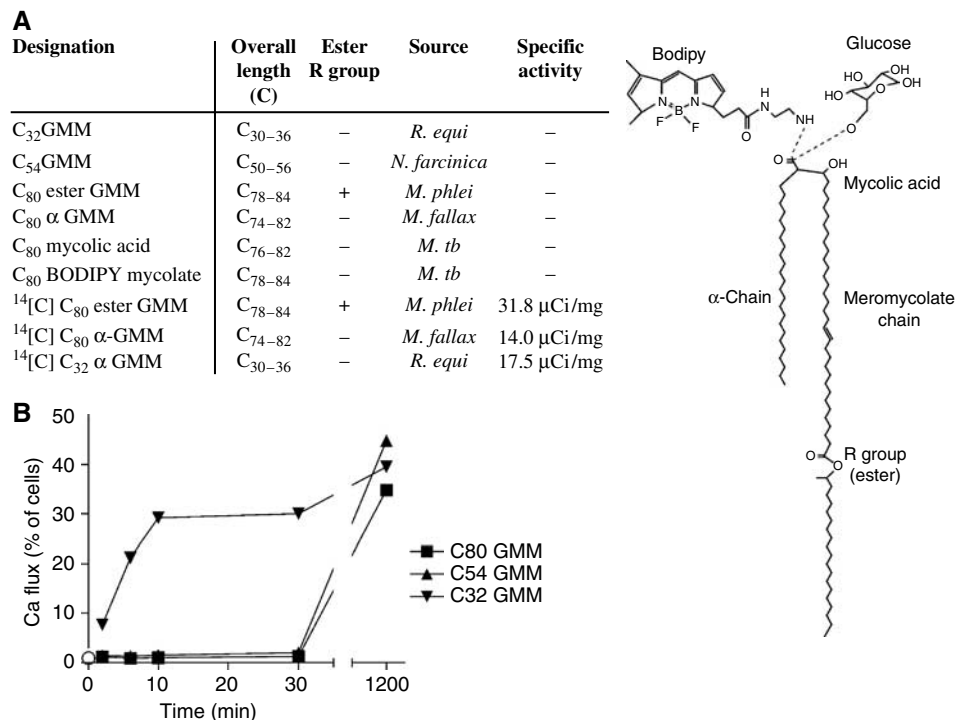


Figure 1 Delayed T-cell recognition of endosomally presented antigens. (A) Free MA and GMM antigens are described as C_x, where x is the combined alkyl chain length of the meromycolate and α -branches of the most abundant species detected by MS. BODIPY or glucose is linked to the carboxyl group as indicated by the dashed line. (B) Human monocyte-derived DCs were preincubated for the indicated time with 20 μ M GMM antigens before adding CD1b-restricted LDN5 T cells and assaying for calcium-induced changes in fluorescence as assessed by flow cytometry.

the intracellular localization of mycolyl lipids, BODIPY-MA and endosomal markers were visualized after a time period that slightly exceeds the minimal time for processing of long-chain antigens (90 min) using confocal microscopy. BODIPY-MA stained discrete compartments and extensively colocalized with MHC class II, which is expressed broadly within the endosomal network (Figure 2). BODIPY-MA also colocalized with compartments that stained with mAb against CD1b or anti-sera against lysosome-associated membrane protein 1 (LAMP-1 CD107a), markers that more selectively label late endosomes and lysosomes. Thus, within a time frame that is relevant to endosomal processing, mycolyl lipids are internalized into the endosomal network of DCs, including CD1b⁺ late endosomes.

Mycolate antigens persist intact within DCs

To determine whether the lipid moieties of antigens undergo chemical alteration after uptake into the endosomal compartments of DCs, we developed thin-layer chromatographic (TLC) autoradiography assays to detect antigen cleavage products. Preliminary studies showed that the ¹⁴[C] label was incorporated into carbohydrate and lipid portions of GMM antigens and that antigens were labeled to a high specific radioactivity (Figure 1A), such that 500 μ g of GMM could be detected using a 72 h autoradiograph (Figure 3A and data not shown). Therefore, this assay detected 1 part per 4000 of lipid added to the culture or approximately 1 part per 200 of lipid taken up by DCs in 72 h. This method was used to study short- and long-chain mycolate and GMM antigens, including two forms of long-chain GMM that differed in the chemical structures of their lipid moieties (Figure 3A). One

form was designated C₈₀ α -GMM because it contained α -MA that lacks oxygen-containing R groups in their mycolate lipid moieties. The other form, C₈₀ ester GMM, is found only in uncommon or non-pathogenic species of mycobacteria such as *Mycobacterium phlei*, but was of interest because it contained an internal ester that might be readily subject to chemical cleavage by acid (Figure 3A).

After 72 h of culture with antigen, DCs were washed to remove non-cell-associated antigens and then subjected to a modified Folch extraction and silica TLC with detection of lipids by charring and autoradiography. The C₈₀ ester GMM, which migrates with a retardation factor (R_f) of 0.62, gave rise to a product that migrated at 0.48 (Figure 3A). This fragment was generated in a time-dependent fashion only when DCs were added to the cultures, demonstrating that it was generated by the DCs (Supplementary Figure 1). Because this cleavage reaction occurred after uptake into cells, we hypothesized that the reaction occurred in acidic endosomal or lysosomal compartments. To more directly test this, we preincubated DCs with concanamycin B, a drug that neutralizes the pH of endosomes by inhibition of proton transport by vesicular ATPases. Concanamycin-treated DCs took up [¹⁴C]C₈₀ ester GMM, but blocked production of the R_f 0.48 lipid product (Figure 3B).

The R_f 0.48 product was not generated from C₈₀ α -GMM. Because the only difference in chemically reactive groups present in ester GMM and α -GMM is the internal ester, we concluded that the DC-mediated alteration involved cleavage at this site. This conclusion was independently supported by experiments carried out in cell-free conditions in which treatment with trifluoroacetic acid (TFA) under harsh condi-

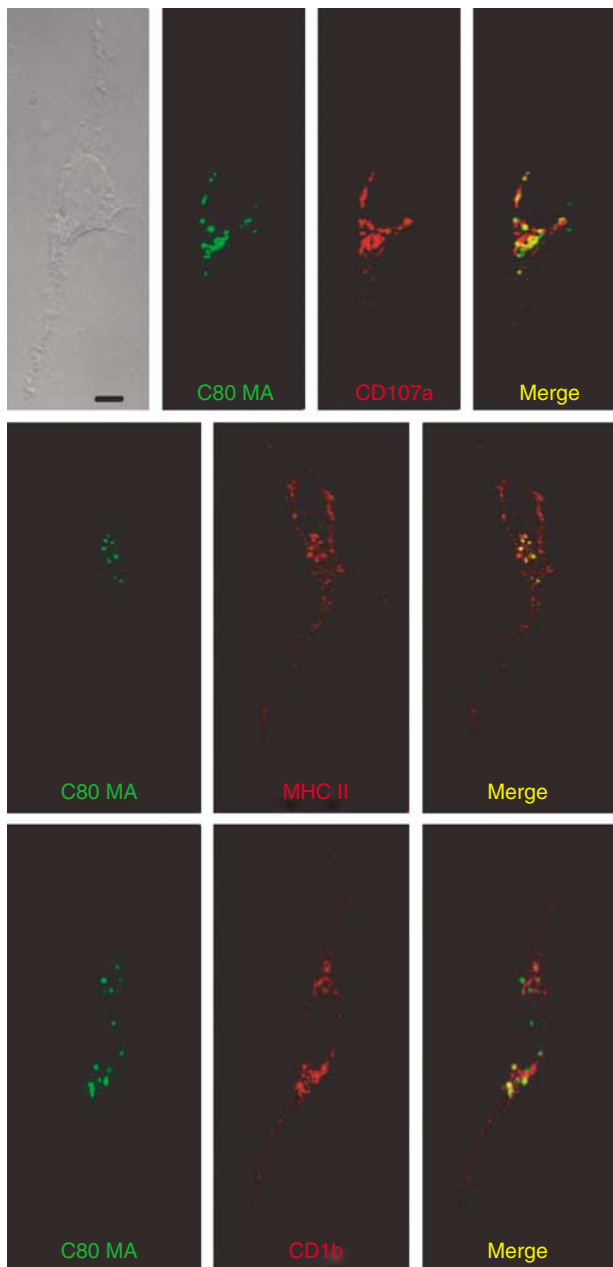


Figure 2 Mycolyl lipids are transported to lysosomes. DCs were incubated with BODIPY-mycolate (green) for 90 min followed by fixation, permeabilization and staining with antibodies against CD1b, MHC II or LAMP-1 (CD107a) and Texas red-conjugated Fab'2 against mouse Ig (red). The phase-contrast light micrograph (upper left) and immunofluorescent confocal micrographs (merge) are shown with a scale bar of 20 μ m.

tions (pH 1.0, 120°C, 120 min) generated a product of R_f 0.48 from C_{80} ester GMM but not from C_{80} α -GMM (Figure 3C). This product was isolated and mass spectrometry (MS), yielding a major ion of m/z 1077.6. This ion corresponded to the mass of a product resulting from a cleavage at the internal ester of the meromycolate chain, GMM with a dicarboxyl C_{60} mycolate (C_{60} GMM), as predicted. Thus, the concanamycin B-inhibitable cleavage product generated within DCs corresponded to C_{60} GMM derived from C_{80} ester GMM. These results indicate that human DCs are capable of altering the lipid length of a model antigen using a selective hydrolysis of an internal ester linkage.

Although this reaction shortened the lipid moiety so that it more closely approximated the CD1b groove volume, this did not account for the ability of long-chain antigens to be loaded onto CD1b proteins in endosomal compartments. Analysis of IL-2 release and calcium flux by T cells in response to the C_{60} GMM fragment showed that the shortened lipid did not enhance its potency compared to full-length C_{80} ester GMM, nor did it allow rapid (<5 min) presentation to T cells, as would be expected if this lipid truncation were fully sufficient to allow loading at the cell surface (Figure 3D–E). Most importantly, other naturally occurring MA and GMM antigens with C_{80} mycolate moieties that lacked the internal ester showed no evidence for antigen cleavage in TLC-autoradiography assays (Figure 3A), even after time periods that greatly exceed those necessary for their presentation to T cells (Figure 1B). After failing to find evidence for lipid trimming, we considered other mechanisms by which endosomal cofactors contribute to processing of long-chain antigens.

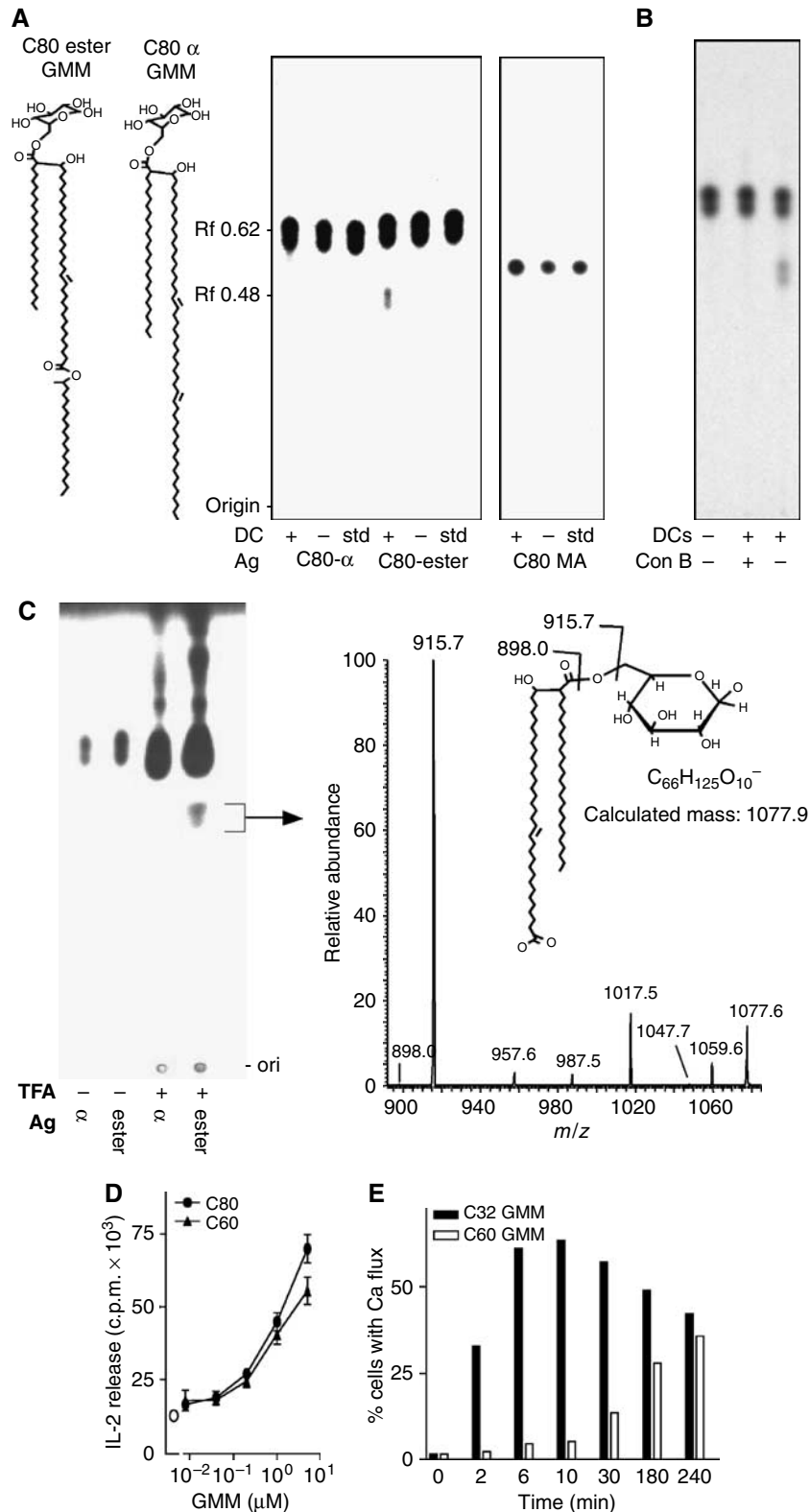
pH is a key regulator of antigen complex formation in cells

Other than trimming, a second factor in the lysosomal microenvironment that contributes to the generation of antigen complexes is acidic pH, which is normally maintained by vesicular ATPases at 4.5–5.5 in DCs (Trombetta *et al*, 2003). Low pH might act indirectly by activating pH-dependent hydrolases or saposins, or might act directly to promote CD1–lipid association, as suggested by *in vitro* binding studies (Ernst *et al*, 1998). Our initial studies of the effects of low pH on GMM and mycolate processing were carried out in intact cells. This approach was guided by the reasoning that if pH could carry out certain effects directly on the CD1b antigen interaction rather than through lysosomally located proteins, then briefly acidifying the extracellular space might be sufficient to bypass the usual endosomal processing requirements.

Figure 3 Generation of cleavage products from GMM by cellular processing in DCs. (A) [14 C] C_{80} α -GMM, [14 C] C_{80} ester GMM or [14 C] C_{80} MA were cocultured with DCs mock-treated by culture at 37°C. After 72 h, DCs were washed to remove extracellular lipids and subjected to lipid extraction. After normalizing for equivalent amounts of antigen-derived radiolabel, lipids were developed with silica TLC. Total lipids were detected by charring (not shown) and labeled lipids were detected by autoradiography in comparison with mock-treated lipids (–) and untreated standards. (B) DCs were treated with concanamycin B for 1 h before adding C_{80} ester GMM and analyzed as in panel A. (C) C_{80} α -GMM (α) or C_{80} ester GMM (ester) was treated with TFA and the material with an R_f of 0.48 was isolated by preparative silica TLC. Mass spectrometry (MS) showed a predominant ion at m/z 1077.7, which corresponded to the predicted mass of an anion produced by hydrolysis at the internal ester of the meromycolate chain to generate a dicarboxylate GMM (C_{60} GMM). CID-MS of the major $[M-H]^-$ ion at m/z 1077.6 showed a dominant loss of 162 units consistent with the presence of an intact hexosyl residue on C_{60} GMM. Additional ions at m/z 957.6, 987.5 and 1017.5 correspond to the incremental loss of CH_2O units from the intact molecule, which arise via through-ring cleavage of glucose and give additional evidence that the carbohydrate portion of the molecule survived the side-chain ester hydrolysis without modification. (D) After isolation of the C_{60} GMM product by preparative TLC, it was compared to the intact precursor, C_{80} GMM, for IL-2 release by CD1b-restricted T cells. (E) Kinetic analysis of GMM presentation using CD1-transfected cells (C1R.CD1b) was performed as in Figure 1B.

In contrast to results obtained at pH 7.4, C₈₀ MA, C₈₀ α-GMM and C₈₀ ester GMM antigens were recognized after 5 min of exposure at pH 4.0 with APCs (Figure 4A), such that their kinetic profiles of recognition were comparable to a non-endosomally presented C₃₂ GMM (Figure 4B). Titration of the pH showed that the transition from slow to rapid presentation

occurred in the pH range 5.5–5.0, which is in the range normally present in late endosomes (Figure 4C). Treating either antigens alone or APCs alone with acid media had no effect on presentation, suggesting that acidic pH did not somehow activate or cleave CD1b proteins or GMMs to generate a form that could be recognized after subsequent interactions at neutral



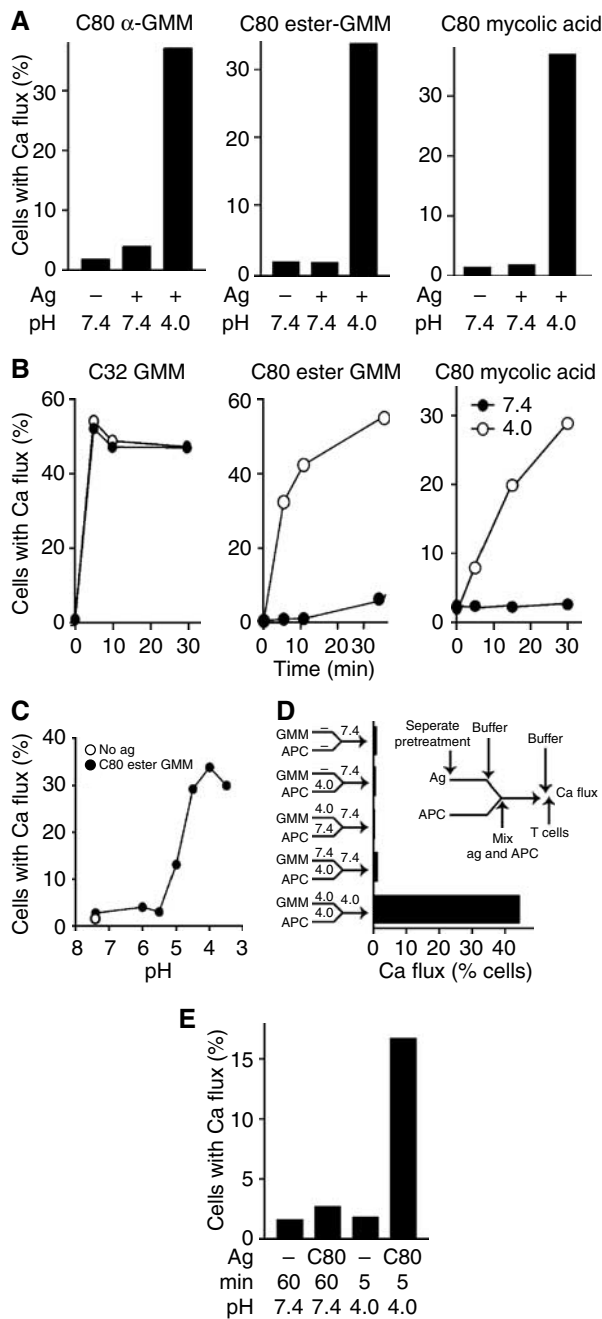


Figure 4 Acidic pH is sufficient to allow rapid presentation of mycolyl glycolipids to T cells. (A–C) B lymphoblastoid cells transfected with CD1b (C1R.CD1b) were preincubated with equipotent doses of C₈₀ α -GMM (0.2 μ M), C₈₀ ester GMM (0.2 μ M) or C₈₀ MA (40 μ M) at either pH 7.4 or 4.0 for 30 min (A), or indicated pH for 15 min (C) or the indicated time (B). The APCs were then neutralized with basic buffers to give pH 7.4 before mixing with T cells and immediately measured by calcium flux. (D) The experimental scheme (inset) shows a two-stage protocol by which cells and antigens were separately incubated at pH 4.0 or 7.4 for 30 min (stage 1), buffered to the indicated pH, mixed, incubated for 5 min (stage 2) and buffered to neutrality before testing T-cell activation by Ca flux. (E) After fixing C1R.CD1b cells under conditions that were previously known to inhibit endosomal uptake (0.02% glutaraldehyde), C₈₀ ester GMM was added for the indicated time before measuring CD1b-mediated Ca flux in T cells.

pH. Instead, two-stage antigen processing assays showed that antigen and APC had to be in physical contact during the acidification to see fast kinetics of recognition (Figure 4D).

Because C1R lymphoblastoid cells have particularly inefficient mechanisms for antigen internalization compared to DCs (Moody *et al*, 2002) and the time periods were short compared to those necessary lysosomal recycling times (Figure 1B), these results were most consistent with pH-induced loading on CD1b proteins at or near the cell surface. However, even in 5 min, antigens or acidic media could be internalized into cells to some degree; so we carried out similar experiments in which cellular membranes were fixed with glutaraldehyde. Although fixation conditions strongly inhibit internalization and recognition of C₈₀ GMM at neutral pH, they did not prevent the acid-mediated presentation of C₈₀ GMM (Figure 4E). These studies suggested that low pH was a key endosomal factor in antigen loading, such that it was sufficient to bypass the need for trafficking of CD1b and antigens to late endosomes and lysosomes, which are normally required for recognition.

Acidic pH promotes CD1b–antigen complex formation in cell-free conditions

To more formally assess whether low pH was sufficient to promote loading in the absence of all cellular factors, we prepared purified, hexahistidine-tagged CD1b proteins and treated them with GMM, recovered them on nickel beads and used GMM-treated CD1b proteins to activate T cells. As seen previously in assays of T-cell activation evaluated with IL-2 release, cytolysis and proliferation (Moody *et al*, 1997, 2002 and data not shown), control experiments showed that measurement of T-cell activation by calcium flux was absolutely dependent on the presence of antigen, CD1b proteins and a brief centrifugation to initiate contact between T cells and beads coated with CD1 and antigen (Figure 5A). CD1b-coated beads incubated with C₃₂ GMM activated T cells to maximum levels when incubated at neutral pH or mildly acidic pH, but no activation was seen with CD1b proteins treated with C₈₀ α -GMM. At pH 5.0, C₈₀ GMM-treated CD1b proteins were able to stimulate a strong T-cell response that was comparable to levels seen with C₃₂ GMM (Figure 5A).

These experiments indicated that CD1b can present GMMs in the absence of all cellular enzymes, so that any possible cleavage reactions would have been chemically mediated. It was unlikely that chemically mediated mycolate cleavage occurred, based on prior experiments in which we had determined that chemical conditions necessary to detectably cleave GMM were significantly more harsh than those used here (Figure 3C). Confirming this, TLC autoradiography of labeled GMMs treated with acid media under conditions that allowed rapid presentation by cells did not detect alterations in GMM mobility (Supplementary Figure 2). Further, we mass spectrometrically analyzed the structures of GMM antigens subjected to the mild conditions that were sufficient for loading in this cell-free assay (pH 5.0, 20°C, 5 h) and the much harsher conditions (pH 1, 120°C, 2 h) necessary to visualize GMM cleavage products in TLC assays (Figure 3C). The chemically susceptible break points in GMM antigens are the internal ester in the meromycolate chain, the ester linkage to glucose and possibly others in glucose moiety or the unsaturation in the meromycolate chain. Unsubstituted alkyl chains are extremely resistant to cleavage. In agreement with these predictions, MS analysis of TFA-treated GMM showed negative ions corresponding to an internal ester-cleaved C₆₀ GMM (m/z 1064.7), free MA (m/z 1190.6) and

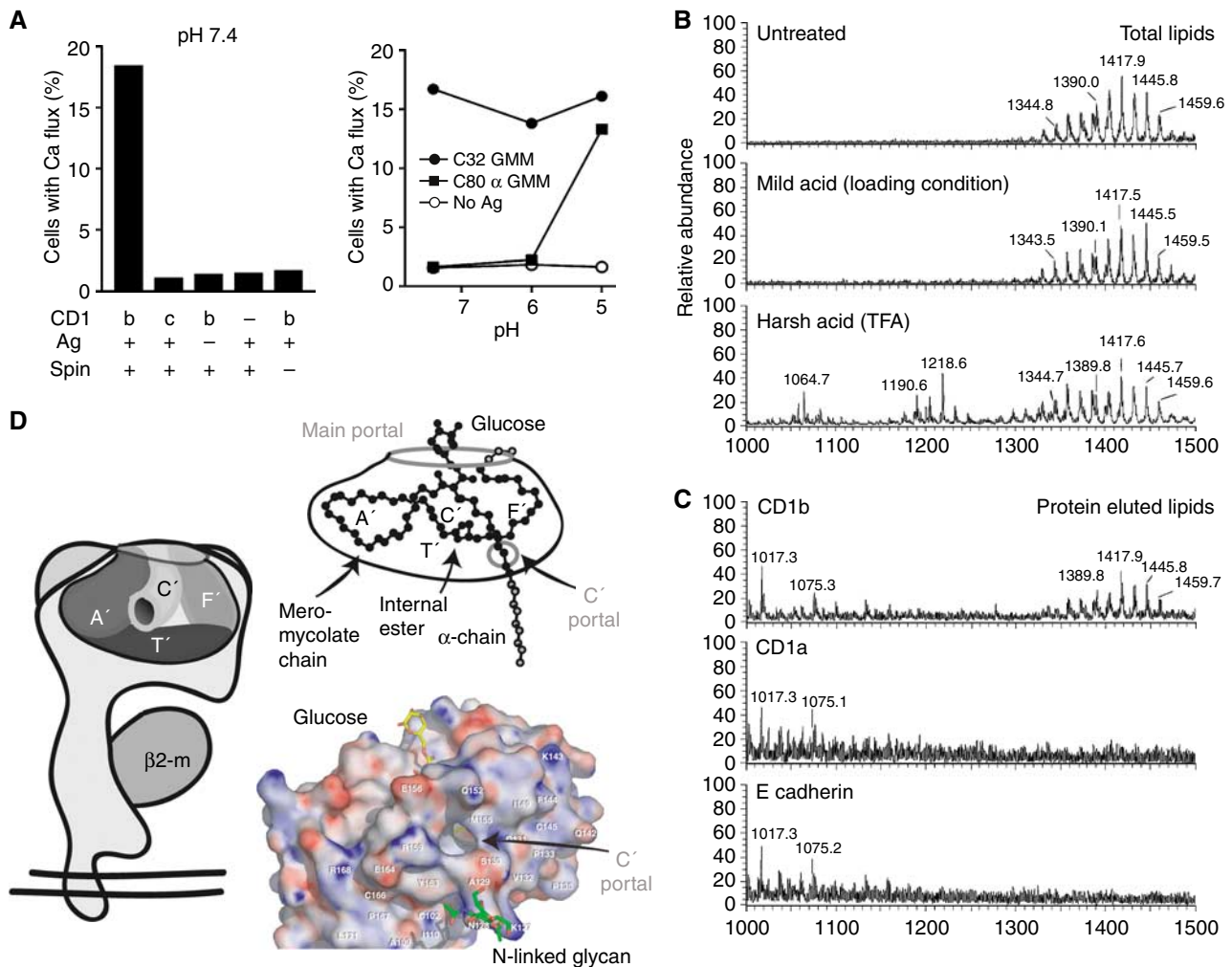


Figure 5 Formation of antigenic CD1b-GMM complexes under cell-free conditions. (A) Recombinant hexahistidine-tagged CD1b or CD1c proteins were coinoculated with C₈₀ α -GMM or C₃₂ GMM or no antigen for 20 h at the indicated pH. The mixture was then neutralized to pH 7.4 before adding nickel beads, washing, adding LDN5 T cells and subjecting the mixture to centrifugation (spin) to initiate bead contact with T cells and measuring calcium flux. This result is representative of four experiments and similar results were obtained for C₈₀ ester GMM (not shown). (B, C) His-tagged CD1a, CD1b or E cadherin proteins were incubated with excess C₈₀ ester GMM and α -GMM at pH 4.0 for 5 h, treated with magnetic nickel beads and recovered with a magnet. Total lipids in the supernatant of this mild acid treatment were compared to untreated lipids and lipids treated with harsh acid conditions as in Figure 3C (TFA, pH 1.0, 120°C, 120 min), revealing negative ions corresponding to C₆₀ dicarboxylate GMM (m/z 1064.7 and others), free mycolate (m/z 1190.6 and others) and acetate adducts of intact α -GMM (m/z 1389.8 and others) and ester GMM (m/z 1445.7 and others). Lipids bound to protein-bead complexes showed ions corresponding to intact GMMs (m/z 1417.9 and others). (D) The CD1b groove can be accessed via the main portal or the smaller C' portal located at the inferior margin of the C' pocket (Gadola *et al*, 2002). Based on the crystal structure of CD1b bound to C₅₆ GMM (Batuwangala *et al*, 2004), this schematic shows how the C₅₆ mycolate moiety (black) lies in the groove with α -branch into the C' pocket, and the longer meromycolate chain lies in the A', T' and F' pockets. The C₇₇₋₈₆ mycolates found in mycobacterial pathogens exceed the interior capacity of the groove, and are predicted to protrude through the portals (gray atoms). An electrostatic surface representation of the CD1b-GMM complex was generated in APBS and illustrates sites of electropositive (blue) and electronegative (red) charge in the range of -15 to $+15$ kT/e. This view shows the adjacent glycan at N128 and two shallow grooves that lead to relatively non-polar (white) areas on the outer surface of the CD1b protein.

ions in an intermediate range ($m/z \sim 1300$), which may correspond to partial loss of glucose. However, antigens treated with mild acid conditions used for loading gave strong mass spectral signals showing that ions corresponding to intact α -GMMs (m/z 1386 and others) and ester GMMs were unaltered compared to starting material (Figure 5B).

After scale up of the *in vitro* loading reaction, we used nano-electro-spray MS to specifically analyze lipids eluted from CD1b, CD1a and E cadherin proteins treated with GMM under mild conditions that are necessary for loading (Figure 5C). MS analysis of highly concentrated lipid eluents showed that CD1b yielded ions corresponding to intact α -GMM and ester GMM (m/z 1389.8–1459.7). Although several ions

that did not match the mass of GMMs (m/z 1017.3, 1075.3), they did not correspond to known products of GMM cleavage, and they were also present in CD1a and E cadherin eluents, suggesting that they are contaminants introduced during the elution and drying process. Thus, we found no evidence for GMM antigens with reduced lipid length, either in the supernatants or bound to CD1b, generated under the mild acid conditions necessary to form antigenic GMM complexes.

Discussion

Both proteins and lipids undergo antigen processing in the endosomes of APCs before their recognition as antigen

complexes by T cells. For peptides, an understanding of the differences in structures of unprocessed proteins and processed peptides was achieved by mass spectrometric detection of peptide fragments eluted from cellular MHC-peptide complexes (Rotzschke *et al*, 1990; Van Bleek and Nathenson, 1990; Rudensky *et al*, 1991). For lipids, it has been unclear as to whether endosomal processing reactions normally involve the alteration of the lipid moieties of antigens. Although lipids can be eluted from cellular CD1 proteins (Joyce *et al*, 1998), this approach has not been widely applied to CD1-lipid complexes formed after antigen processing in intact cells because detergents used to extract the transmembrane domains of CD1 proteins from cellular membranes can disrupt CD1-lipid complexes. Therefore, we sought to produce antigenic complexes *in vitro* with antigens whose lipid moieties exceed the volume of CD1b grooves and analyze the structure of bound lipids. In parallel, we measured lipid cleavage reactions in intact DCs using radiolabeled antigens. The latter approach was considered feasible because mammalian cells cannot trim lipids from the alkyl terminus using ω -oxidation. Therefore, any modification of lipid structure would likely occur by β -oxidation at the carboxylate or chemical attack at discernable chemical substitutions on alkyl chains, resulting in relatively simple and interpretable patterns of antigen-derived breakdown products.

Our results show that human DCs rapidly internalize labeled mycolyl lipids and deliver them to lysosomes that coexpress LAMP-1, CD1b or MHC class II. One of the antigens studied, C₈₀ ester GMM, was cleaved by hydrolysis of an internal ester in its meromycolate chain to yield a product with a C₆₀ lipid moiety and an intact TCR epitope. Thus, DCs can take up lipids into endosomes and modify their length by selectively cleaving them in a way that does not otherwise degrade the antigen. The cleavage reaction does not occur at pH 4.0 outside of cells (Figure 5C; Supplementary Figure 2), suggesting that it is not generated by low pH alone, but instead is carried out by an as yet unknown lysosomal enzyme. This ester hydrolysis reaction is equivalent of that which is necessary for deacylation of diacylglycerols or sphingolipids; so this kind of reaction may be of importance in altering the lipid moieties of other antigens. However, among various types of mycolates, wax-ester forms are known to be present in *M. phlei* and other saprophytes, but not *Mycobacterium tuberculosis* and *Mycobacterium leprae*, two pathogens that generate CD1-mediated responses *in vivo* (Moody *et al*, 2000a). Therefore, the cleavage of C₈₀ ester GMM provides a serendipitous positive control, indicating that all tested GMM and free mycolic antigens entered into cells and were exposed to the hydrolytic environment within late endosomal and lysosomal compartments. However, the more general conclusion is that MA and GMM antigens are not detectably altered in chemical structure when they are taken up into DCs for time periods that greatly exceed the 5–30 min necessary to generate antigenic complexes (Figures 1B and 3A) or under conditions needed to form antigenic complexes in cell-free conditions. Even a sensitive cellular assay might have failed to detect all fragments generated by cellular metabolism, but the generation of antigenic CD1b-GMM complexes under cell-free conditions rules out enzymatic cleavage reactions to alter lipid length before loading (Figure 5).

Instead of lipid trimming, low pH is necessary and sufficient to promote the formation of antigen complexes of CD1b with each of three long-chain bacterial lipid antigens. Low pH might act through cellular factors such as lipid transfer proteins (Kang and Cresswell, 2004; Zhou *et al*, 2004a), especially saposin C and apolipoprotein E, which are normally located in endosomes and have been shown to enhance mycolyl lipid presentation by CD1b (Winau *et al*, 2004; van den Elzen *et al*, 2005). Whereas some of the effects of these two lipid transfer proteins occur at neutral pH, the lipid-transferring properties of saposins are enhanced at low pH (Zhou *et al*, 2004a). Our results make clear that low pH, acting as a single variable, mediates all or nothing differences in antigen recognition under cell-free conditions. Thus, low pH carries out key effects directly on the CD1b-antigen interaction that are separate from any pH-mediated effects on lipid transfer proteins.

Because C₈₀ mycobacterial mycolates exceed the volume of the CD1b groove, a basic question arises as to what happens to any excess lipid that cannot fit within the groove. These results and the crystal structure of the CD1b-GMM complex point to an explicit and plausible mechanism (Figure 5D). In the crystal structure, the carbohydrate moiety of GMM rests near the entrance of the groove and the C₅₆ mycolate lipid is positioned so that the shorter α -branch occupies the small C' pocket and the longer meromycolate branch lies in the A'T'F' superchannel (Batuwangala *et al*, 2004). If cellular CD1b proteins also bind antigens in this way, then loading of intact C₈₀ mycolate moieties indicates that the termini of the meromycolate chain and the α -chain protrude through the main portal and C' portal, respectively (Figure 5D, gray atoms). This model predicts that certain CD1b-presented lipids are bound entirely within the confines of the groove, but the C' portal functions as an escape hatch that allows CD1b proteins to bind particularly long bacterial lipids such that their termini protrude to the outer surface of the protein.

This more complex binding mechanism may allow CD1b to mediate T-cell recognition of highly diverse classes of lipids by re-routing excess lipid away from the TCR interaction site. The position of the protruding lipid is not yet known from crystal structures, but two small concavities on the outer surface of CD1b near the C' portal could guide the lipid toward isoleucine 149 or tyrosine 163 (Figure 5D). Such excess lipid may have no substantial effect on recognition, akin to the mechanism by which the ragged ends of long peptides extend beyond the MHC II platform. Alternatively, the protruding lipid may interact with the outer surface of CD1 proteins in ways that affect lipid retention within the groove, as suggested by studies in which long-chain lipid antigens show particularly high potency and long half-lives compared to short-chain antigens (Beckman *et al*, 1994; Shamshiev *et al*, 1999, 2002; Moody *et al*, 2002; Gilleron *et al*, 2004). These considerations point to a testable model whereby the CD1b protein is a pH-regulated lipid clamp. CD1b likely binds low-affinity, shorter chain self-lipids during its transit through the secretory pathway, but then pH-induced relaxation of the $\alpha 1$ and $\alpha 2$ domain structure allows more extensive access to the groove by intact long-chain lipids during trafficking through acidic endosomes, which are then retained more effectively in post-endosomal compartments. Because long chain length distinguishes mycolyl and phthioceranoate lipids present in the cell wall of myco-

bacteria from smaller mammalian lipids, the ability of endosomally localized CD1b proteins to clamp down on long-chain mycobacterial lipids could serve to enhance self-non-self lipid antigen discrimination.

Materials and methods

Lipid antigens

M. phlei, *Nocardia farcinica*, *Rhodococcus equi* and *M. fallax* were cultured in 7H9 medium supplemented with 0.5 g/l Tween 80 and 10 g/l D-glucose. ^{14}C -labeled lipids were produced by adding 1 mCi/l of [^{14}C]acetate in early log phase for 24 h. GMMs were purified as described previously (Moody *et al*, 1997) or by one-dimensional preparative silica TLC (Scientific Adsorbents Inc., Atlanta, GA) resolved with chloroform/methanol/water (60/16/2). *R. equi*, *N. farcinica*, *M. phlei* and *M. fallax* produced C_{32} GMM, C_{54} GMM, C_{80} ester GMM and C_{80} α -GMM, respectively. *M. tuberculosis* MA (C_{80} MA) was purchased from Sigma or purified from saponificates of *M. tuberculosis*.

T-cell cultures and cellular assays

LDN5 and DN1 T cell lines (Porcelli *et al*, 1992; Beckman *et al*, 1994; Moody *et al*, 1997) were activated by C1R B lymphoblastoid cells transfected with full-length human CD1b (C1R.CD1b) or human monocyte-derived DCs prepared from the peripheral blood by centrifugation over Ficoll-Hypaque, adherence to tissue-culture flasks, culture of adherent cells with 300 U/ml GM-CSF and 200 U/ml IL-4 for 72–96 h, followed by irradiation (5000 rad). IL-2 release from LDN5 T cells or J.RT-3 cells transfected with the α -chains of LDN5 T cells (10 ng/ml PMA) was measured by culturing T cells with DCs or C1R (1:1 ratio) cells plus antigen in 200 μl /well. After 24 h, 50 μl of supernatant was transferred to wells containing 125 μl of media and 10^4 HT-2 cells, which were cultured for 24 h before adding 1 μCi [^3H]thymidine for an additional 6–24 h of culture, followed by harvesting and counting β emissions.

To determine the kinetic profiles for T-cell activation, T cells were loaded with fluorophores, Fura-red (4 μM) and Fluo-4 (2 μM), in Hank's balanced salt solution (HBSS) and subjected to flow cytometric (BD FacSort) analysis of calcium-induced changes in their fluorescence emission spectra. Antigen loading onto APCs at neutral pH was accomplished by incubating 10^6 cells/ml for 1 min to 24 h, followed by mixing 1:1 with antigen-loaded C1R cells or DCs, centrifugation (~1000 g) for 60 s, vortexing and FACS analysis for 20 s or 2000 events (Moody *et al*, 2002). For fixation experiments, C1R.CD1b was fixed with 0.02% glutaraldehyde for 30 s and then the reaction was quenched with excess lysine (0.2 M) as described (Moody *et al*, 2002).

Antigen loading was measured after either one- or two-stage protocols for adjusting the pH. For one-stage loading, APCs (2×10^6 /ml) and antigens were mixed together at a 1:1 ratio in HBSS with 1% BSA, 0.35 g sodium bicarbonate and 20 ml Hepes that had been adjusted to pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 or 7.4 using HCl or NaOH and confirmed by a electronic pH meter. Before adding T cells, the medium was neutralized by adding an equal volume of basic medium to adjust pH to 7.4. Two-stage experiments were carried out by first incubating the APCs alone and antigen alone in separate tubes under defined pH conditions for 30 min before mixing the APCs and antigen (stage 1). The mixture of APCs and antigens were adjusted to pH to 7.4 or 4.0 followed by an additional incubation for 5 min (stage 2), pH neutralization, adding T cells and immediately measuring T-cell activation by calcium flux.

Recombinant protein-antigen complexes

The extracellular domains of CD1a, CD1b and CD1c heavy chains linked to $\beta_2\text{M}$ were amplified from their corresponding cDNA by PCR and separately cloned into the expression vector pMT/BiP/V5-His B (Invitrogen), which carries a C-terminal hexahistidine tag sequence. A stop codon was introduced at the 3' end of the $\beta_2\text{M}$ sequence via the PCR primer and the resulting plasmids were cotransfected at equimolar ratios together with a 1:40 molar ratio of a blasticidin resistance gene into *Drosophila melanogaster* S2 cells using Effectene (Sigma). CD1- $\beta_2\text{M}$ was purified from by Ion Metal Affinity Chromatography on a Ni-NTA resin (Qiagen), anion exchange chromatography on MonoQ HR5/5 in 10 mM Tris-HCl pH 8.0 and size-exclusion chromatography on Superdex S200 16/60

in 50 mM Hepes pH 7.5 and 150 mM NaCl using a fast-performance liquid chromatography system (GE Healthcare). Recombinant His-tagged proteins comprised of the first two extracellular domains of E cadherin were the gift of Emilio Parisini and Jonathon Higgins.

For cell-free presentation of antigens to T cells, His-tagged CD1 or E cadherin was incubated for 5–20 h with 40- to 75-fold molar excess of GMM in 100 μl HBS at pH values ranging from 5 to 7.4. Acidic buffers were neutralized and incubated with 60 μl of Ni-NTA agarose bead for 20–45 min, and pilot experiments using flow cytometry showed that the level of CD1b adherent to beads was not affected by pH treatments. The resulting complexes were added to T cells for measuring calcium flux (6 μg , 1.3 μM) or used in larger amounts (100 μg) for elution for MS. CD1b-bound lipids were separated from total lipids by centrifugation and magnetic capture of nickel beads, followed by three washes, extraction in chloroform/methanol (2:1), drying under nitrogen and re-dissolving in 1/20th of the starting volume of 100 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ of C/M (2:1) solution for nanospray MS. Total lipids subjected to mild acid but not bound to CD1b were analyzed in the same way except that supernatants were subjected to Folch extraction.

Mass spectrometry

Nanospray ESI-MS (ThermoFinnigan LCQ Advantage) was performed using borosilicate glass pipettes pulled to a 2- μm orifice. Preparative TLC eluates were analyzed by CID-MS/MS using 8:2 (V/V) isopropyl alcohol:120 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ aqueous solution.

Lipid cleavage assays

Immature DCs (600 000) were incubated with 1 μM of [^{14}C]C₃₂ GMM, [^{14}C]C₈₀ α -GMM, [^{14}C]C₈₀ ester GMM or [^{14}C]C₈₀ MA for 4–72 h, washed with PBS (5% FBS), detached with 0.05% trypsin and 0.53 mM EDTA in PBS in a total volume of 300 μl and then transferred to a 15 ml glass tube. $\text{CHCl}_3/\text{MeOH}$ (2:1; 700 μl) was added to the tube followed by vortexing and centrifugation (2000 g for 10 min). The upper aqueous phases lack detectable scintillation. The lower organic phase was collected and washed twice with an excess volume of water and developed on silica TLC with $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ 60/16/2 (V/V/V) and exposed to film for 24–72 h. [^{14}C]MA-treated cells were analyzed in the same way except that silica plates were resolved with petroleum ether/ether/acetic acid 65/35/1 (V/V/V). Plates were charred with cupric acetate solution (3% cupric acetate in 8% phosphoric acid) at 160°C for 15 min. In selected experiments, concanamycin B (10 nM) was added 1 h before adding antigens. For cell-free antigen cleavage under harsh conditions, 400 μg of C₈₀ ester GMM or C₈₀ α -GMM was dried under a stream of nitrogen in a 15 ml glass tube. A 1 ml volume of 2 M TFA (Sigma-Aldrich) was added and heated at 120°C for 2 h and cooled. Then, 1 ml of chloroform was added, extensively vortexed and subjected to centrifugation (2500 r.p.m., 10 min) to give two layers. The lower layer was dried on the silica TLC plate and resolved with chloroform:methanol:water (60:16:2).

Confocal microscopy

DCs were cultured in complete medium with 4 μM BODIPY-GMM at 37°C for 30 min, adhered to fibronectin (20 $\mu\text{g}/\text{ml}$)-coated coverslips for 1 h before fixing with 2% paraformaldehyde in Hank's media for 2 min at 20°C. Cells were permeabilized with 0.2% saponin and stained with P3, BCD1B.3 (anti-CD1b), L243 (anti-MHC II) or antisera against LAMP-1 followed by Texas red-conjugated donkey F(ab')₂ anti-rabbit IgG or Texas red conjugated donkey F(ab')₂ anti-mouse IgG and analyzed with a Nikon Eclipse TE 2000-U confocal microscope.

Computer modeling of CD1b

Surfaces and electrostatic potentials of the CD1b-GMM complex were generated with the programs PyMol (Delano Scientific, San Carlos, CA, 2002) by Delano and APBS (Baker *et al*, 2001) using CD1b coordinates from 1UQS (Batuwangala *et al*, 2004).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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