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CD1a and MHC Class I Follow a Similar Endocytic Recycling Pathway

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Abstract

CD1 proteins are a family of Major Histocompatibility Complex (MHC) class I-like antigen-presenting molecules that present lipids to T cells. The cytoplasmic tails (CT) of all human CD1 isoforms, with the exception of CD1a, contain tyrosine-based sorting motifs, responsible for the internalization of proteins via the clathrin-mediated pathway. The role of the CD1a CT, which does not possess any sorting motifs, as well as its mode of internalization are not known. We investigated the internalization and recycling pathways followed by CD1a and the role of its CT. We found that CD1a can be internalized via a clathrin- and dynamin-independent pathway and that it follows a Rab22a- and ARF6-dependent recycling pathway, similarly to other cargo internalized independently of clathrin. We also found that the CD1a CT is S-acylated. However, this post-translational modification does not determine the rate of internalization or recycling of the protein, or its localization to detergent-resistant membrane microdomains (DRM), where we found CD1a to be enriched. We also show that plasma membrane DRM are essential for efficient CD1a-mediated antigen presentation. These findings place CD1a closer to MHC class I in its trafficking and potential antigen-loading compartments, among CD1 isoforms. Furthermore, we identify CD1a as a new marker for the clathrin- and dynamin-independent and DRM-dependent pathway of internalization as well as the Rab22a- and ARF6-dependent recycling pathway.

Keywords

internalization; recycling; S-acylation; clathrin; dynamin; CD1; MHC class I; cytoplasmic tail

INTRODUCTION

CD1 proteins are a family of glycosylated molecules that present self and foreign lipid antigens to T cells. In humans, this family of proteins can be divided according to sequence and functional criteria into group 1 – CD1a, CD1b and CD1c; group 2, which includes

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CD1d and, finally, group 3 that includes CD1e. CD1 molecules share sequence and structural homology with MHC class I and are thought to have evolved from classical MHC class I by gene duplication and neofunctionalization early in vertebrate evolution (1). Similar to MHC class I, CD1 molecules are heterodimers comprised of a heavy chain that includes three extracellular domains (1, 2 and 3) followed by a transmembrane domain and a short cytoplasmic tail (CT). The heavy chain is non-covalently bound to β_2 -microglobulin (β_2m) light chain. However, the antigen-binding groove shows striking differences between CD1 molecules and MHC class I. The CD1 antigen-binding groove is deeper and narrower than MHC class I and the amino acids that line it are predominantly non-polar, resulting in a hydrophobic antigen-binding channel [reviewed in (2)]. This feature of CD1 results in binding of lipid, glycolipid and lipopeptide antigens that can then be presented to a T-cell receptor. In recent years, a variety of lipid antigens were found to be bound by CD1 molecules, such as diacylglycerols, polyketide lipids, sphingolipids, lipopeptides and mycobacterial lipids such as mycolic acids [reviewed in (3)]. In addition, it has been shown that lipids with different chemical and physical properties traffic to different intracellular compartments. A study using lipid analogues that differed only in the length and unsaturations of the respective alkyl chains revealed that lipids with short or unsaturated alkyl chains traffic to the endocytic recycling compartment (ERC), while lipids with long and saturated tails are delivered to late endocytic compartments (4). Indeed, glucose monomycolate, a microbial lipid antigen presented by CD1b, with long alkyl chains (C₈₀) preferentially accumulates in lysosomes when compared to an analog of the same antigen with shorter alkyl chains (C₃₂) (5).

CD1 molecules have been shown to follow different intracellular trafficking pathways [reviewed in (6)]. For instance, CD1b prominently localizes to late endosomes and lysosomes, while CD1a does not traffic to late endocytic compartments and CD1c and CD1d show a more promiscuous distribution within the endocytic pathway (7). Therefore, CD1 is functionally similar to MHC class II, in regard to the ability of both molecules to survey endocytic compartments and, specifically, late endosomes/lysosomes for antigen acquisition.

The CT of CD1 proteins has been shown to be critical for CD1 intracellular localization and its antigen-presenting function. The CTs of human CD1b, CD1c and CD1d, and also murine CD1d, all possess a tyrosine-based motif of the YXX ϕ type, in which Y is a tyrosine, X any amino acid and ϕ a bulky hydrophobic residue. In contrast, CD1a does not contain any recognizable sorting motifs. The deletion of the CTs of CD1b and murine CD1d reduces or abolishes the internalization and trafficking of the respective proteins to late endocytic compartments (8-12). Moreover, tail-deleted CD1b and CD1d mutants display an impaired antigen-presenting capacity (9, 11, 13). In addition, some CD1 proteins possess putative dileucine-based sorting motifs (14), although it has not been shown if these play any role in trafficking or function. Tyrosine- and dileucine- based motifs are known to bind adaptor protein complexes (AP), such as AP-2, which is involved in endocytosis [reviewed in (15)]. In fact, human CD1b, CD1c and murine CD1d CTs have been shown to bind AP-2 or one of its subunits (8, 12). This last finding implies that CD1b, CD1c and CD1d are internalized in an AP-2-dependent manner, a pathway that is also mediated by clathrin, which coats the endocytic vesicles, and dynamin, which is thought to be involved in membrane fission. Indeed, CD1b internalization has been shown to be dynamin-dependent (8). Despite lacking any known sorting motifs in its CT, CD1a is internalized from the plasma membrane into endosomal compartments and recycles back to the plasma membrane, through the ERC (7, 16). It is not clear, however, if the CD1a CT determines the intracellular trafficking pathways followed by this molecule.

Here, we investigated the internalization and recycling pathways followed by CD1a and the role of the CD1a CT. Our results suggest that CD1a and MHC class I follow similar trafficking pathways within the endocytic system.

RESULTS

CD1a follows a clathrin-, AP-2- and dynamin-independent pathway of internalization

CD1a was previously found in clathrin-coated pits and clathrin-coated vesicles (7, 16). However, unlike other CD1 isoforms, CD1a does not possess a tyrosine-based motif or other known sorting motifs that can bind AP-2 and mediate clathrin-dependent internalization (Table 1). Therefore, we investigated the pathway of internalization followed by CD1a. We first utilized a knock-down approach directed at the clathrin-heavy chain (CHC) and the $\mu 2$ subunit of AP-2 using siRNA. For this, HeLa cells stably expressing CD1a or CD1b were transfected with siRNA oligos and analyzed for the presence of CD1 on the surface by flow cytometry (Figure 1, A and B). As a control, transferrin receptor (TfR), a molecule known to follow a clathrin-mediated pathway of internalization was used. The TfR showed a marked increase in surface expression when either CHC or $\mu 2$ were knocked-down. Strikingly, CD1a surface expression did not show any changes, contrary to CD1b which is known to bind AP-2 and follow a clathrin-mediated internalization pathway (Figure 1, A and B) (8). We also disrupted the clathrin-mediated pathway by using a dominant-negative mutant of dynamin 2. HeLa cells stably transfected with CD1a or CD1b and transiently transfected with dynamin 2 K44A dominant-negative mutant expectedly showed an increase in TfR surface expression (Figure 1, C and D). In accordance with the results obtained with siRNA, the surface expression of CD1a did not show significant changes, while CD1b surface levels were upregulated. These results suggest that CD1a follows a clathrin-, AP-2- and dynamin-independent internalization pathway. The experiments directly measuring CD1a internalization in dynamin 2 K44A transfectants were not fully conclusive as they showed a small but non-significant reduction (data not shown).

Importantly, we also analyzed human immature monocyte-derived dendritic cells (DCs), which normally express all CD1 isoforms. We detected increased surface levels of TfR when AP-2 $\mu 2$ was knocked-down by shRNA transduction, whereas no increase in CD1a surface levels could be detected (Figure 1E). Moreover, we detected a decrease in CD1a surface expression, further suggesting that CD1a and TfR follow different pathways of internalization.

CD1a is S-acylated in the cytoplasmic tail

In a search of a possible role for the CD1a cytoplasmic tail (CT), in internalization and trafficking of the molecule, we analyzed the presence of post-translational modifications that can occur in the CT. Palmitoylation consists in the covalent attachment of a fatty acid (which can be palmitate or another fatty acid, hence the more appropriate designation S-acylation) to a cysteine. Contrary to myristoylation, another type of post-translational lipid modification, there is no consensus sequence for S-acylation and it does not always occur at the N-terminus. Interestingly, CD1a possesses two cysteines in its CT (Table 1), which is striking considering that it only has six residues in total. We therefore investigated if CD1a could be S-acylated, by incubating HeLa:CD1a stable transfectants with radiolabeled palmitic acid, followed by lysis and immunoprecipitation of CD1a (Figure 2). As a control, we were able to detect labeled TfR, which is a known S-acylated protein. Strikingly, we also detected labeled CD1a, indicating that, indeed, CD1a is S-acylated. We predicted that, if CD1a is S-acylated in its CT, a truncation of the tail would abolish the lipid modification. Therefore, we generated HeLa cells that stably express a CD1a tail-truncated mutant, with no cytoplasmic cysteines (HeLa:CD1a TD, Table 1). Notably, we failed to detect labeled

CD1a in HeLa:CD1a TD cells (Figure 2), indicating that CD1a is S-acylated on one or both cysteines of the CT.

CD1a partitions to detergent-resistant membrane microdomains and these are necessary for efficient CD1a-restricted antigen presentation

Many palmitoylated proteins, such as kinases and some G proteins (17), are known to localize to detergent-resistant membrane microdomains (DRM), or lipid rafts, which are domains enriched in cholesterol and sphingolipids [reviewed in (18)]. Also, GPI-anchored proteins (GPI-AP), which follow a nonclathrin, dynamin-independent pathway of internalization (19) partition into DRM establishing a dynamin-independent and DRM-dependent pathway of internalization. During the preparation of this article, another study found that MHC class II invariant chain (Ii) can associate with a fraction of CD1a molecules in immature dendritic cells and that this association is dependent on DRM integrity, where CD1a is partially found (20). Therefore, we investigated if CD1a partitions into DRM also in HeLa cells, which do not express Ii. We extracted the DRM by lysing HeLa:CD1a stable transfectants in cold Triton X-100 and fractionating the lysate using a sucrose step gradient. Under these conditions, the DRM float and can be isolated from other membranes. As shown in Figure 3, A and B, CD1a partitions together with a DRM marker, caveolin, concentrating specifically in fraction number 10. Nevertheless, most of the CD1a protein is detected in fractions that do not float (fractions numbers 1, 2, 3 and 4), although a significant amount of caveolin also partitions to these fractions. As a control, TfR which is a non-DRM protein is virtually undetectable in DRM-containing fractions (Figure 3C). These results suggest that, indeed, a fraction of the CD1a present in the cell is associated with DRM, independently of Ii.

To confirm that DRM are important for CD1a-dependent antigen presentation, we tested the efficiency of presentation of a CD1a-dependent antigen in cholesterol-depleted immature monocyte-derived dendritic cells (DCs). We treated DCs with Methyl- β -cyclodextrin (M β CD), fixed the cells and incubated with dideoxymycobactin (DDM) and CD8-2 T cells (21). We found a striking decrease in the efficiency of presentation of this antigen, which in these experimental conditions has to be surface-loaded (Figure 3E). As a control, we saw that non-fixed cells, in which M β CD is washed away after treatment, do not present any defect in DDM presentation (data not shown). This indicates that the cells are not dead or functionally impaired after cholesterol-depletion. These results suggest that plasma membrane DRM are essential for CD1a-restricted antigen presentation in DCs, confirming previous studies (20).

Tail-deleted CD1a localizes to DRM and shows normal trafficking and function

Since CD1a was shown to partition to DRM and is S-acylated, we investigated if the CD1a tail-deleted mutant (CD1a TD), which is not S-acylated, also partitions into DRM. Interestingly, we were able to detect this mutant in DRM fractions, suggesting that the S-acylation does not determine the partition of this protein into DRM (Figure 3D).

S-acylation of the CT of transmembrane proteins is known to influence their trafficking. For instance, S-acylation of one cysteine in the CT of the cation-dependent mannose 6-phosphate receptor is critical for the correct trafficking and function of this receptor (22). We therefore investigated whether the trafficking of CD1a TD is altered, compared to CD1a wt. We first studied the expression of CD1a TD on the cell surface. We analyzed by flow cytometry the levels of CD1a on the surface of transiently transfected HeLa cells and saw no apparent difference in the surface expression of CD1a TD mutant when compared with CD1a wt (Figure 4A). We also analyzed the internalization and recycling rates of CD1a TD. Surprisingly, the CD1a internalization rate was relatively fast and reached equilibrium after

20 minutes (Figure 4B). This result was unexpected since CD1a does not have any sorting motif in its CT, like other CD1 isoforms which possess tyrosine-based motifs (Table 1), and it is not internalized in a clathrin-dependent manner (Figure 1). Notably, when we compared the internalization rates of CD1a wt and CD1a TD we saw no difference (Figure 4B). To study the recycling rates, we analyzed by flow cytometry the reappearance of CD1a on the cell surface after internalizing the protein bound to an antibody and stripping the antibody bound to non-internalized proteins. Similar to the internalization and surface expression studies, no differences between the recycling of CD1a wt and CD1a TD were detected (Figure 4C). Therefore, these results identified no differences in trafficking between the CD1a wt and CD1a TD. To confirm this, we compared the intracellular localization of CD1a wt and CD1a TD by transfecting these constructs into HeLa cells and performing confocal microscopy analysis. CD1a is known to recycle through the ERC without trafficking to late endocytic compartments (7). Therefore, we used transferrin ligand (Tf), which also recycles through the ERC, as a marker for this compartment. As shown in Figure 4D, CD1a wt displays punctate staining after internalization and accumulation in the perinuclear region, where the ERC is localized. As expected, there is colocalization with Tf (Figure 4F). Similarly, CD1a TD shows perinuclear accumulation and colocalization with Tf (Figure 4I), suggesting that there are no differences in trafficking, after internalization, between the two molecules, at this level of resolution. Together, these results indicate that the S-acylation of the CD1a CT does not play a significant role in the internalization or recycling of the protein, or its localization to DRM.

CD1a TD presents antigens efficiently to T cells

All the assays described thus far failed to show any differences in localization and trafficking between CD1a wt and CD1a TD. However, differences below the detection level of our assay systems could still lead to functional defects. We therefore compared the ability of the CD1a TD to present the antigen sulfatide to specific CD1a-restricted T cells (23) and compared this to CD1a wt. As shown in Figure 5, when transfected HeLa cells were pulsed with sulfatide antigen, recognition by CD1a-restricted, sulfatide-specific T cells revealed no significant difference between CD1a wt and CD1a TD. This suggests that the CD1a CT does not appear to influence the antigen-presenting function of CD1a.

CD1a follows an ARF6 and Rab22a-dependent recycling pathway, similar to MHC class I

As shown in Figure 1, the CD1a internalization pathway differs from the one followed by cargo internalized in a clathrin-dependent manner, such as TfR. Recently, the small GTPase Rab22a was shown to be involved in the recycling of proteins that are not dependent on clathrin for internalization (24). This recycling pathway, which can be distinguished from the classical slow recycling pathway followed by TfR, is inhibited by the expression of Rab22a constitutively-active or dominant-negative mutants (24). However, a different study found significant inhibition in the recycling of TfR by a Rab22a constitutively-active mutant (25). This disparity could be due to the difference in the species origin of Rab22a used (25). We used the same constructs as Weigert et. al, in HeLa cells, and saw striking colocalization between CD1a and Rab22a (Figure 6D) and CD1a and Rab22a-Q64L constitutively-active mutant (Figure 6H) in the tubular structures that represent tubular recycling endosomes (24), suggesting that CD1a follows a Rab22a-dependent recycling pathway. Importantly, TfR could not be detected in these tubular recycling endosomes (Figure 6, C and F), consistent with the results obtained by Weigert et al. When we used a Rab22a dominant-negative mutant (Rab22a S22N) we detected a marked reduction in the CD1a recycling rate, strongly suggesting that CD1a follows a Rab22a-dependent recycling pathway (5.07 fold change in CD1a surface expression after 30 minutes in the control vs. 2.43 fold change in cells transfected with the dominant-negative mutant Rab22a S22N). The residual recycling

observed in the dominant-negative mutant could be due to an incomplete block of Rab22a-dependent recycling or the existence of an alternative recycling pathway.

Proteins of the CD1 family show similarity to MHC class I at the sequence and structural level. Interestingly, MHC class I also follows a clathrin- and dynamin-independent internalization pathway and a Rab22a-dependent recycling pathway (24, 26), similar to what we observed with CD1a. Therefore, we determined if MHC class I and CD1a colocalize under steady-state conditions. As shown in Figure 7, we observed striking colocalization between these two proteins, including in the perinuclear area. These data further suggest that CD1a and MHC class I follow similar intracellular trafficking pathways.

CD1a endosomal recycling has been shown to be ARF6-dependent (7). Interestingly, MHC class I is also a marker for this pathway (27). We therefore made use of an ARF6 constitutively-active mutant (ARF6-Q67L), which blocks membrane trafficking shortly after internalization and sequesters cargo that normally traffics through the ARF6-dependent endosomal recycling pathway (28), such as MHC class I. HeLa cells transfected with the ARF6-Q67L mutant showed typical enlarged vacuolar structures where MHC class I accumulated (Figure 8A). Strikingly, CD1a, but not TfR (which is internalized in a clathrin-dependent manner), accumulated in the same structures. As expected, CD1b, which is internalized in an AP-2- and dynamin-dependent manner (8), also could not be detected in these enlarged vacuolar structures (Figure S1). These results are in agreement with the finding that CD1a follows an AP-2- and dynamin-independent internalization pathway (Figure 1) and strongly suggest that CD1a follows a similar recycling pathway to MHC class I. Further, they indicate that these two proteins not only share structural similarities but are also similar in their intracellular trafficking.

DISCUSSION

Most cytoplasmic tails (CTs) from different CD1 isoforms across species have well characterized tyrosine-based sorting motifs or, alternatively, putative dileucine-based sorting motifs (14). Primates (including humans) CD1a CTs are the shortest known with only three residues downstream of the three basic residues present in general in all CD1 proteins (14) (Chris C. Dascher, unpublished data). We investigated possible lipid modifications of the cysteines of the CD1a CT and showed that the CD1a CT is S-acylated, on one or both cysteines. Interestingly, we were also able to detect S-acylation of CD1c, which has a cysteine next to the third residue of the CT like CD1a, but not CD1b which does not have any cysteines in its CT (DCB and MBB, unpublished data) (Table 1). Protein S-acylation is known to be important for membrane targeting, localization to membrane microdomains, signaling and protein trafficking [reviewed in (29)]. It is therefore surprising that the truncation of the last three residues of the CD1a CT did not affect trafficking, including the rate of internalization and recycling. This suggests that the pathway followed by CD1a does not require any specific motifs in the CT. In the absence of specific motifs to direct intracellular trafficking, the pathway followed by CD1a could represent what might be described as a default recycling pathway for cell-surface proteins. Alternatively, CD1a could interact with an unknown protein that regulates its trafficking along the endocytic pathway. Further, we did not detect any defects in the antigen-presenting capacity of CD1a TD, compared to CD1a wt, suggesting that the S-acylation of the CD1a CT does not play a significant role in the function of the protein. However, CD1a has been shown to be capable of loading antigens on the cell surface (30), which could mask changes in intracellular loading of antigens onto CD1a.

While this article was being submitted, another study found that MHC class II invariant chain (Ii) associates with CD1a and may influence CD1a trafficking (20). Our findings

complement that study by examining directly the CD1a internalization and recycling pathway and the role of CD1a CT in a cell type that does not express Ii. Furthermore, we confirmed that CD1a localizes to detergent-resistant membrane microdomains (DRM) in HeLa cells but this was not dependent on the S-acylation of its CT. We also confirmed that cholesterol-depleted immature monocyte-derived dendritic cells (DCs) are less efficient in presenting a CD1a-dependent exogenous antigen (dideoxymycobactin) loaded on the cell surface. This suggests that plasma membrane DRM are essential for CD1a-restricted antigen presentation in DCs and is in agreement with the published results (20).

We found that CD1a and MHC class I follow similar intracellular trafficking pathways. Both molecules can be detected in the Rab22a-dependent recycling pathway and recycle in an ARF6-dependent manner, contrary to TfR and other cargo internalized in a clathrin-dependent fashion. Indeed, we show in HeLa cells and DCs that the internalization of CD1a is essentially clathrin-, AP-2- and dynamin-independent. The reduction in CD1a surface levels, seen only in DCs when the AP-2 μ 2 subunit was knocked-down, was unexpected. One possibility is that DCs might upregulate clathrin-independent pathways of internalization, such as the one followed by CD1a, when the clathrin-mediated pathway is blocked. Our study looked for the first time at the internalization of CD1a, and we were surprised to find that the kinetics were very fast, reaching equilibrium after 20 minutes, a rate consistent with receptor-mediated internalization. This implies that the clathrin-independent pathway followed by CD1a mediates fast internalization, similar to the clathrin-dependent pathway.

It is generally accepted that CD1 evolved from a classical MHC class I early in vertebrate evolution (1). In this context, CD1a could represent a more primordial CD1 isoform, when compared for example with CD1b, since it has a smaller antigen-binding groove and binds lipids with short alkyl chains (e.g. sulfatide) (23, 31). It also can bind lipids on the surface of the cell, contrasting with CD1b, which can accommodate lipids with C₈₀ alkyl chains in its antigen-binding groove and can only load these long lipids in acidic compartments such as late endosomes and lysosomes (5, 30). A model has been proposed where, after gene duplication, different CD1 isoforms acquired different trafficking patterns which then led to changes in the antigen-binding groove, in order to accommodate lipids that were present in the newly surveyed intracellular compartments (32). Our results fit this model in the sense that CD1a shows a similar trafficking pattern to MHC class I. We and others recently reported the presence of two CD1 genes in chicken, which makes these the most ancient known so far (33, 34). Interestingly, one of these proteins shows an intracellular distribution similar to human CD1a (DCB, MBB and Cristopher C. Dascher, unpublished data).

Therefore, our study describes another level of similarity between MHC class I and CD1a, namely their intracellular trafficking, and these molecules essentially identify an internalization and recycling pathway that is only partially characterized. Yet, this pathway may be followed by a number of cell surface molecules with important immunological functions.

MATERIALS AND METHODS

Cell culture

Cell culture reagents were from GIBCO (Invitrogen, Carlsbad, CA). HeLa epithelial cell line was cultured in DMEM, 10% fetal calf serum heat-inactivated, 100 U/ml penicillin G, 100 μ g/ml streptomycin, 2 mM L-glutamine and 20 mM HEPES.

CD14⁺ monocyte isolation

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat preparations from healthy human donors, by overlaying them on a Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) cushion and spinning at 2800 rpm for 20 minutes. After washing 3 times with PBS, CD14⁺ monocytes were positively selected from PBMC using CD14 microbeads (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions.

Monocyte-derived dendritic cells

Differentiation of CD14⁺ monocytes into immature monocyte-derived dendritic cells (DCs) was done by culturing in 300 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF, Sargramostim, Immunex, Seattle, WA) and 200 U/ml of IL-4 (PeproTech, Rocky Hill, NJ) for 4 days in complete medium (RPMI, 10% fetal calf serum heat-inactivated, 100 U/ml penicillin G, 100 µg/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES, 1 mM sodium pyruvate, 55 µM 2-mercaptoethanol and essential and nonessential amino acids). The cytokines were replenished at day 2.

Antibody labeling

Monoclonal antibodies 10H3 (anti-CD1a) and W6/32 (anti-MHC class I) were conjugated to Alexa Fluor 488 or 546 using monoclonal antibody labeling kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Constructs and HeLa transfection

EGFP-Rab22a constructs were a kind gift from Dr. J. Donaldson [NHLBI, NIH, Bethesda, MD; (24)], GFP-Dynamin 2 wt and K44A were kindly provided by Dr. M. McNiven (Mayo Clinic, Rochester, MN) and HA-ARF6-Q67L was a kind gift from Dr. V. Hsu [(BWH, Harvard Medical School, Boston, MA; (35))].

cDNAs encoding human CD1a wt and CD1a TD (without the last three aminoacids of the cytoplasmic tail) were generated by PCR using human CD1a in pSR⁺-neo (36) as a template DNA. The primers used were 5'-CGCGGATCCGCGCCGCCACCATGCTGTTTTTGCTACTTCCATTGT-3' (sense), 5'-CCGCTCGAGCGGTTAACAGAAACAGCGTTTCCTGAA-3' (antisense for CD1a wt) and 5'-CCGCTCGAGCGGTTAGCGTTTCCTGAACCAAAGCGC-3' (antisense for CD1a TD). Following digestion with *Bam*HI and *Xho*I, the PCR product was cloned into pcDNA3 (Invitrogen, Carlsbad, CA) and confirmed by sequencing.

To generate HeLa:CD1a stable transfectants, HeLa cells (1×10^7) were electroporated with 10 µg DNA at 250 V and 960 µF in a Gene Pulser II (Bio-Rad, Hercules, CA). After 24 h, 1 mg/ml G418 was added to the medium and 3 weeks later, HeLa:CD1a wt and HeLa:CD1a TD cells with similar mean fluorescence intensities were FACS-sorted and clones grown in the presence of G418.

For transient transfections, HeLa cells were transfected using Fugene 6 (Roche, Basel, Switzerland) according to the manufacturer's instructions.

RNA-Mediated Interference

RNA interference of the clathrin heavy chain (CHC) and the µ-subunits of the AP-2 complex was performed by using small interfering RNA (siRNA) duplexes (Qiagen, Valencia, CA; target sequences GUGGAUGCCUUUCGGGUCA for µ2 and UCCAAUUCGAAGACCAAUU for CHC) or an AP-2 µ2 subunit (NM_004068) directed short hairpin RNA (shRNA), cloned into the lentiviral expression vector PLKO.1 [obtained

from the Broad Institute TRC consortium, TRC ID#: TRCN0000060238/39/41/42 (Open Biosystems, Huntsville, AL); target sequences 5'-GTGGTCATCAAGTCCAACCTT-3', 5'-CACCAGCTTCTTCCACGTAA-3', 5'-GCTGGATGAGATTCTAGACTT-3' and 5'-CATTTATGAACTCGCTGCTA-3', respectively]. 293T cells were co-transfected with the shRNA lentiviral vector and appropriate packaging plasmids (d8.9, VSV-G), and viral supernatants harvested after 48 and 72 h and pooled. CD14⁺ monocytes were spin-inoculated with virus for 30 minutes at 2000 rpm. Twenty four hours later, granulocyte-macrophage colony-stimulating factor (GM-CSF, Sargramostim, Immunex, Seattle, WA) and IL-4 (PeproTech, Rocky Hill, NJ) were added at 300 U/mL and 200 U/mL, respectively to generate immature monocyte-derived DCs. After 48 hours, stably shRNA expressing cells were selected using continuous culture in 2 µg/mL puromycin for a minimum of 4 days. Cells were then analyzed on day 6 for surface expression of CD1a and Tfr. HeLa cells were transfected twice at 72 h intervals with the siRNAs by using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cells were then analyzed by flow cytometry 48–72 h after the second round of transfection.

Flow cytometry analysis

Transfected HeLa cells were washed with PBS and subsequently harvested with PBS and 5 mM EDTA or 0.5% trypsin-EDTA (GIBCO, Invitrogen) and incubation at 37°C. shRNA-transduced monocyte-derived dendritic cells (DCs) were collected, overlaid on a Ficoll-Paque (GE Healthcare) cushion and spun to exclude dead cells. Primary antibodies 10H3 (anti-CD1a) and BCD1b3.2 (anti-CD1b) were added at a concentration of 10 µg/ml and anti-Tfr clone 5E9 (ascites; ATCC, Manassas, VA) at a dilution of 1:300. The cells were incubated on ice, washed and phycoerythrin (PE)- or fluorescein-conjugated secondary anti-mouse antibodies added. After incubating on ice, the cells were washed and analyzed using a three-color FACSCalibur or a FACScan flow cytometer (BD Biosciences, San Jose, CA).

Surface biotinylation

HeLa:CD1a stable transfectants were washed with ice-cold PBS and surface biotinylated on ice with 0.5 mg/ml of Sulfo-NHS-LC-Biotin or Sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in PBS (pH=8.0) and 20 mM HEPES for 40 minutes at 4°C, adding 1 vol. of 1 mg/ml biotin after 20 minutes. The cells were washed twice with PBS and 100 mM glycine to quench the excess biotin.

Immunoprecipitation

Samples were pre-cleared by incubation with Protein G sepharose (GE Healthcare, Piscataway, NJ) and monoclonal isotype antibody control for 1 h at 4°C. The supernatant was saved, incubated with 2–4 µg of 10H3 (anti-CD1a) monoclonal antibody for 1 h, on ice, and Protein G beads were added. After incubating at 4°C overnight with rotation, the beads were washed 4 times with TNE and 1% Triton X-100, washed once with 50 mM Tris (pH=6.8) and resuspended in SDS sample buffer (62.5 mM Tris pH=6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 179 mM β-mercaptoethanol).

Immunoblotting

SDS-PAGE gels were transferred to polyvinylidene fluoride membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 1 h 30 minutes at 21V in a Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). After drying, membranes were blocked with PBS, 0.2% Tween-20 (PBS_T) and 0.5% BSA for 1 h at room temperature. Antibodies (rabbit polyclonal anti-caveolin, BD Biosciences, San Jose, CA, 125 ng/ml; goat polyclonal anti-Tfr, R&D Systems, Minneapolis, MN, 0.5 µg/ml; HRP-conjugated anti-rabbit and HRP-conjugated anti-goat, Jackson ImmunoResearch Lab., Inc., West Grove, PA, 40 ng/ml) or

HRP-conjugated streptavidin (ExtrAvidin, Sigma, St. Louis, MO, 1:20,000) in PBS_T were incubated in the same conditions and blots developed with Western Lightning Chemiluminescence Reagent (Perkin Elmer, Boston, MA) according to the manufacturer's instructions.

Palmitoylation assay

HeLa:CD1a stable transfectants (~60-80% confluent) in 10 cm dishes were labeled overnight with 250 μ Ci/ml palmitic acid [9,10-³H] (Perkin Elmer, Boston, MA) in complete medium with 1 mM sodium pyruvate. After washing with ice-cold PBS, cells were lysed in RIPA buffer (50 mM Tris pH=7.5, 150 mM NaCl, 1% Triton X-100, 0.05% deoxycholic acid, 0.1% SDS and protease inhibitors) and the lysate spun for 10 minutes at 13,000 rpm, at 4°C. The supernatant was then saved and TfR and CD1a immunoprecipitated using 2 μ g of the monoclonal antibodies MEM-189 (Abcam, Cambridge, MA) and 10H3, respectively. The immunoprecipitates were resuspended in SDS sample buffer (62.5 mM Tris pH=6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 20 mM DTT), boiled for 5 minutes and analyzed by SDS-PAGE. The gel was then washed with DMSO (2 $\times$ 20 minutes), once with 2,5-diphenyloxazole (PPO, 22.5% in DMSO) for 1 h and, finally, with water (2 $\times$ 30 minutes). The gel was then dried and exposed to a pre-flashed (using a Sensitize TM pre-calibrated flash from GE Healthcare, Piscataway, NJ) X-ray film.

Isolation of detergent-resistant membrane microdomains (DRM)

HeLa:CD1a wt and HeLa:CD1a TD stable transfectants (~90% confluent) were washed with ice-cold PBS and surface biotinylated (using Sulfo-NHS-LC-Biotin). Cells were then washed with TNE buffer (25 mM Tris pH=7.4, 150 mM NaCl and 5 mM EDTA) and lysed in this buffer containing 1% Triton X-100, 0.1 M sodium carbonate (pH=11) and protease inhibitors, on ice. After passing the lysate several times through a 22-G needle, the lysates were mixed with 1 vol. of 80% sucrose in TNE buffer (with protease inhibitors). A sucrose step gradient was made by overlaying 35% and 5% sucrose steps and the gradients centrifuged overnight at 100,000 g, at 4°C. One ml fractions were then collected from the bottom and the final concentration of Triton X-100 adjusted to 0.5% in the fractions corresponding to the 35% and 5% sucrose steps.

Internalization assay

Confluent or near confluent HeLa:CD1a wt and HeLa:CD1a TD stable transfectants on 6 cm dishes were washed with ice-cold PBS and surface biotinylated with Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) as described above. Cells were left to internalize surface proteins at 37°C for different periods of time (time 0' was left on ice), washed with ice-cold PBS and the surface biotin cleaved by incubation at 4°C for 20 minutes with 10 mM of the reducing agent sodium 2-mercaptoethanesulfonate (MesNa) in 50 mM Tris (pH=8.6), 150 mM NaCl, 1 mM EDTA and 0.2% BSA. After this first incubation, 10% vol. of 100 mM MesNa in same buffer was added and after a further 20 minutes, 12.5% vol. of 100 mM MesNa was added. The cells were incubated with 16% vol. of 500 mM iodoacetamide to quench the reducing agent, then washed with ice-cold PBS and lysed in RIPA buffer with protease inhibitors.

CD1a ELISA

Plates were coated with 5 μ g/ml of 10H3 antibody (anti-CD1a), blocked with PBS, 0.05% Tween-20 and 5% skimmed milk and incubated with the lysates from the internalization assay. To detect the total CD1a protein, anti- γ m rabbit polyclonal antibody (Rockland, Gilbertsville, PA; 0.8 μ g/ml) was used. After incubating with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Jackson ImmunoResearch Lab., Inc., West Grove, PA; 0.4

µg/ml), the detection was performed with ABTS (30% w/v in 0.1 M citric acid, pH=4.35 with 1×10^{-3} vol. H_2O_2). To detect internalized CD1a, plates were incubated with alkaline phosphatase (AKP)-conjugated streptavidin (BD Biosciences, San Jose, CA; diluted 1:1000) after incubating with the lysates. The AKP was developed with p-nitrophenyl phosphate (pNPP, 1 mg/ml in Tris buffered saline pH=9.8, 1 mM $MgCl_2$). The O.D. at 405 nm was read in a plate reader (Molecular Devices, Sunnyvale, CA).

Recycling assay

HeLa cells were transiently co-transfected with CD1a or EFGP-Rab22a S22N constructs and pEGFP-C1 (BD Biosciences, San Jose, CA) in 6 well plates with Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions and 24 h later cells were trypsinized, washed with complete medium, counted and 250,000 cells plated in each well of 96 well plates. Anti-CD1a monoclonal antibody 10H3 (10 µg/ml) was then incubated with the cells on ice in complete medium for 30 minutes and the excess antibody washed with cold complete medium. Cells were left to internalize the antibody-CD1a complexes for 25-30 minutes at 37°C and were then chilled on ice, washed with ice-cold PBS and the surface antibody stripped by washing for 1 minute with stripping buffer (0.5% acetic acid pH=3.0, 0.5 M NaCl) at R.T. The cells were then incubated for different periods of time at 37°C with complete medium to allow for proteins to recycle (time 0' was left on ice) and were then washed and processed for flow cytometry analysis.

Immunofluorescence staining

Cells were plated onto glass coverslips pre-incubated with serum and left adhering overnight. Cells were then either transfected with Fugene 6 (Roche, Indianapolis, IN) or processed for staining. Where internalized transferrin (Tf) was observed, cells were serum-starved by incubation at 37°C for 30 minutes in minimal medium and 0.5% BSA, then chilled on ice, washed once with the same medium (ice-cold) and incubated with anti-CD1a monoclonal antibody (10H3, 1 µg/ml) and/or labeled Tf (Alexa Fluor 546- or Alexa Fluor 647-conjugated, Invitrogen, 10 µg/ml) for 30 minutes on ice. After washing three times with ice-cold medium, cells were incubated at 37°C for 30-45 minutes for the internalization to occur. In the case of Tf internalization, labeled Tf was kept in the medium in order to saturate the recycling pathway. After washing with PBS, the surface antibody was stripped by washing for 1 minute with stripping buffer (0.5% acetic acid pH=3.0, 0.5 M NaCl) at R.T. Cells were then washed again with PBS, fixed in 3% paraformaldehyde in PBS for 15-20 minutes at R.T., quenched with 50 mM NH_4Cl in PBS and blocked / permeabilized with PBS, 0.5% BSA and 0.1% saponin for 15 minutes at R.T. Primary and secondary antibodies (anti-mouse Alexa Fluor 488- and Alexa Fluor 546-conjugated, Invitrogen; 4 µg/ml and anti-mouse Cy5-conjugated, Jackson ImmunoResearch Lab., Inc.; 2.8 µg/ml) were incubated in the same buffer for 30 minutes at R.T. and washed five times after each incubation. Coverslips were finally mounted in mounting medium (15% w/v vinol 205, 33% v/v glycerol, 0.1% azide in PBS) and analyzed in a Nikon C-1 confocal microscope with EZ C1 software. Images were processed with Adobe Photoshop CS v8.0, adjusting the levels of each channel up to a maximum threshold defined by the absence of signal in the negative controls.

Antigen presentation assays

HeLa cells (2.5×10^4 /well) in RPMI-1640 medium containing 10% fetal calf serum were pulsed for 1 h or incubated during the whole assay, at 37°C, with sonicated purified synthetic C24:1 (nervonoyl-) sulfatide [kindly provided by Dr. L. Panza, Università del Piemonte Orientale, Novara, Italy (37)]. After 3 h, sulfatide-specific human K34 B9.1 T cells (23) were added (1×10^5 /well) and the supernatants harvested after 48 h. Immature monocyte-derived dendritic cells (DCs) (2.5×10^4 /well) were irradiated at 5000 rad and

incubated in serum-free RPMI-1640 medium with 10 mM Methyl- β -cyclodextrin (Sigma, St. Louis, MO) for 45 minutes at 37°C. They were then fixed for 2 minutes with 0.04% glutaraldehyde, quenched for the same time with 0.2M L-lysine (pH=7.4) and washed in complete medium. Finally, these cells were incubated with a sonicated *Mycobacterium tuberculosis* H37Ra lipid fraction (acetone-soluble fraction after chloroform/methanol extraction, kind gift of Dr. D.B. Moody, BWH, Harvard Medical School, Boston, MA) and CD8-2 T cells [5×10^4 /well, (21)] for approximately 21h.

IFN- γ and TNF- α ELISA

Plates were coated with 1 μ g/ml MAb1 (anti-human TNF- α , BD Biosciences), 3 μ g/ml HB-8700 (anti-human IFN- γ , ATCC) or 1 μ g/ml anti-human IFN- γ (2G1, Thermo Scientific, Waltham, MA) monoclonal antibodies; blocked with PBS, 0.05% Tween-20 and 10 mg/ml BSA or PBS, 1% BSA and incubated with the supernatants from the antigen presentation assays. For detection, monoclonal antibodies MAb11 (anti-human TNF- α biotin labeled, 0.5 μ g/ml, BD Biosciences), 69 [anti-human IFN- γ biotin labeled, 0.72 μ g/ml, kindly provided by Dr. G. Garotta, F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland (38)] and anti-human IFN- γ (0.5 μ g/ml, Thermo Scientific) were used. After incubating with horseradish-conjugated streptavidin (Invitrogen, Carlsbad, CA; 0.3 μ g/ml) or alkaline phosphatase (AKP)-conjugated streptavidin (BD Biosciences, San Jose, CA; diluted 1:1000), the detection was performed using o-Phenylenediamine dihydrochloride (OPD, Sigma, St. Louis, MO) or p-nitrophenyl phosphate (pNPP, 1 mg/ml in Tris buffered saline pH=9.8, 1 mM MgCl₂), respectively. The OPD reaction was stopped by adding 0.5 vol. of 10% sulphuric acid and the O.D. at 490 nm or 405 nm was read in a plate reader (Molecular Devices, Sunnyvale, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CD1a Surface Expression is Not Affected by Inhibition of the Clathrin-Mediated Pathway

HeLa:CD1a (Panels A and C) or HeLa:CD1b (Panels B and D) stable transfectants were transiently transfected with clathrin-heavy chain (CHC) or $\mu 2$ -subunit of AP-2 ($\mu 2$) siRNA oligos, twice at 72 h intervals (Panels A and B), or with EGFP-Dynamin 2 wt or EGFP-Dynamin 2 K44A for 42 h (Panels C and D), then stained with anti-CD1a, anti-CD1b or anti-TfR antibodies and analyzed by flow cytometry. Error bars represent the standard deviation of three independent experiments. Panel E. CD14⁺ monocytes were differentiated into immature monocyte-derived dendritic cells and transduced (or not) with GFP or $\mu 2$ shRNA encoding lentivirus for 6 days and analyzed in the same way. The Mean Fluorescence Intensity (MFI) is represented in all cases and the error bars represent the standard deviation of four different shRNA for $\mu 2$.

Figure 2. CD1a wt, but Not CD1a TD, is Post-Translationally Modified by S-Acylation

HeLa:CD1a wt or HeLa:CD1a TD stable transfectant cells were labeled overnight with ^3H -palmitic acid and, after lysing the cells, the indicated proteins were immunoprecipitated with appropriate monoclonal antibodies. The immunoprecipitates were analyzed by 12.5% SDS-PAGE and the gels subjected to fluorography. The results are representative of two independent experiments.

Figure 3. CD1a Partitions to Detergent-Resistant Membrane Microdomains, which are Necessary for Efficient CD1a-Restricted Exogenous Antigen Presentation

HeLa:CD1a wt (Panel A) or HeLa:CD1a TD (Panel D) stable transfectant cells were surface biotinylated, lysed and fractionated in a sucrose step gradient. Fractions were collected from the bottom and CD1a immunoprecipitated (Panels A and D). Samples were resolved on 12.5% SDS-PAGE and immunoblotted with HRP-conjugated streptavidin (Panels A and D) or with rabbit polyclonal anti-caveolin (Panel B) or goat polyclonal anti-TfR (Panel C), followed by HRP-conjugated anti-rabbit or anti-goat antibodies, respectively. In Panel A, associated β 2-microglobulin (β 2m) is also shown. Results are representative of at least two independent experiments. Panel E. CD14⁺ monocytes from human donors were differentiated into immature monocyte-derived dendritic cells (DCs) and cholesterol-depleted by treatment with Methyl- β -cyclodextrin (M β CD). DCs were then fixed and incubated with indicated concentrations of sonicated antigen and CD8-2 T cells, which recognize dideoxymycobactin (DDM) presented by CD1a. After 21h, the release of IFN- γ was measured by ELISA. Error bars represent standard deviation of triplicate measurements and the results are representative of three independent experiments.

Figure 4. CD1a TD Shows Normal Surface Expression, Internalization and Recycling

Panel A. HeLa cells were transiently co-transfected with CD1a wt and EGFP or CD1a TD and EGFP, stained for CD1a and analyzed by flow cytometry, gating on EGFP+ cells. Panel B. HeLa:CD1a wt and HeLa:CD1a TD stable transfectants were surface biotinylated with a cleavable biotin at 4°C, shifted to 37°C for the indicated periods of time (to allow for internalization), after which cells were placed on ice to stop internalization. Surface biotin was cleaved with a reducing agent (on ice) and cells were then lysed and lysates probed for different proteins by ELISA with each condition performed in duplicate. The results (in arbitrary units) are normalized for the total CD1a protein present in each lysate and the internalization at time zero. Panel C. HeLa cells were transiently co-transfected with CD1a wt and EGFP or CD1a TD and EGFP, stained for CD1a and incubated at 37°C to allow for internalization. After stripping the surface-bound antibody with a brief acidic wash, cells were incubated at 37°C for different periods of time to allow for recycling. Cells were then

stained with PE-labeled anti-mouse secondary antibody and analyzed by flow cytometry, gating on EGFP⁺ cells. The results (in arbitrary units) are normalized for the recycling at time zero. Error bars in panels A, B and C represent the standard deviation of three independent experiments. Panels D-I. HeLa cells were transiently transfected with CD1a wt (Panels D, E and F) or CD1a TD (Panels G, H and I), serum-starved for 30 minutes and incubated with anti-CD1a and Alexa 546-conjugated transferrin (Tf) on ice for 30 minutes. Cells were then washed and incubated with Alexa 546-conjugated Tf for 30 minutes at 37°C. After stripping the surface-bound antibody with a brief acidic wash, cells were fixed, permeabilized and labeled with Alexa 488-conjugated anti-mouse (Panels D and G) antibody. In the merge panels F and I, colocalization is indicated by the yellow color. Scale bars, 10 μ m.

Figure 5. CD1a TD Efficiently Presents Sulfatide to T cells

Nervonoyl sulfatide, at the indicated concentrations, was incubated with HeLa:CD1a wt or HeLa:CD1a TD and the CD1a-restricted sulfatide-specific human T-cell clone K34 B9.1, during the whole assay (Panels A and B) or pulsed for 1 h at 37°C (Panels C and D). Supernatants were collected after 48 h and release of human IFN- γ (Panels A and C) and human TNF- α (Panels B and D) was measured by ELISA. Error bars represent the standard deviation of triplicate measurements.

Figure 6. CD1a Follows a Rab22a-Dependent Recycling Pathway

HeLa:CD1a stable transfectants were transiently transfected with EGFP-Rab22a (wt or Q64L constitutively-active mutant). After 24 h, cells were serum-starved for 30 minutes and incubated with Alexa 647-conjugated transferrin (Tf, Panel C) or Alexa 546-conjugated Tf (Panel F) and anti-CD1a monoclonal antibody (Panels B and G), for 30 minutes on ice. Cells were then washed and incubated with Alexa 647-conjugated Tf for 45 minutes at 37°C (Upper panels) or switched only to 37°C for 30 minutes (Lower panels). After stripping the surface-bound antibody with a brief acidic wash, cells were fixed, permeabilized and labeled with Alexa 546-conjugated anti-mouse (Panel B) or Cy5-conjugated anti-mouse (Panel G) antibodies. Arrowheads depict tubular structures that contain both EGFP-Rab22a and CD1a. In the merge panel D, colocalization is indicated by the yellow color, while in panel H it is indicated by the color cyan. Scale bars, 10 μ m.

Figure 7. CD1a Colocalizes with MHC Class I at Steady-State

HeLa:CD1a stable transfectant cells were fixed, permeabilized and stained with Alexa-488-conjugated anti-MHC class I (Panel A) and Alexa 546-conjugated anti-CD1a (Panel B) antibodies. In the merge panel, colocalization is indicated by the yellow color. Scale bars, 10 μm .

Figure 8. CD1a is Internalized and Recycled via an ARF6-Dependent Pathway

HeLa:CD1a stable transfectants were transiently transfected with ARF6-Q67L constitutively-active mutant. After 24 h, cells were serum starved for 30 minutes and incubated with Alexa 647-conjugated transferrin (Tf, Panel C) for 30 minutes on ice. Cells were washed, incubated with Alexa 647-conjugated Tf for 30 minutes at 37°C and then fixed, permeabilized and incubated with Alexa 488-conjugated anti-MHC class I (Panel A) and Alexa 546-conjugated anti-CD1a (Panel B) antibodies. Arrowheads depict typical enlarged vacuolar structures induced by ARF6-Q67L expression. Scale bar, 10 μ m.

Table 1

Cytoplasmic domains of different CD1 isoforms and the tail-truncated mutant CD1a TD.

Human CD1a	.RKRCFC
CD1a TD	.RKR
Human CD1b	.RRRS YQ NIP
Human CD1c	KKHCS YQD IL
Human CD1d	KRQTS YQG VL
Mouse CD1d	RRRS AYQD IR

Tyrosine-based motifs are in bold.