Regulation of intracellular trafficking of human CD1d by association with MHC class II molecules

Suk-Jo Kang and Peter Cresswell

Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06520-8011, USA

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Introduction

CD1 family members are antigen-presenting molecules capable of presenting bacterial or synthetic glycolipids to T cells. Here we show that a subset of human CD1d molecules are associated with major histocompatibility complex (MHC) class II molecules, both on the cell surface and in the late endosomal/lysosomal compartments where class II molecules transiently accumulate during transport. The interaction is initiated in the endoplasmic reticulum with class II–invariant chain complexes and appears to be maintained throughout the class II trafficking pathway. A truncated form of CD1d which lacks its cytoplasmic YXXZ internalization motif is transported to late endosomal/lysosomal compartments in the presence of class II molecules. Furthermore, the same CD1d deletion mutant is targeted to lysosomal compartments in HeLa cells expressing class II molecules and invariant chain by transfection. The deletion mutant was also found in lysosomal compartments in HeLa cells expressing only the p33 form of the invariant chain. These data suggest that the intracellular trafficking pathway of CD1d may be altered by class II molecules and invariant chain induced during inflammation.

While CD1b accumulates prominently in late endosomes and lysosomes (Sugita et al., 1996; Jackman et al., 1998), CD1c is localized throughout the endosomal system. It is expressed at higher levels on the plasma membrane and in early endosomes and less intensely in late endocytic compartments than CD1b (Briken et al., 2000; Gebo et al., 2000; Schaible et al., 2000; Sugita et al., 2000). CD1a, lacking a tyrosine-based motif, is sorted to recycling endosomes and the plasma membrane (Sugita et al., 1999). Studies of CD1a, b and c support the notion that the antigen repertoire they present reflects the different intracellular compartments which they survey. However, what precisely is responsible for their different intracellular distribution remains unclear.

The molecular mechanisms of antigen presentation and processing by MHC class I and class II molecules have been well elucidated (reviewed by Pamer and Cresswell, 1998; Pieters, 2000). However, there is no comprehensive understanding of the mechanisms regulating lipid association with CD1d molecules. As an initial approach to this problem, we looked for proteins which physically associate with human CD1d molecules and which therefore might facilitate lipid association or exchange. In the course of these experiments, we were surprised to find that CD1d
associates with MHC class II molecules. The interaction appears to be initiated in the endoplasmic reticulum (ER) and to be maintained in MIIcs and on the plasma membrane. Here we document these findings and examine the possibility that the class II interaction provides a secondary targeting signal directing CD1d within the endocytic pathway.

**Results**

**CD1d is associated with MHC class II molecules**

To search for CD1d-associated molecules, we expressed human CD1d in the B-lymphoblastoid cell line, C1R (see Table I for a listing of the cell lines used in this study). C1R.CD1d cells were labeled with [35S]methionine for 15 min and chased at 37°C for various times. In preliminary experiments, several proteins co-precipitated specifically with CD1d in the detergent Brij 98 but not in a variety of other detergents, including Triton X-100 or polyoxyethylene nonyl ether (C12E9). Therefore, after extraction in Brij 98, CD1d-β2m heterodimers were immunoprecipitated using the monoclonal antibody (mAb) 51.1.3 and protein A-Sepharose and analyzed by SDS-PAGE followed by fluorography. Three dominant proteins of 90, 35 and 30 kDa appeared to associate specifically with CD1d throughout its maturation (Figure 1A), although the intensity of the 35 kDa band peaked at 2 h and waned thereafter. To identify the proteins, a Brij 98 extract of C1R.CD1d cells was applied to a 51.1.3 mAb affinity column, and CD1d-associated proteins were eluted using 1% C12E9, ethanol precipitated, separated by SDS-PAGE and stained with Coomassie Blue (Figure 1A). The bands corresponding to p35 and p30 in (A) were identified as DRα- and β-subunits by mass spectrometry (see Materials and methods).

These findings were confirmed by co-immunoprecipitation experiments. [35S]methionine-labeled C1R.CD1d cells were extracted in Brij 98, and CD1d and its associated proteins were immunoprecipitated. After heating the immunoprecipitates at 100°C in 1% SDS containing 5 mM dithiothreitol (DTT), the eluates were diluted in 1% Triton X-100/10 mM iodoacetamide (IAA) and the released DRα chain identified by subsequent immunoprecipitation with the mAb DA6.147 (Figure 2A, lane 2). The DRβ chain was also identified by immunoprecipitation with mAb HB10A (data not shown). DR-associ- ated CD1d was detected by first precipitating with the mAb L243, specific for DRαβ dimers, or DA6.147, followed by the D5 mAb to precipitate denatured CD1d heavy chain (Figure 2A, lanes 4 and 5). The CD1d-class II interaction was also clearly present when CD1d was expressed in a second B-lymphoblastoid cell line, .221 (Figure 5C, upper panels, lanes 1–5). To verify that the association was not occurring after cell lysis, labeled C1R cells were mixed with unlabeled C1R.CD1d cells and the mixture extracted in Brij 98. As a positive control, the same number of labeled C1R.CD1d cells was extracted in parallel. Co-precipitation of CD1d-associated class II molecules was seen in the control extract but not in the mixed samples (Figure 2B).

![Identification of CD1d-associated proteins](image-url)
Since the tetraspanins CD82, CD63 and CD81 have been shown to associate with class II molecules (Szollosi et al., 1996; Hammond et al., 1998), we asked whether they mediate the CD1d–class II association. CD1d, FLAG-tagged at the C-terminus, was expressed in 221 cells and Brij 98 lysates passed through an anti-FLAG mAb (M2)–conjugated column. The CD1d–class II complexes were eluted by competition with FLAG peptide and used for immunoprecipitation with a variety of antibodies. Figure 2C clearly shows that, although CD82 is associated with some class II–CD1d complexes, there are far more class II molecules precipitated by the anti-DR mAb L243 than by the anti-CD82 mAb, indicating that CD1d molecules can interact with class II independently of CD82. Both antibodies were used in excess (Figure 2C, bottom panels). CD63, previously shown to interact with DR molecules (Hammond et al., 1998), is associated with class II in the column flow-through (Figure 2C, lower panels) but not with the class II–CD1d complexes. CD81 does not interact detectably with class II molecules in Brij 98 even though it was solubilized efficiently (data not shown).

We also wished to determine whether the CD1d–class II interaction exists in normal antigen-presenting cells. Dendritic cells (DCs) were produced from monocytes isolated from normal peripheral blood mononuclear cells by incubation with granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 as described (Sallusto and Lanzavecchia, 1994). These cells, and the residual monocyte-depleted lymphocytes, were lysed in Brij 98. HLA-DRα chain was detected by western blotting with R.DRAB (anti-HLA-DRαβ). The bands corresponding to DRα are indicated on the left. (D) Monocyte-derived DCs (top panels) and monocyte-depleted lymphocytes (middle panels) were lysed in 2% Brij 98, and the extracts were incubated sequentially with beads conjugated with 28-8-6s (C1), a negative control mAb and with L243. The DR-associated CD1d was eluted with 1% C12E9, ethanol precipitated, separated by SDS–PAGE and detected by western blotting with biotinylated D5 and HRP-conjugated streptavidin (lanes 1 and 2). To identify free CD1d, the supernatants from the initial L243 immunoprecipitations (the total for DCs, 1/20 of the total for monocyte-depleted lymphocytes) were re-immunoprecipitated with either a negative control mAb, 28-14-8s (C2) or 51.1.3 and detected as above (lanes 3 and 4). As a positive control, C1R CD1d cells (bottom panels) were subjected to the same procedure. The bands corresponding to CD1d heavy chain are indicated on the left.
CD1d associates with the class II–invariant chain complex

MHC class II molecules are synthesized in the ER where they associate with trimers of the invariant chain (I) to form a nonamer complex (αβ)3I3. During Golgi transport, the class II–associated invariant chain acquires O-linked glycans that increase its apparent Mr from 33 to 35 kDa (Machamer and Cresswell, 1984). After sorting into the endocytic pathway, the invariant chain is degraded, leaving the CLIP (class II–associated invariant chain peptide) fragment in the peptide-binding groove. In Figure 1A, p33, seen at the 0 and 30 min time point, disappeared by 2 h. At the same time, the p35 band became much more intense before fading at the later time points. CD1d could also be co-precipitated with the mAb DA6.147, which preferentially recognizes the HLA-DR–invariant chain complex (Figures 2A and 5B and C). These considerations suggested that p33 might be immature invariant chain and that the increased p35 signal at 2 h might be due to superimposition of O-glycosylated invariant chain on the DRα chain band. To examine this, anti-CD1d immunoprecipitates from radiolabeled C1R.CD1d cells extracted in Brij 98 were disrupted with SDS/DTT. Invariant chain was immunoprecipitated successfully from the eluates with PIN1.1, which is specific for the cytoplasmic tail (Figure 3A, lane 2). The same result was obtained with .221.CD1d (Figure 5C, upper panels, lane 7). To confirm that CD1d–invariant chain association occurs in the ER, C1R.CD1d cells were pulse-labeled with [35S]methionine for 15 min and chased for up to 2 h. After lysis in Brij 98, anti-CD1d immunoprecipitates were disrupted with SDS/DTT and the CD1d-associated invariant chain precipitated with PIN1.1 and subjected to endoglycosidase H (Endo H) digestion. CD1d-associated invariant chain initially was Endo H-sensitive but became resistant (Figure 3B), consistent with the initiation of the CD1d–invariant chain interaction in the ER and maintenance during transport through the Golgi apparatus.

The data suggest that CD1d associates with the class II–invariant chain complex in the ER and moves with it through the Golgi and into the endocytic pathway, remaining associated with the class II αβ dimer after invariant chain degradation. An alternative, perhaps less likely, hypothesis is that CD1d associates with free invariant chain in the ER, moves with it through the Golgi into the endocytic pathway, and only associates with class II molecules after invariant chain degradation (see Discussion).

CD1d is associated with mature class II αβ dimers in MIICs and on the plasma membrane

Class II αβ dimers are located in the MIICs/lysosomes and on the cell surface. To determine whether the CD1d–class II association exists at both sites, we performed subcellular fractionation experiments. C1R.CD1d cells were disrupted using a ball-bearing homogenizer and the MIICs/lysosomes were isolated by Percoll density gradient fractionation. Western blotting of the fractions showed that this procedure successfully separated light organelles such as the ER and plasma membrane (low density fractions, left) from heavy organelles such as lysosomes (including MIICs; high density fractions, right) (Figure 4A). MIICs were detected using a rabbit anti-HLA-DM serum (fractions 13 and 14) whereas ER-containing fractions were detected using MaP.ERp57, an mAb recognizing the ER resident protein, ERp57 (fractions 1–4), ER and plasma membrane are not separated by this procedure. Class II molecules were located in the top and bottom fractions, corresponding to plasma membrane and MIICs, respectively (Figure 4A). CD1d was distributed similarly, although a greater proportion was in the top fractions (Figure 4A, top panel). This almost certainly represents CD1d in the plasma membrane rather than in the ER because the band is diffuse, indicating extensive glycan modification.

To determine whether the CD1d–class II complexes are present in MIICs and/or in the plasma membrane, each gradient fraction was solubilized with Brij 98, immunoprecipitated with L243, and co-precipitated CD1d heavy chains detected by immunoblotting. CD1d was class II–associated in fractions corresponding both to MIICs and the plasma membrane (Figure 4B). Since L243 reacts with mature DRαβ dimers but not DR–invariant chain complexes, CD1d in the light fractions (1–4) cannot be associated with class II molecules in the ER. Association on the cell surface was confirmed further by biotinylation of surface proteins. After labeling with [35S]methionine and chasing for 0.5 or 5 h to allow class II and CD1d maturation, the cell surface proteins of C1R.CD1d were biotinylated with a membrane-impermeable biotinylating agent, sulfo-NHS-SS-biotin. The cells were extracted in...
Brij 98, and CD1d or DR molecules were immunoprecipitated with 51.1.3 or L243, respectively. The immune complexes were dissociated by boiling in SDS and the biotinylated molecules re-precipitated with streptavidin-agarose and analyzed by SDS–PAGE. The results shown in Figure 4C clearly demonstrate the presence of surface CD1d-associated class II molecules (left panel, lane 2) and class II-associated CD1d (right panel, lane 4).

**Class II molecules mediate lysosomal delivery of CD1d**

Based on the above data, we hypothesized that class II–invariant chain complexes might drive associated CD1d molecules into MHCII independently of the CD1d endocytic motif. To examine this question, a truncated form, CD1dt, lacking the six C-terminal cytoplasmic amino acids encompassing the YXXZ sequence, was constructed and expressed in C1R cells. C1R clones expressing comparable amounts of mutant and wild-type CD1d were selected for analysis (Figure 5A). To determine whether CD1dt could associate with class II molecules, the transfectants were labeled with [35S]methionine, and DR molecules were precipitated from Brij 98 extracts using the mAb W6/32 (lanes 1 and 4), L243 (lane 2), DA6.147 (lane 3) or 51.1.3 (lanes 5 and 7). SDS/DTT-eluted material was re-immunoprecipitated with D5 (lanes 4 and 5) or PIN.1 (lanes 6 and 7) and analyzed by 12% SDS–PAGE. The bands corresponding to CD1d, DRα and invariant chain, are marked on the right of the gel.
sufficient for detectable association (Figure 5C, lower panels).

To determine whether CD1dt can be delivered to MIICs in the presence of class II and invariant chain, we examined its intracellular distribution by immunofluorescence microscopy in HeLa cells expressing various elements of the class II–invariant chain complex. As a marker for MIICs and lysosomes, we used CD63 (Metzelaar et al., 1991). In normal HeLa cells, wild-type CD1d was present both on the cell surface and in perinuclear structures (Figure 6A, a–c). The perinuclear structures also contained CD63, confirming that they are lysosomes. CD1dt was

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**Fig. 6.** Role for class II molecules in the intracellular localization of CD1d. (A) Double-label immunofluorescence micrographs of HeLa.CD1d and HeLa.CD1dt cells. Cells were fixed, permeabilized and co-immunostained for CD1d (a) or CD1dt (d) and CD63 (b and e). Merged images (red, CD1d or CD1dt; green, CD63) are shown in (c) and (f). (B) Lysosomal sorting of CD1dt in the presence of class II–invariant chain complexes. Deconvoluted immunofluorescence images of HeLa.I.CD1dt.ab cells (I+/class II DR+). Cells were fixed, permeabilized and co-immunostained for CD1dt (a) and CD63 (b), CD1dt (d) and class II (DR1) (c), and CD63 (g) and transferrin receptor (h). Merged images [CD1dt, red; class II and transferrin receptor, green; CD63, green in (c), red in (i)] are shown in (c), (f) and (i). Conventional immunofluorescence images are shown in the insets.
present mostly at the cell surface, with very little in internal structures (Figure 6A, d–f). To determine whether CD1dt can be sorted to MIICs by the associated class II–invariant chain complex, we expressed it in HeLa.I cells, which express HLA-DR and the p33 form of human invariant chain (see Table I). Vesicular co-localization of CD1dt and CD63 was almost perfect (Figure 6B, a–c), as was co-localization with intracellular class II molecules (Figure 6B, d–f). Transferrin receptor, an early endosomal marker, does not co-localize with CD63 (Figure 6B, g–i). A similar lysosomal distribution of CD1dt was observed in HeLa.CD1dt cells treated with IFN-γ, which induces the expression of class II, DM and invariant chain genes, among others (Supplementary data, figure 1).

Recent findings have indicated that mouse CD1d associates with invariant chain and that this affects the intracellular distribution of CD1d (Jayawardena-Wolf et al., 2001). We examined this possibility for human CD1d by expressing CD1dt in HeLa.I cells, positive for the p33 form of human invariant chain. When CD1dt was co-expressed with invariant chain alone, significant co-localization with CD63 was observed (Figure 7a–c). CD1dt also co-localized with invariant chain in perinuclear vesicles (Figure 7d–f). The transferrin receptor did not co-localize with CD63 (Figure 7g–i). The findings argue that, in the presence of the class II–invariant chain complex, intact CD1d molecules might gain access to lysosomes directly from the TGN rather than by endocytosis from the plasma membrane.

**Discussion**

The data presented show that human CD1d associates with MHC class II molecules both in B-lymphoblastoid cell lines and in normal DCs and CD1d-positive lymphocytes, probably B cells. Association with known tetraspanins appears not to be responsible. CD1d binds to MHC class II–invariant chain complexes in the ER, and the CD1d–class II association is maintained in late endosomal/lysosomal MIICs as well as on the plasma membrane. The tyrosine-based endocytic signal in the cytoplasmic domain is not required for CD1d transport to MIICs in B-lymphoblastoid cell lines, nor is it required in HeLa cells expressing class II and invariant chain. The results suggest that CD1d can be sorted to MIICs by association with class II–invariant chain complexes.

The precise role of the invariant chain in the sorting of CD1dt is unclear. One can imagine three ways in which it could affect the distribution of CD1dt. First, invariant chain trimers could associate directly with CD1dt in the ER and transport them to the endocytic pathway. Immunofluorescence studies indicate that the CD1dt mutant sorts to lysosomes in HeLa cells expressing the p33 form of the invariant chain (Figure 7), consistent with

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**Fig. 7.** The p33 form of the invariant chain can deliver CD1dt to MIICs. Deconvoluted immunofluorescence images of HeLa.I.CD1dt cells (I+/class II–). Cells were fixed, permeabilized and co-immunostained for CD1dt (a) and CD63 (b), CD1dt (d) and invariant chain (e), and CD63 (g) and transferrin receptor (h). Merged images [CD1dt, red; invariant chain and transferrin receptor, green; CD63, green in (c), red in (i)] are shown in (c), (f) and (i). Conventional immunofluorescence images are shown in the insets.
this view. Similar results were obtained with mouse CD1d (Jayawardena-Wolf et al., 2001). However, we have not been able to convincingly show the interaction biochemically in the absence of class II molecules (data not shown), whereas we can show interaction of class II α- and β-chains and invariant chain with CD1d in the ER of C1R.CD1d cells (Figures 1–3). In other experiments, we were unable to show localization of CD1d in MIICs of the class II-negative, invariant chain-positive B-lymphoblastoid cell line .174, while co-expression of DR did induce MIIC localization (Supplementary data, figure 2). A possible explanation for this discrepancy is that in normal cells, a fraction of the invariant chain expressed has an ER retention motif as a result of alternative initiation of translation (Strubin et al., 1986). This also applies to the alternatively spliced form of the invariant chain, p41, and four forms generally are produced (p33, p35, p41 and p43). Because of the formation of mixed trimers which include the ER-retained forms, release of invariant chain from the ER is significantly impaired in normal cells (Lamb and Cresswell, 1992; Arunachalam et al., 1994). Thus, a direct interaction of CD1d with free invariant chain trimers in human cells may be less important than in mouse cells, which lack the ER-retained variants.

A second potential mechanism may involve the association of CD1d with invariant chain trimers which are also bound to one or two class II dimers. The formation of complete nonamers, (αβ)3I3, is thought to be essential for transport from the ER (Anderson and Cresswell, 1994), but this idea is compatible with the data presented here. Thirdly, complete class II–invariant chain nonamers could associate with CD1d via the class II molecules, in an interaction perhaps cooperatively involving the invariant chain. This hypothesis has the satisfying aspect that it does not require one to postulate the transfer of the CD1d molecules to class II dimers in the MIIC after invariant chain degradation.

Wild-type CD1d molecules accumulate in lysosomes independently of the expression of class II molecules and invariant chain. Thus, a key question is whether the class II-dependent targeting mechanism is biologically significant. The CD1d–class II interaction is clearly observed in normal antigen-presenting cells (Figure 2D). Approximately 5–10% of the total CD1d is class II-associated in the C1R.CD1d cell line (data not shown), and it appears that a similar level of interaction is detectable in DCs (Figure 2D). Thus class II-associated and free CD1d molecules appear to co-exist. A rationalization for the observed interaction is that the route of access to the endocytic pathway may be different for the two populations of CD1d molecules, which may have important immunological consequences.

Free CD1d molecules probably traffic directly to the cell surface from the TGN and subsequently are internalized into late endosomes/lysosomes via early endosomes. CD1d molecules associated with class II–invariant chain complexes are likely to be segregated from the constitutive pathway at the TGN and directly deposited into the endocytic pathway without access to the plasma membrane or early endosomes. Degradation of the invariant chain in MIICs would release class II–CD1d complexes that are then transported to the cell surface. If CD1d molecules are capable of binding lipids in any vesicular environment, the two populations could have a different profile of associated lipids. CD1a molecules successfully bind lipids, even though they only have access to the plasma membrane and early endosomes. CD1d molecules internalized from the plasma membrane would have access to the same pool of lipids as CD1a before encountering the lipids available deeper in the endocytic pathway, whereas class II-associated CD1d molecules would not.

Class II molecules in multivesicular MIICs are found predominantly on the membranes of the internal vesicles, whereas other membrane proteins such as LAMP-1, LAMP-2 and HLA-DM are found on the limiting membrane (Escola et al., 1998). CD1d molecules associated with class II might be targeted similarly to the internal vesicles, while free CD1d molecules might not. There is no obvious reason why this should result in access to a different lipid pool, but the subsequent route of access to the plasma membrane of the two CD1d subsets could be different. Class II-associated CD1d molecules could also be incorporated into the ‘exosomes’ released from antigen-presenting cells by fusion of multivesicular MIICs with the plasma membrane. Exosomes have been shown to be capable of sensitizing class II-restricted CD4+ T cells (Raposo et al., 1996).

CD1d could also affect the maturation or intracellular trafficking of associated class II molecules. The rate of ER egress of class II molecules was not altered noticeably by CD1d as determined by the rate of acquisition of Endo H resistance by DRβ chains, nor was the rate of invariant chain proteolysis affected (Figure 1A; our unpublished observations). We also found no change in the surface expression of class II–CLIP complexes in the presence of CD1d (our unpublished observations), although effects on class II peptide loading could be masked by the excess of class II molecules which are not CD1d-associated. Finally, class II molecules associated with CD1d on the cell surface could function as co-receptors for CD4 molecules expressed on CD1d-restricted CD4+ T cells, thus enhancing their sensitivity. The answers to these questions await further experimentation.

Materials and methods

Cell lines

The B-LCL C1R and .221 lines, and their CD1d transfectants, were maintained in Iscove’s medium (IMDM; Gibco-BRL, Gaithersburg, MD) containing 5% bovine calf serum (BCS) or 10% fetal bovine serum (FBS) at 37°C. CD1d-transduced, invariant chain-expressing, DR-expressing and normal HeLa cells were maintained in IMDM containing 10% FBS. HeLa.1 cells were a gift from Dr M.Marks (Marks et al., 1995).

Antibodies

The mouse mAbs to human CD1d, 51.1.3 (Exley et al., 1997, 2000; Kim et al., 1999) and D5 (Kim et al., 1999; Rodionov et al., 1999; Exley et al., 2000) were gifts from Dr S.Porcelli (Albert Einstein College of Medicine) and Dr S.Balk (Harvard Medical School), respectively. The mAbs L243 (anti-HLA-DRβ1; Lampson and Levy, 1980; Blum and Cresswell, 1988), DA6.147 (anti-HLA-DRα chain; Guy et al., 1982), HB10A (anti-HLA-DRβ chain; Clark and Yakoski, 1984), R.DRAB (anti-HLA-DRβ; Marks et al., 1990), GAP.A3 (anti-HLA-A3; Berger et al., 1982), PIN1.1 (anti-I chain N-terminus; Roche et al., 1991), MaP.ERG57 (anti-ERG57; Diedrich et al., 2001), MaP.CD82 (anti-CD82; Hammond et al., 1998) and H5C6 (anti-CD68; Metzel et al., 1991) have been described previously. The mAbs, 28-8-6S (anti-H-2Kb and H-2Db) and 28-14-8S (anti-H-2Dα and H-2Lα) were provided by Dr J.Frelinger (University of
North Carolina, Chapel Hill, NC). Rabbit anti-recombinant HLA-DM sera, DM323 and K589 (Sloan et al., 1995; Denzin et al., 1996) were provided by Drs H.Zweerink (Merck Research Laboratories, Rahway, NJ) and L.Karlsson (The R.W.Johnson Pharmaceutical Research Institute, San Diego, CA), respectively. The mAbs J5-81 (anti-CD81), fluorescein isothiocyanate (FITC)-conjugated anti-CD95 (transferrin receptor) and FITC-conjugated anti-CD63 were from BD Pharmingen (San Diego, CA). FITC- or Texas Red (TR)-conjugated secondary antibodies were from Vector Laboratories Inc. (Burlingame, CA).

**Construction of CD1d wild-type and deletion mutant expression vectors**

The vector CD1d/Srstr-neo was a gift of Dr S.Porcelli. PCR was used with various primers to construct from this cDNA encoding FLAG-tagged full-length CD1d, secreted soluble CD1d and the CD1d deletion mutant, CD1dt. pCDM8-DRA and pCDM8-DRB1*0101, which express DR1 and \( \beta \) chains, respectively, were a generous gift of Drs M.Marks and E.Long (Long et al., 1994; Marks et al., 1995).

**Generation of stable transfected cell lines**

C1R and .221 cells were transfected by electroporation. They were screened for CD1d expression by flow cytometry. Expression vectors encoding DR\( \alpha \) and DR\( \beta \) were co-transfected into HeLa.l.CD1d and HeLa.l.CD1dt cells (see Table I) using Lipofectamine 2000 (Gibco-BRL), selected by 750 ng/ml purycin and sorted for CD1d expression.

**Generation of monocyte-derived dendritic cells**

DCs were derived from monocytes as described (Sallusto and Lanzavecchia, 1994). Briefly, monocytes from peripheral blood mono-nuclear cells (8 \( \times \) 10\(^6\) cells) (AllCells, LLC, Berkeley, CA) were attached to tissue culture flasks in 20% FBS/RPMI 1640 twice and incubated for 7 days with 800 U/ml GM-CSF (Research & Diagnostic Systems Inc., Minneapolis, MN) and 1000 U/ml IL-4 (R&D systems) in 10% FBS/RPMI 1640.

**Generation of stable cell lines transfused by retrovirus**

HeLa cells were retrovirally transfused following described protocols (http://www.stanford.edu/group/nolan/phx_helper_free.html).

**Metabolic labeling and immunoprecipitation**

Labeling with \(^{35}\)S)methionine and cysteine (ICN, Costa Mesa, CA), immunoprecipitations and Endo H digestions were performed as previously described (Denzin et al., 1996). For re-immunoprecipitation experiments, washed primary immunoprecipitates were boiled for 5 min in 100 \( \mu \)l of 1% SDS/5 mM DTT/Tris-buffered saline (TBS) before diluting with 1 ml of 1% Triton X-100/10 mM IAA/TBS. The beads were centrifuged and the supernatants were used for second immunoprecipitations. The samples were boiled with Laemmli SDS sample buffer and separated by 12% SDS-PAGE prior to autoradiography.

**Purification of CD1d-associated proteins from C1R.CD1d**

C1R.CD1d cells (6 \( \times \) 10\(^5\)) were lysed in 200 ml of 2% Brij 98/Tris/phenylmethylsulfonyl fluoride (PMSF)/IAA pH 7.4 for 1 h on ice. After removing nuclei and debris by centrifugation, the extracts were passed through columns of mouse IgG-conjugated BioGel A15m beads and 51.1.3-conjugated BioGel A15m beads sequentially at 4°C. After washing with 0.1% Brij 98/Tris pH 7.4, CD1d-associated proteins were eluted with 1% C\(_2\)H\(_5\)/TBS and ethanol precipitated at -70°C. The proteins were separated by SDS-PAGE, and detected using Coomassie Blue.

**Mass spectrometry analysis**

Mass spectrometry analysis was performed by Yale Cancer Center Mass Spectrometry Resource & HHMI Biopolymer Laboratory/W.M.Keck Foundation Biotechnology Resource Laboratory. In brief, the samples were digested in situ with trypsin, subjected to nanospray MS–MS analysis on a Q-TOF mass spectrometer. The proteins matching with the MS–MS spectra were searched using the SEQUEST search program.

**Isolation of CD1d-class II complexes for tetraspanin analysis**

A total of 1 \( \times \) 10\(^6\) .221.TMD1d.f cells were lysed in 30 ml of 2% Brij 98/TBS/PMSF/IAA for 1 h on ice. The nuclei and debris were removed by centrifugation. The extract was passed first through a mouse IgG-conjugated agarose column (Sigma) and then through an anti-FLAG mAb (M2)-conjugated bead column (Sigma). After washing with 0.1% Brij 98/TBS, the CD1d-class II complexes were eluted by incubation with 200 \( \mu \)g/ml FLAG peptide/0.1% Brij 98/TBS. FLAG peptide was removed by repetitive filtering using an Ultrafree-15 Centrifugal Filter device (Millipore, Bedford, MA).

**Purification of DR-associated CD1d from normal cells**

Monocyte-derived DCs (45 \( \times \) 10\(^6\) cells) and monocyte-depleted lymphocytes (6.8 \( \times \) 10\(^6\) cells) were lysed in 2.5 and 14 ml of 2% Brij 98/TBS/PMSF/5% FCS, respectively. After centrifugation the extracts were incubated with mouse IgG-conjugated beads and then with 28-8-6S, a negative control mAb, conjugated to BioGel A15m beads, for 3 h at 4°C. The supernatant was then incubated with L243-conjugated A15m beads. After washing with 0.1% Brij 98/TBS, the beads were incubated in 1% C\(_2\)H\(_5\)/TBS at 4°C overnight to elute associated CD1d which was ethanol precipitated. To detect free CD1d, the supernatants from the initial L243 immunoprecipitations were re-immunoprecipitated with a negative control mAb, 28-14-8S, or 51.1.3 and protein G-Sepharose. As a positive control, C1.R.CD1d cells were subjected to the same procedure except that the Brij 98 lysates were used directly for immunoprecipitation. To detect CD1d on SDS-PAGE, D5 mAb, purified using protein A-Sepharose, was conjugated with sulfo-NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) according to the manufacturer’s instructions and used for western blotting with HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and enhanced chemiluminescence (Pierce).

**Subcellular fractionation**

Subcellular fractionation by Percoll density gradients was performed as previously described (Hammond et al., 1998). Fourteen fractions (785 ml) were collected and 350 ml from each was mixed with Brij 98 (final concentration, 1%)/TBS containing PMSF and IAA, incubated for 1 h on ice and immunoprecipitated with L243. The precipitates were separated by non-reducing SDS–PAGE and electrophoretically transferred to an Immobilon P membrane (Millipore). CD1d heavy chains were detected with the mAb D5 followed by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and visualized with enhanced chemiluminescence (Pierce). A 20 ml aliquot of each fraction was mixed with 10\(^\times\) non-reducing sample buffer, separated by 12% SDS–PAGE, and CD1d, HLA-DR, HLA-DM and ERp57 were detected by western blotting.

**Cell surface biotinylation**

Cells were metabolically labeled for 15 min and chased for 30 min or 5 h. They were washed twice with phosphate-buffered saline (PBS) containing 0.1 mM CaCl\(_2\) and 1 mM MgCl\(_2\) and incubated in PBS with 2 mM sulfo-NHS-SS-biotin (Pierce) at 4°C for 30 min. After washing, the cells were extracted with 1% Brij 98 and immunoprecipitated with either 51.1.3 or L243. After boiling in 1% SDS for 5 min, the eluates were diluted with 1% Triton X-100/TBS prior to re-precipitation with streptavidin–agarose (Sigma) and SDS–PAGE.

**Flow cytometric analysis**

Cells (0.5 \( \times \) 10\(^6\)) were stained as described previously (Denzin et al., 1994) and analyzed using a Becton Dickinson FACScan (Mountain View, CA).

**Immunofluorescence microscopy**

Immunofluorescence was performed based on methods previously described (Hammond et al., 1998). HeLa cells, CD1d-expressing HeLa cells and HeLa transfecants were allowed to adhere to sterile coverslips. Cells were fixed prior to staining in 3.7% formaldehyde, permeabilized with permeabilization buffer (PB: PBS containing 10% FBS, 0.1% saponin, 10 mM glycine, 0.02% sodium azide). For co-staining of CD1d and CD63 in Figure 6A, samples were labeled sequentially with 51.1.3 followed by TR-conjugated secondary antibody and then with FITC-conjugated anti-CD63 antibody. In Figures 6B and 7, samples were co-stained with 51.1.3 conjugated with Alexa594 (Molecular Probes Inc., San Diego, CA) and examined using a Zeiss Axioplan2 microscope (63\(^\times\)). Images were collected by Openlab (Improvision, Coventry, UK) at the indicated wavelengths. Stacks of optical sections were obtained, deconvoluted and examined as single sections.
Supplementary data

Supplementary data for this paper are available at The EMBO Journal Online.

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References


Exley, M. et al. (2000) CD1d structure and regulation on human thymocytes, peripheral blood T cells, B cells and monocytes. Immunology, 100, 37–47.


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