Immunity, Vol. 11, 743–752, December, 1999, Copyright ©1999 by Cell Press

Separate Pathways for Antigen Presentation by CD1 Molecules

Masahiko Sugita,* Ethan P. Grant,*
Elly van Donselaar,† Victor W. Hsu,* Rick A. Rogers,‡
Peter J. Peters,† and Michael B. Brenner*§
*Division of Rheumatology, Immunology, and Allergy
Brigham and Women's Hospital
Harvard Medical School
Boston, Massachusetts 02115
†The Netherlands Cancer Institute
Plesmanlaan 121-H4
1066 CX Amsterdam
The Netherlands
‡Department of Environmental Health
Harvard School of Public Health
Boston, Massachusetts 02115

Summary

The ability to sample relevant intracellular compartments is necessary for effective antigen presentation. To detect peptide antigens, MHC class I and II molecules differentially sample cytosolic and endosomal compartments. CD1 constitutes another lineage of lipid antigen-presenting molecules. We show that CD1b traffics deeply into late endosomal compartments, while CD1a is excluded from these compartments and instead traffics independently in the recycling pathway of the early endocytic system. Further, CD1b but not CD1a antigen presentation is dependent upon vesicular acidification. Since lipids and various bacteria are known to traffic differentially, either penetrating deeply into the endocytic system or following the route of recycling endosomes, these findings elucidate efficient monitoring of distinct components of the endocytic compartment by CD1 lipid antigen-presenting molecules.

Introduction

In order to initiate specific T lymphocyte–mediated immune responses against viral and microbial infection, accurate information of invading pathogens must be conveyed to the host immune system. Presenting foreign molecular components of the pathogen or their fragments to T cells is a pivotal step in initiating the adaptive immune response. The immune system has evolved at least three major types of molecules capable of presenting antigens to T cells; two types (class I and class II) encoded by the major histocompatibility complex (MHC) and one type encoded by the CD1 complex located outside of the MHC region. These antigen-presenting molecules present peptide and lipid antigens, respectively, and thus appear to mediate distinct but complementary pathways of host defense against infection (Germain and Margulies, 1993; Porcelli and Modlin, 1999).

§To whom correspondence should be addressed (e-mail: mbrenner@rics.bwh.harvard.edu).

Given the variety of pathways for entry of pathogens into host cells and for intracellular delivery of their products, each of these antigen-presenting molecules must be elaborately directed for sampling the intracellular compartments that may contain relevant antigens. In the endogenous pathway of protein antigen presentation, cytosolic peptides, such as those derived from viral proteins synthesized in virus-infected cells, are transported into the endoplasmic reticulum (ER) through an ER membrane–associated peptide transporter encoded by the *TAP* genes. The peptides are then sampled in the ER by MHC class I molecules and presented to $CD8^+$ cytotoxic T cells capable of eliminating the virus-infected cells (Heemels and Ploegh, 1995). In contrast, MHC class II molecules traffic to late endosomal compartments for sampling internalized exogenously derived peptides and mediate activation of $CD4^+$ helper T cells to produce immunoregulatory cytokines and to induce specific humoral responses (Cresswell, 1994). Thus, MHC class I and class II molecules coordinately orchestrate immune responses against the peptide antigens of pathogens by surveying distinct intracellular compartments.

The CD1 family of β2-microglobulin-associated, nonpolymorphic glycoproteins mediate an MHC-independent presentation of lipid and glycolipid antigens (Beckman et al., 1994; Sieling et al., 1995; Kawano et al., 1997; Moody et al., 1997; Rosat et al., 1999) as well as glycosylphosphatidylinositol (GPI)-anchored proteins (Schofield et al., 1999) to a variety of T cells that function against infection with pathogens. Mycobacteria-infected cells are detected and killed by group 1 CD1 (CD1a, CD1b, and CD1c)-restricted T cells that recognize lipid and glycolipid antigens derived from phagocytosed intracellular mycobacteria (Stenger et al., 1997), and a direct pathway for elimination of released microbes has been proposed through granulysin produced by these cells (Stenger et al., 1998). Group 2 CD1 (CD1d)-restricted immune responses have been shown to control humoral immunity against parasites (Schofield et al., 1999). Most of these CD1 isoforms appear to monitor infection with pathogens by sampling antigens in endosomal compartments. They contain a cytoplasmic tail tyrosine-based sequence that directs their trafficking to endosomes to which some CD1-restricted lipid antigens are known to be delivered (Sugita et al., 1996; Prigozy et al., 1997; Brossay et al., 1998; Jackman et al., 1998; Chiu et al., 1999; Rodionov et al., 1999). They also require endosomal acidification in order to present lipid antigens to T cells (Porcelli et al., 1992; Kawano et al., 1997).

CD1a molecules are prominently expressed on some types of professional antigen-presenting cells (APCs), such as epidermal Langerhans cells (Caux et al., 1992), bronchial dendritic cells (Tazi et al., 1993), and monocyte-derived dendritic cells (Porcelli et al., 1992). CD1a-restricted T cells express T cell receptor (TCR) α and β chains and CD8 α and β chains and recognize and kill microbe infected dendritic cells (Stenger et al., 1998; Rosat et al., 1999). However, CD1a is unique compared

to all the other CD1 isoforms, as it lacks the tyrosine-based cytoplasmic tail motif (Calabi and Milstein, 1986) that has been demonstrated to mediate endosomal localization of other CD1 isoforms (Sugita et al., 1996; Prigozy et al., 1997; Brossay et al., 1998; Jackman et al., 1998; Chiu et al., 1999; Rodionov et al., 1999). Thus, we hypothesized that CD1a might survey distinct intracellular compartments and mediate an antigen presentation pathway fundamentally separate from that mediated by the other CD1 and MHC molecules.

In the present study, we demonstrate that unlike CD1b, which requires endosomal acidification for its efficient binding and presentation of mycobacterial lipid antigens, CD1a-mediated antigen presentation was independent of endosomal acidification. Consistent with this functional difference, immunofluorescence microscopic studies as well as electron microscopic analysis revealed anatomical separation of intracellular CD1a and CD1b molecules. Whereas CD1b was found in late endosomal and lysosomal vesicles that expressed lysosome-associated membrane protein-1 (LAMP-1), CD1a-containing intracellular vesicles were completely negative for this lysosomal marker protein. Furthermore, these CD1a-containing vesicles were identified as part of the recycling vesicle compartment of the early endocytic system, since accumulation of the T27N mutant of ARF6, a small GTPase that regulates transport of recycling vesicles to the cell surface (Peters et al., 1995a; D'Souza-Schorey et al., 1998), was observed in CD1a-containing but not CD1b-containing vesicles. We propose that these differences between CD1a and CD1b allow the CD1 system to efficiently monitor phagocytosed mycobacteria by surveying distinct subcompartments of the endocytic system.

Results

Difference in Steady State Intracellular Localization between CD1a and the Other CD1 Isoforms

We and others showed previously that a tyrosine-based endosomal targeting sequence (YXXZ, where Y is tyrosine, X is any amino acid, and Z is a hydrophobic amino acid) that is present in the short cytoplasmic domain of most CD1 proteins is crucial for their localization to endosomes and for their presentation of endocytosed lipid antigens (Sugita et al., 1996; Brossay et al., 1998; Jackman et al., 1998; Chiu et al., 1999). CD1a is the only member of the human CD1 family that lacks this endosomal targeting motif (Calabi and Milstein, 1986; Balk et al., 1989), and yet mycobacterial lipid antigen-specific, CD1a-restricted T cells as well as CD1b- and CD1c-restricted T cells can reconize and kill mycobacteria-infected dendritic cells (Stenger et al., 1997, 1998; Rosat et al., 1999), implying that CD1a may encounter and bind antigens derived from endosome-resident mycobacteria in a site distinct from that for the other CD1 isoforms. Immunofluorescence microscopic analysis of HeLa cell transfectants expressing each of the CD1 isoforms revealed a striking difference in the localization of CD1a compared with the other CD1 molecules (Figure 1). CD1b prominently localized in peripherally distributed vesicles (Figure 1B) that we previously showed belonged to the late endocytic system (Sugita et al., 1996). CD1c and CD1d were also found in peripherally distributed vesicles (Figures 1C and 1D, respectively), albeit less prominently than CD1b. In contrast, prominent plasma membrane staining was observed for CD1a, and the peripherally distributed vesicles seen for the other CD1 molecules were not apparent (Figure 1A). To further detect intracellular staining, either CD1a-transfected or CD1b-transfected HeLa cells were double-labeled with antibodies to the relevant CD1 isoforms and LAMP-1, a marker of late endosomes and lysosomes, and viewed by confocal microscopy (Figure 2). CD1b was found in numerous intracellular vesicles (green vesicles in Figure 2D), and a nearly identical vesicular distribution was observed for endogenous LAMP-1 expression (red vesicles in Figure 2E). The CD1b- and LAMP-1-containing vesicles were almost entirely superimposable (yellow vesicles in Figure 2F), confirming the prominent localization of CD1b in late endosomal and lysosomal compartments. In contrast, there was virtually no colocalization of CD1a and LAMP-1 (Figure 2C). Instead, a cluster of CD1a-positive, LAMP-1-negative intracellular vesicles was noted prominently in a juxtanuclear position (Figure 2A, shown with arrowheads). This striking difference in intracellular steady state localization between CD1a and CD1b suggested that these molecules might mediate distinct pathways for antigen presentation.

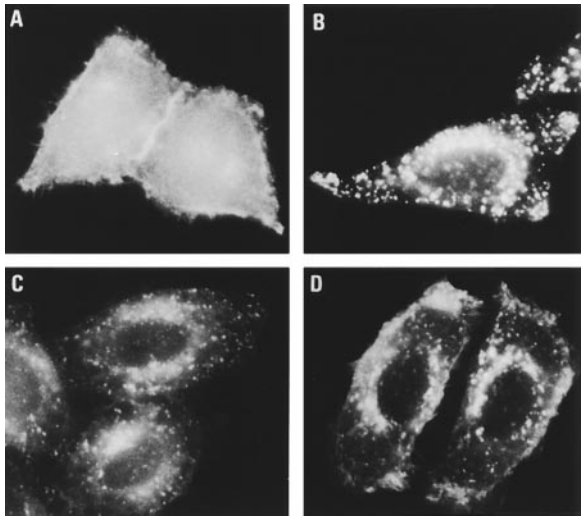

Figure 1. Intracellular Localization of CD1a Was Distinct from that of the Other CD1 Isoforms

HeLa cell transfectants expressing either CD1a (A), CD1b (B), CD1c (C), or CD1d (D) were fixed with 2% formaldehyde and permeabilized with 0.2% saponin. The cells were then labeled with mouse monoclonal antibodies to the relevant CD1 isoforms followed by incubation with fluorescein isothiocyanate (FITC)-conjugated donkey antibody to mouse IgG. Magnification, 600×.

Differential Effect of Blockers of Endosomal Acidification on CD1a- and CD1b-Mediated Antigen Presentation

CD1b-restricted exogenous glycolipid antigens, such as lipoarabinomannan, are taken up by APCs and delivered to LAMP-1$^+$ late endosomal compartments, where they colocalize with CD1b (Prigozy et al., 1997). Thus, one

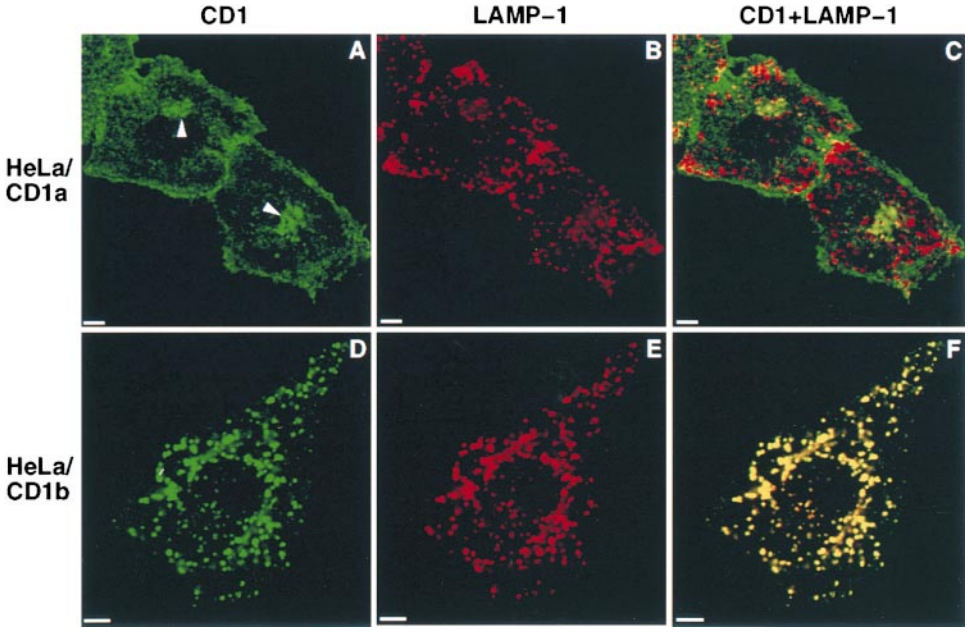

Figure 2. CD1a Was Almost Excluded from LAMP-1$^+$ Late Endosomes and Lysosomes

HeLa cell transfectants expressing either CD1a (upper panels) or CD1b (lower panels) were fixed and permeabilized. The cells were then double-labeled with mouse monoclonal antibodies to the relevant CD1 isoforms (detected with FITC-conjugated donkey anti-mouse IgG [green]) and a rabbit antiserum against human LAMP-1 (detected with Texas red–conjugated donkey anti-rabbit IgG [red]) and analyzed by cofocal microscopy. Fluorescent confocal images of CD1a$^+$ (upper panels) and CD1b$^+$ (lower panels) HeLa cells were obtained for CD1 expression (green, [A] and [D]) and endogenous LAMP-1 (red, [B] and [E]). The two images were then superimposed to detect any cellular compartments expressing both CD1 and LAMP-1 (yellow, [C] and [F]). Scale bars, 5 μm.

can hypothesize that CD1b encounters and binds lipid antigens in these acidic endosomal compartments. Consistent with this hypothesis, binding of lipid antigens to CD1b requires an acidic environment both in vitro and in vivo (Porcelli et al., 1992; Ernst et al., 1998). Since CD1a appears to be virtually excluded from these CD1b$^+$ highly acidic late endosomes and lysosomes (Figure 2), we reasoned that CD1a-mediated antigen presentation might not require endosomal acidification. Thus, we compared the effect of concanamycin B, which specifically inhibits the vacuolar proton ATPase in endocytic compartments (Benaroch et al., 1995; Sugita and Brenner, 1995), on antigen presentation mediated by CD1a and CD1b. In order to detect antigen presentation by these CD1 isoforms, we used TCR-deficient Jurkat cells (J.RT3) reconstituted with the specific TCR α and β chains derived from two mycobacterial lipid antigen-specific T cell lines. CD8-2/J.RT3 was obtained by transfection of J.RT3 cells with the TCR α and β chain cDNAs isolated from the CD8-2 CD1a-restricted T cell line, whereas LDN5/J.RT3 was obtained by transfection with the TCR α and β chain cDNAs isolated from the LDN5 CD1b-restricted T cell line (Grant et al., 1999). In both cases, reconstitution with the relevant pair of specific TCR α and β chains was sufficient to transfer both antigen specificity and CD1 restriction. Thus, dose-dependent, specific mycobacterial lipid antigen recognition by CD8-2/J.RT3 and LDN5/J.RT3 occurred only when JY transfectant cells expressing the correct CD1 isoform were used as APCs (Figure 3A). As expected, the CD1b-mediated antigen presentation to LDN5/J.RT3 was completely blocked by concanamycin B, emphasizing the requirement for endosomal acidification (Figure 3B, right). In contrast, little if any effect of this drug was detected, under identical conditions, on the CD1a-mediated antigen presentation to CD8-2/J.RT3 cells (Figure 3B, left).

Given that these distinctive features of CD1a were more comparable to those of MHC class I than class II, we initially hypothesized that CD1a, like MHC class I, might bind antigens in the ER. However, this did not appear to be the case, since CD1a-restricted recognition of lipid antigen by CD8-2/J.RT3 was not affected by treatment of APCs with brefeldin A (Figure 3C, right). The complete blocking effect of this drug on protein transport from pre-Golgi to post-Golgi compartments was confirmed by total blockade of lysozyme secretion (Figure 3C, left). Radiolabeled lysozyme was retained intracellularly in the presence of brefeldin A, whereas it was secreted into the medium in the absence of the drug. These results suggested that CD1a antigen loading would occur in a post-Golgi compartment. To examine the possibility that endocytosis might be required for CD1a antigen presentation in this in vitro model, APCs that were fixed with glutaraldehyde to block endocytosis were tested for their ability to present lipid antigen to T cells. Recognition of lipid antigen by LDN5/J.RT3 occurred when APCs were antigen pulsed and then fixed, but not when antigen pulsed after fixation, indicating that uptake of lipid antigen and its intracellular delivery were required for CD1b-mediated antigen presentation (Figure 3D, lower panel). Similarly, only a marginal recognition of lipid antigen by CD8-2/J.RT3 was detected when APCs were fixed and then pulsed with

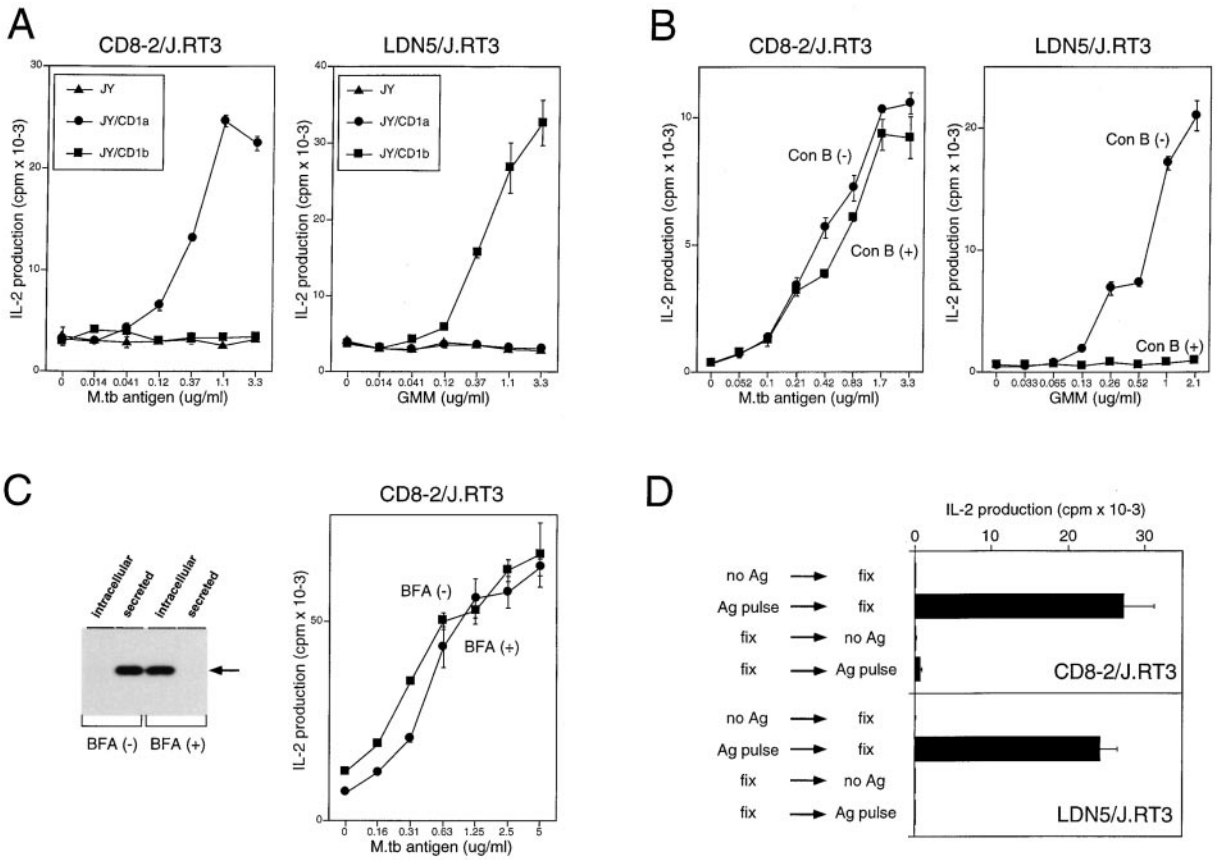

Figure 3. CD1a-Mediated Antigen Presentation Was Resistant to Concanamycin B and Brefeldin A

(A) Untransfected JY cells and JY cells stably transfected with either CD1a (JY/CD1a) or CD1b (JY/CD1b) were incubated overnight with indicated concentrations of either the semipurified *Mycobacterium tuberculosis* (M.tb) lipid antigen preparation (for CD8-2/J.RT3) (Rosat et al., 1999) or purified glucose monomycolate (GMM) (for LDN5/J.RT3) (Moody et al., 1997) and fixed with 0.08% glutaraldehyde. The TCR transfectant cells were cultured with these fixed APCs, and IL-2 production was measured as described in Experimental Procedures.

(B) JY/CD1a and JY/CD1b were preincubated with 10 nM concanamycin B (Con B) for 30 min and then washed and incubated overnight with indicated concentrations of specific antigen preparations either in the presence or absence of 10 nM concanamycin B. The cells were then washed, fixed, and incubated with CD8-2/J.RT3 (left) and LDN5/J.RT3 (right), respectively, and IL-2 release into the media was measured.

(C) HeLa cells transiently transfected with CD1a were preincubated with 10 μg/ml brefeldin A (BFA) for 30 min and then washed and incubated with indicated concentrations of the semipurified M.tb antigen preparation either in the presence or absence of 10 μg/ml brefeldin A. After 5.5 hr, cells were washed, fixed, and incubated with CD8-2/J.RT3, and IL-2 release was measured (right). To confirm the blocking effect of brefeldin A, HeLa cells transiently transfected with lysozyme cDNA were metabolically pulse-radiolabeled with [^{35}S]methionine for 5 min and then chased in regular media for 6 hr either in the presence or absence of 10 μg/ml brefeldin A. After the chase, secreted and intracellularly retained lysozyme were detected by immunoprecipitation with anti-lysozyme antibody from the supernatant and the cell lysate, respectively. The immunoprecipitated proteins were analyzed on a 12% SDS-PAGE gel. The position of lysozyme is indicated with an arrow.

(D) Peripheral blood monocytes stimulated with GM-CSF and IL-4 were incubated for 4 hr with specific antigen preparations (0.75 μg/ml of the semipurified M.tb lipid antigen preparation for CD8-2/J.RT3 or 1 μg/ml of GMM for LDN5/J.RT3) either before or after fixation with 0.1% glutaraldehyde. The cells were then washed and incubated with CD8-2/J.RT3 (upper panel) or LDN5/J.RT3 (lower panel), and IL-2 release into the media was measured.

antigen (Figure 3D, upper panel), suggesting that the cell surface was not the major site for CD1a antigen binding. In addition, the observation that phagocytosed intracellular mycobacteria were readily detected by the CD8-2 T cells (Stenger et al., 1997) led us to the hypothesis that CD1a might bind antigen at an intracellular site that belonged to the post-Golgi compartment.

Detection of CD1a$^+$ Vesicles in Professional APCs

To gain further insight into the intracellular trafficking of CD1a, we studied untransfected physiological APCs. Epidermal Langerhans cells are key professional APCs involved in skin immunity (Streilein et al., 1990) that express high levels of CD1a molecules but little CD1b (Caux et al., 1992). We employed immunogold-labeled transmission electron microscopy to analyze Langerhans cells for the localization of CD1a expression. CD1a was expressed abundantly on the plasma membrane (p) of Langerhans cells but not on the plasma membrane of adjacent keratinocytes (Kc), indicating the specificity of the labeling (Figure 4). In addition, prominent intracellular CD1a labeling was detected in small vesicular or tubular structures clustered mainly at a juxtanuclear position, some of which were identical in shape and in size to Birbeck granules (Kashihara et al., 1986) (Figure 4, arrows). Although the origin and the function of these Langerhans cell–specific vesicles is only partially understood, several lines of evidence suggest that Birbeck

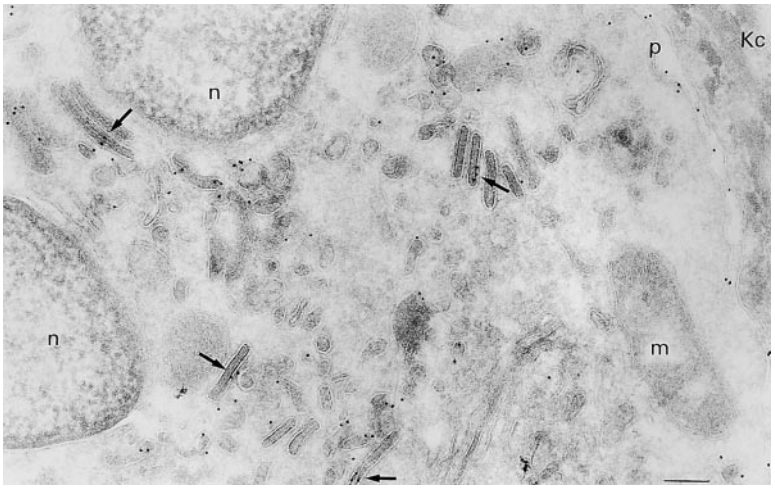

Figure 4. Immunoelectron Micrograph of Thin Cryosections of Human Epidermal Langerhans Cells In Situ

A skin specimen from punch biopsy was analyzed for CD1a expression. In epidermal Langerhans cells, CD1a was found in small intracellular vesicles, including typical Birbeck granules (shown with arrows), as well as on the plasma membrane (p). Abbreviations: m, mitochondria; n, nucleus. Scale bars, 200 nm.

granules are formed by invaginations of the plasma membrane and mediate an endocytic function (Hanau et al., 1987; Ray et al., 1989; Bartosik, 1992). Thus, we speculated that CD1a might be internalized from the cell surface and transported intracellularly in Langerhans cells via Birbeck granules.

In order to examine the possibility of CD1a internalization from the cell surface more directly, we obtained monocyte-derived CD1a$^+$ dendritic cells in vitro by stimulating human peripheral blood monocytes with granulocyte-macrophage colony–stimulating factor (GM-CSF) and interleukin-4 (IL-4) (Porcelli et al., 1992). Following immunogold labeling with an antibody to CD1a, transmission electron microscopy readily detected CD1a in plasma membrane–associated clathrin-coated pits (Figure 5, arrowheads) and vesicles (Figure 5, inset), indicating that delivery via clathrin-coated pits and vesicles was likely a prominent pathway for CD1a internalization. Consistent with the lack of CD1a expression in LAMP-1$^+$ endocytic compartments in HeLa cells (Figure 2), few CD1a molecules were detected in endosomal and lysosomal compartments of these dendritic cells (data not shown), including the MHC class II compartment or MIIC (Peters et al., 1991, 1995b) in which MHC class II and CD1b are proposed to bind peptide and lipid antigens, respectively (Sanderson et al., 1994; Tulp et al., 1994; Sugita et al., 1996; Prigozy et al., 1997). Interestingly, similar to epidermal Langerhans cells, we detected small intracellular vesicles that expressed CD1a molecules in these monocyte-derived dendritic cells (Figure 5, shown with arrows). These CD1a$^+$ vesicles lacked an electron-dense coat structure and thus were distinct from clathrin-coated vesicles. Given that CD1a was internalized in clathrin-coated pits from the cell surface and that CD1a was not significantly transported to late endosomes and lysosomes, we reasoned that these CD1a$^+$ vesicles might be recycling vesicles delivering internalized proteins back to the cell surface instead of transporting them more deeply into the endocytic system.

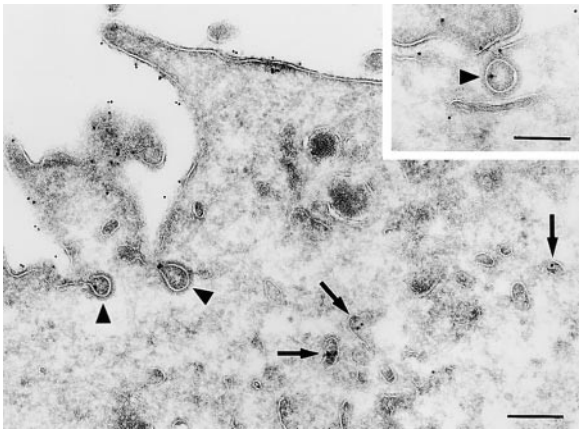

Figure 5. Immunoelectron Micrographs of Thin Cryosections of In Vitro–Derived Dendritic Cells

Peripheral blood monocytes stimulated with GM-CDF and IL-4 were analyzed for CD1a expression. CD1a was found in plasma membrane–associated clathrin-coated pits (shown with arrowheads) and clathrin-coated vesicles (inset, shown with an arrowhead) as well as in small noncoat vesicles (shown with arrows). Scale bars, 200 nm.

Expression of CD1a but not CD1b in Recycling Vesicles

Recycling vesicles that contain recycling proteins accumulate at a juxtanuclear position before trafficking to the cell surface and are collectively referred to as recycling endosomes (Daro et al., 1996). A specific marker for these recycling vesicles has not been established. However, we and others previously demonstrated that a small GTPase, ARF6, regulates transport of recycling vesicles from recycling endosomes to the cell surface and that overexpression of the T27N mutant of ARF6 (ARF6-T27N) perturbs the GTPase cycle, which results in its accumulation in recycling vesicles (D'Souza-Schorey et al., 1995, 1998; Peters et al., 1995a). Thus, we tested whether CD1a$^+$ intracellular vesicles could be labeled with ARF6-T27N. HeLa cells stably transfected with either CD1a or CD1b were transiently supertransfected with ARF6-T27N, double-labeled with antibodies to the relevant CD1 isoforms and ARF6, and analyzed by confocal microscopy. ARF6-T27N was detected in a cluster of vesicles at a juxtanuclear position as well as in some peripherally distributed vesicles (Figures 6B and 6E). A similar vesicular distribution was

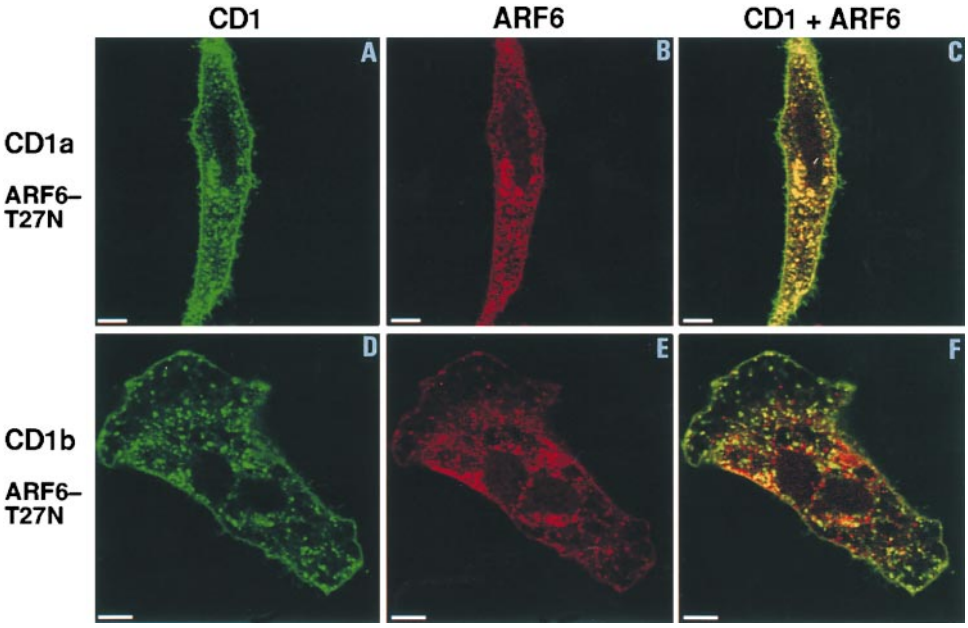

Figure 6. CD1a-Containing Intracellular Vesicles but Not CD1b-Containing Vesicles Were Labeled with ARF6/T27N

HeLa cell transfectants expressing either CD1a (upper panels) or CD1b (lower panels) were grown on coverslips and transiently supertransfected with ARF6/T27N cDNA. The cells were then fixed and permeabilized, and double-labeling with mouse antibodies to either CD1a or CD1b (detected with FITC-conjugated donkey anti-mouse IgG [green]) and rabbit anti-ARF6 antibody (detected with Texas red–conjugated donkey anti-rabbit IgG [red]) was performed. Fluorescent confocal images were obtained for CD1a (green, [A]), CD1b (green, [D]) and ARF6 (red, [B] and [E]) expression. The corresponding two images were then superimposed to detect vesicles expressing both CD1 and ARF6 (yellow, [C] and [F]). Scale bars, 5 μm.

detected for CD1a, and the CD1a- and the ARF6-T27N-containing intracellular vesicles were almost entirely superimposable (Figure 6C). In contrast, the distribution of CD1b-containing intracellular vesicles (Figure 6D) differed significantly from that of ARF6-T27N-containing vesicles (Figure 6E), demonstrated by the presence of numerous separate green and red vesicles after superimposition of the two images (Figure 6F). Thus, endosomal trafficking pathways for CD1a and CD1b were clearly separated, based on their colocalization with either ARF6-T27N or LAMP-1. Whereas CD1b, like MHC class II, trafficked deeply into the LAMP-1^+ late endocytic system, CD1a followed a recycling pathway of the early endocytic system via ARF6-regulated recycling vesicles.

Discussion

Here, we functionally and anatomically separate the antigen presentation pathways mediated by CD1a and CD1b molecules. The CD1b pathway resembles that of MHC class II in localization to late endosomal and lysosomal compartments and in the requirement for endosomal acidification in order to function. However, blockade of endosomal acidification as well as prevention of phagosome-lysosome fusion are well-known invasion mechanisms employed by phagocytosed microbes, which may abrogate both CD1b- and MHC class II–mediated pathways (Kaufmann, 1993; Sturgill-Koszycki et al., 1994). For peptide antigens, access to the cytosol by escape from endosomes and their subsequent delivery to the ER enables the effective elicitation of MHC class I–restricted, $CD8^+$ cytotoxic T cells that can eliminate the infected cells (Brunt et al., 1990; Bouwer et al., 1992; Mazzaccaro et al., 1998), although this pathway has often proved difficult to detect for many intracellular bacteria (Kaufmann, 1993). Here, we show that CD1a molecules avoid entry into late endosomal and lysosomal compartments and do not require endosomal acidification or delivery of antigens to the ER for their antigen presentation, predicting the ability to offer the immune system an alternate opportunity to recognize phagocytosed microbes separate from the other CD1 isoforms and MHC class I and class II molecules.

Mycobacteria reside in endosomes by preventing phagosome-lysosome fusion (Gordon et al., 1980; Hart and Young, 1991). However, lipids and glycolipids derived from endosome-resident mycobacteria can escape from phagosomes and distribute to other subcellular compartments (Xu et al., 1994). Interestingly, Mukherjee et al. (1999) recently showed that a pathway for endosomal trafficking of lipids is controlled by the basic structure of their hydrophobic acyl chains. Whereas lipids with saturated long (and thus rigid) acyl chains are sorted at the sorting endosomes toward lysosomes, those with unsaturated short (and thus more mobile) acyl chains are preferentially transported to recycling endosomes. Indeed, CD1b-restricted antigens so far identified are lipids and glycolipids with extremely long acyl chains (Beckman et al., 1994; Sieling et al., 1995; Moody et al., 1997). In addition, shortening the acyl chain and the sphingosine base of α-galactosylceramide, which is presented by CD1d in an endosomal acidification–dependent manner like CD1b-restricted antigens, diminishes specific T cell responses (Kawano et al.,

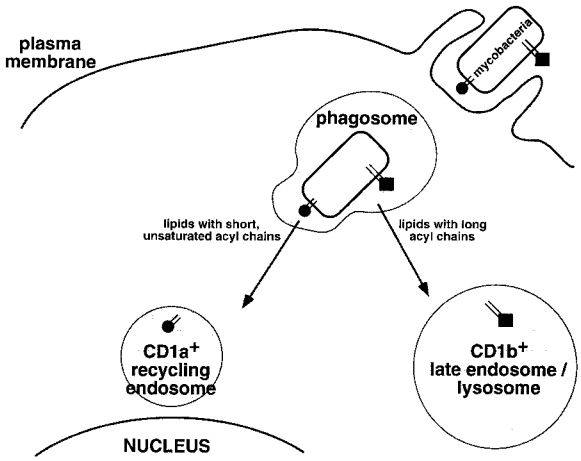

Figure 7. Intracellular Trafficking of CD1 Molecules and Mycobacteria-Derived Lipid Antigens in Mycobacteria-Infected Cells

1997; Brossay et al., 1998). Although the structural basis for lipid antigens presented by CD1a is not fully characterized, it seems likely that certain lipid antigens derived from phagocytosed mycobacteria are delivered, based on the chemical nature of their hydrophobic portion, to either recycling endosomes or late endosomes and lysosomes and sampled by CD1a or CD1b molecules, respectively (Figure 7). These features of distinct intracellular trafficking and intracellular requirements for lipid antigen presentation by CD1a and CD1b enable the immune system to efficiently monitor intracellular pathogens.

CD1a molecules are prominently expressed on activated monocyte-derived dendritic cells as well as epidermal Langerhans cells and dendritic cells in epithelia of certain tissues, such as the bronchus (Tazi et al., 1993), the gingiva (Crawford et al., 1989), the conjunctiva (Yoshida et al., 1997), the vagina, and the cervix (Miller et al., 1992). This distribution suggests that CD1a may play a role in antigen presentation at sites constantly exposed to external pathogens. CD1a and CD1b are not necessarily expressed in a coordinate fashion. Probably the best characterized difference in expression of these two molecules is seen on epidermal Langerhans cells. Freshly isolated Langerhans as well as cytokine-induced Langerhans cells express high levels of CD1a with virtually no CD1b on the cell surface (Caux et al., 1992). We examined epidermal Langerhans cells in situ for their intracellular and surface expression of CD1b by immunogold-labeled electron microscopy, but no CD1b expression was detected in these cells (data not shown). Thus, CD1a may play a dominant role compared to other CD1 isoforms on certain cells or in particular locations based on its differential cellular expression.

The ability to sample cytosolic and endosomal compartments by MHC class I and class II molecules, respectively, is essential for the generation of effective host responses to peptide antigens. Here we show that lipid antigen-presenting CD1 isoforms traffic in distinct components of the endocytic system in a manner that predicts comprehensive antigen sampling. The differential trafficking of exogenous lipids into the recycling pathway or the deep endocytic pathway correlates with the differential trafficking of CD1a and CD1b molecules. Moreover, the maneuvering of bacteria to prevent their trafficking into acidified, deep endocytic compartments points to the importance of sampling both early and late pathways of endocytic trafficking. The ability of the immune system to present the universe of foreign lipid, glycolipid, and lipoprotein antigens of microbes offers an important opportunity to detect and eliminate pathogens. The differential trafficking of the CD1 isoforms allows these antigen-presenting molecules to broadly sample vesicular compartments and detect microbes that constantly attempt to evolve evasive mechanisms. It seems likely that MHC and CD1 antigen-presenting molecules offer complementary opportunities that function in host defense.

Experimental Procedures

Cell Lines and Antibodies

The J.RT3 cells reconstituted with the mycobacterial lipid antigen–specific, CD1-restricted TCRs (CD8-2/J.RT3 and LDN5/J.RT3) have been described (Grant et al., 1999). These TCR-reconstituted cells were cultured in RPMI 1640 (GIBCO–BRL) complete medium (10% heat-inactivated fetal calf serum, 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 55 μM 2-mercaptoethanol [all from GIBCO–BRL]), containing G418 (GIBCO–BRL) (1 mg/ml) and hygromycin B (GIBCO–BRL) (500 μg/ml). A human B lymphoblastoid cell line, JY (Russell et al., 1996), was transfected with either CD1a cDNA in p3-9 or CD1b cDNA in p3-9 (Sugita et al., 1996) by electroporation and cultured in RPMI 1640 complete medium containing hygromycin B (200 μg/ml). The high-expressing cells were collected by fluorescence-activated cell sorting and used as APCs. IL-2-depenent HT-2 cells (Grant et al., 1999) were cultured in RPMI complete medium supplemented with 1 nM recombinant human IL-2 (Ajinomoto, Kawasaki, Japan). HeLa cell transfectants expressing CD1 molecules (Sugita et al., 1997; Spada et al., 1998) were grown in Dulbecco's modified essential medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. The mouse monoclonal antibodies 10H3 (anti-CD1a) (Olive et al., 1984), 4A7.6 (anti-CD1b) (Olive et al., 1984), F10/21A3.1 (anti-CD1c) (Grant et al., 1999), CD1d51 (anti-CD1d) (Exley et al., 1997; Spada et al., 1998), and F10.6.6 (anti-lysozyme) (Aoe et al., 1997) as well as rabbit polyclonal antisera against LAMP-1 (Carlsson et al., 1988) and ARF6 (D'Souza-Schorey et al., 1998) have been described.

T Cell Transfectants Stimulation Assay

Untransfected JY cells and JY cells stably transfected with either CD1a (JY/CD1a) or CD1b (JY/CD1b) were incubated overnight with indicated concentrations of either the semipurified *Mycobacterium tuberculosis* lipid antigen preparation (for CD8-2/J.RT3) (Rosat et al., 1999) or purified glucose monomycolate (GMM) (for LDN5/J.RT3) (Moody et al., 1997), washed and fixed with 0.08% glutaraldehyde. The TCR transfectant cells (5×10^4/well) were cultured with these fixed APCs in the presence of 10 ng/ml phorbol myristate acetate (PMA) using 96-well, flat-bottomed microtiter plates (200 μl media/well). Aliquots of the culture supernatants were collected after 24 hr, and the amount of interleukin-2 (IL-2) released into the supernatants was measured as described (Grant et al., 1999) using the HT-2 indicator cells, whose proliferation is dependent on IL-2.

To examine the effect of concanamycin B, JY/CD1a and JY/CD1b were preincubated with 10 nM concanamycin B (gift of Dr. Hidde Ploegh) for 30 min and then washed and incubated overnight with indicated concentrations of specific antigen preparations either in the presence or absence of 10 nM concanamycin B. Subsequently, the JY/CD1a and JY/CD1b cells were washed, fixed, and incubated with CD8-2/J.RT3 and LDN5/J.RT3, respectively, and IL-2 production by the T cells was measured as described above. To examine the effect of brefeldin A, HeLa cells were transfected with CD1a cDNA in pSRαneo (Porcelli et al., 1992) by a standard calcium phosphate precipitation method (Chen and Okayama, 1988). Two days after transfection, cells were preincubated with 10 μg/ml brefeldin A (Epicentre Technologies) for 30 min and then washed and incubated with indicated concentrations of the semipurified *M. tuberculosis*

antigen preparation either in the presence or absence of 10 μg/ml brefeldin A. After 5.5 hr, cells were washed, fixed, and incubated with CD8-2/J.RT3, and IL-2 release was measured, using HT-2 cells as described above.

Pulse-Chase Experiments

To examine the blocking effect of brefeldin A on protein transport from the pre-Golgi to the post-Golgi compartments, HeLa cells were transfected with lysozyme cDNA and metabolically pulse-radiolabeled with [^{35}S]methionine for 5 min as described (Sugita and Brenner, 1994). The radiolabeled cells were then washed and chased in regular media for 6 hr either in the presence or absence of 10 μg/ml brefeldin A. After the chase, secreted and intracellularly retained lysozyme were detected by immunoprecipitation with anti-lysozyme antibody from the supernatant and the cell lysate, respectively. The immunoprecipitated proteins were analyzed on a 12% SDS-PAGE gel under reducing conditions as described (Sugita and Brenner, 1994).

Immunofluorescence and Confocal Microscopy

CD1$^+$ HeLa cell transfectants grown on glass coverslips were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, permeabilized with 0.2% saponin, and then processed as previously described (Sugita et al., 1997). Briefly, the permeabilized cells were labeled with mouse monoclonal antibodies to the relevant CD1 isoforms, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated donkey F(ab')$_2$ antibody to mouse IgG (Jackson ImmunoResearch Laboratories) in PBS containing 5% normal donkey serum. Coverslips were mounted for observation on glass slides in Vectashield mounting medium (Vector Laboratories) as an antifading agent. The stained cells were viewed and photographed using a Nikon Optiphot-2 fluorescence microscope with an FITC filter set.

For confocal microscopy, HeLa cells expressing either CD1a or CD1b were fixed and permeabilized as described above and double-labeled with mouse monoclonal antibodies to the relevant CD1 isoforms and rabbit polyclonal antiserum against LAMP-1, followed by incubation with FITC-conjugated donkey F(ab')$_2$ antibody to mouse IgG and Texas red–conjugated donkey F(ab')$_2$ antibody to rabbit IgG (both from Jackson ImmunoResearch Laboratories) in PBS containing 5% normal donkey serum. In some experiments, CD1$^+$ HeLa cell transfectants grown on coverslips were supertransfected with ARF6-T27N cDNA (D'Souza-Schorey et al., 1998) by a standard calcium phosphate precipitation method. One day after transfection, the cells were washed three times with PBS, fixed, and permeabilized. The cells were then double-labeled with mouse monoclonal antibodies to the relevant CD1 isoforms and rabbit polyclonal antiserum against ARF6, followed by incubation with the secondary antibodies as described above. The labeled cells were examined using a Leica TCS-NT confocal laser scanning microscope fitted with krypton and argon lasers as described (Rogers et al., 1993; Jackman et al., 1998).

Immunogold-Labeled Electron Microscopy

A skin specimen from punch biopsy and peripheral blood monocytes stimulated with GM-CSF (Immunex) and IL-4 (Schering-Plough) (Porcelli et al., 1992) were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde (both from Polysciences), respectively, and processed for ultrathin cryosectioning as described (Peters et al., 1995a; D'Souza-Schorey et al., 1998). Cryosections were incubated with the 10H3 anti-CD1a antibody for 45 min, washed, and then incubated with protein-A gold (EM Laboratory, Utrecht University) for 30 min. Labeled sections were viewed with a JOEL 1010 electron microscope.

Acknowledgments

We thank Drs. H. Ploegh, M. Fukuda, D. B. Moody, S. A. Porcelli, D. Olive, and C. Mawas for their gifts of reagents; Immunex for GM-CSF; Schering-Plough Corporation for IL-4; and Jean Lai for expert help with confocal microscopy. This work was supported by grants from the National Institutes of Health.

Received September 1, 1999; revised November 8, 1999.

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