

# Recycling CD1d1 Molecules Present Endogenous Antigens Processed in an Endocytic Compartment to NKT Cells<sup>1</sup>

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Mouse CD1d1 molecules present endogenous glycolipids to NKT cells. Although glycolipid presentation requires CD1d1 transport through the endocytic pathway, the processing requirements for such endogenous Ag presentation by CD1d1 molecules are undefined. We examined CD1d1 Ag presentation to NKT cells by disrupting endocytic trafficking and function in cells expressing normal and mutated CD1d1 expressed by recombinant vaccinia viruses. Consistent with previous studies, we found that preventing CD1d1 localization to endosomes by altering its cytoplasmic targeting sequences abrogated recognition by V $\alpha$ 14J $\alpha$ 281<sup>+</sup> NKT cells without affecting recognition by V $\alpha$ 14<sup>-</sup> NKT cells. Increasing the pH of acidic compartments by incubating cells with chloroquine or bafilomycin A1 blocked CD1d1 recognition by V $\alpha$ 14<sup>+</sup> (but not V $\alpha$ 14<sup>-</sup>) NKT cells without reducing levels of cell surface CD1d1. Similar results were obtained with primaquine, which interferes with the recycling of cell surface glycoproteins. These results suggest that the loading of a subset of glycolipid ligands onto CD1d1 molecules entails the delivery of cell surface CD1d1 molecules and an acidic environment in the endocytic pathway. *The Journal of Immunology*, 2002, 168: 5409–5414.

The CD1 molecules exhibit an eclectic set of characteristics selected from the properties of MHC class I and class II molecules (reviewed in Ref. 1). Like MHC class I molecules, CD1 molecules associate with  $\beta_2$ -microglobulin ( $\beta_2m$ )<sup>3</sup> and require  $\beta_2m$  association for their recognition by T cells, yet they function independently of the MHC-encoded TAP peptide transporter (2–6). Like MHC class II molecules, most CD1 molecules traffic through the endocytic pathway (7–9). In contrast to class II molecules, which utilize sequences present within the invariant chain to traffic to endosomes (10, 11), CD1 molecules contain a Tyr-based endosomal targeting motif (TyrXX $\Phi$ , where X = any amino acid and  $\Phi$  = a hydrophobic amino acid (12)) in their cytoplasmic domains, which is required for trafficking to the endocytic pathway (7–9, 13).

Considering the structural similarity of CD1 to MHC class I and class II molecules, it was not surprising that T cells recognize CD1 molecules. There are two mouse CD1 genes, *cd1d1* and *cd1d2*: CD1d1 molecules are recognized by a unique set of T cells known as NKT cells (described below; Refs. 5, 14, and 15), whereas the

function of CD1d2 molecules is unclear (16–19). Humans also possess NKT cells (13, 20). In contrast to MHC class I or II molecules, which present peptide ligands to the immune system, CD1d1 molecules present glycolipids. In fact, in collaboration with Joyce et al. (21), we have found that a major natural ligand of CD1d1 molecules is a glycolipid, GPI. Additionally, a number of studies have shown that  $\alpha$ -galactosylceramide, a synthetic form of a glycolipid found predominantly in marine sponges, can bind to CD1d1 molecules and trigger NKT cells (22–31). Certain NKT cells have also been reported to recognize CD1d1-bearing cellular lipids such as phosphatidylinositol (23, 32). These studies extend prior findings that human CD1b presents natural glycolipids (e.g., GM1) (33, 34) or those derived from mycobacterial cell wall components (e.g., mycolic acid, lipoarabinomannan) to T cells (35–39).

These and other exogenously added glycolipids are most likely processed in late endosomes or lysosomes (4, 26, 36, 37, 39, 40). A lysosomal enzyme,  $\alpha$ -galactosidase A, was recently shown to process exogenously added glycolipids for presentation by CD1d1 molecules to NKT cells (41). This raises the question of whether presentation of endogenous glycolipids by CD1d1 molecules to NKT cells, for example, also entails endosomal processing and/or loading onto CD1d1.

To address this question, we have generated recombinant vaccinia viruses (rVVs) that express wild-type (WT) CD1d1 or mutants with alterations in their endosomal targeting sequence and examine the effect of agents known to disrupt endosomal function and intracellular trafficking to endosomal compartments.

## Materials and Methods

### Cell lines, Abs, and other reagents

P13.9 fibroblasts were kindly provided by Dr. R. Germain (National Institutes of Health, Bethesda, MD) and were cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. The V $\alpha$ 14<sup>+</sup> mouse CD1-specific NKT cell hybridomas, DN32.D3 (14, 42), N38-2H4 (23), and N38-3C3 (23), and the V $\alpha$ 5<sup>+</sup> N37-1A12 NKT cell hybridoma (23) have all been described. The cells were cultured in the presence of IMDM supplemented with 10% FBS and L-glutamine. FITC-conjugated rabbit anti-mouse IgG antiserum was purchased from DAKO (Carpinteria, CA). PE-conjugated 1B1 (mouse CD1 specific) (43) or rat IgG2b isotype control mAb, purified

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<sup>3</sup> Abbreviations used in this paper:  $\beta_2m$ ,  $\beta_2$ -microglobulin; LAMP-1, lysosome-associated membrane protein 1; MOI, multiplicity of infection; TD, tail deleted; VV, vaccinia virus; WT, wild type.

rat anti-mouse lysosome-associated membrane protein 1 (LAMP-1), as well as purified and biotinylated anti-mouse IL-2 mAb were purchased from BD Pharmingen (San Diego, CA). Mouse rIL-2 used as standards in the ELISA described below was obtained from PeproTech (Rocky Hill, NJ). A Texas Red-labeled donkey anti-rat Ig antiserum was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The 1H6 is a new mouse CD1-specific mAb (mouse IgG2a) that was generated in our laboratory for this study by immunization of CD1d1-deficient mice (19), kindly provided by Dr. L. Van Kaer (Vanderbilt University, Nashville, TN) with a rVV encoding the CD1d1 cDNA (5), followed 7 mo later with an NS/0 cell line transfected with the CD1d1 cDNA (3D3; a kind gift from Dr. S. Joyce, Vanderbilt University). Generation of Ab-secreting hybridomas was performed using standard methods. Supernatants were tested for CD1d1 specificity by cytofluorography using CD1d1-transfected L cells (44), kindly provided by Dr. W. Paul (National Institutes of Health). Chloroquine, bafilomycin A1, primaquine, and saponin were purchased from Sigma-Aldrich (St. Louis, MO).

#### rVV generation

To generate rVV-encoding mutations in the cytoplasmic tail of CD1d1 molecules, the *AvrII-NotI* fragment in WT CD1d1 cDNA in pSC11-mod (5) was PCR amplified to modify the cytoplasmic domain of CD1d1 by either removing the last 8 aa (tail deleted, TD) or changing the Y at position 322 to an A (Y322A). A unique *NruI* site not present in either the pSC11 rVV vector or CD1d1 cDNA was engineered into the mutants (3' of the transcriptional stop site) to assist in the identification of mutants. The oligonucleotides used for the generation of the mutants were the following: 5'-CAG CAG CCT AGG AGG ACA GGA TAT C-3' (positive strand for both mutants); 5'-TAC GTG CGG CCG CTC GCG ACT ATT ACC TTC TCC AGA TAT A-3' (negative strand, TD); and 5'-TAC GTG CGG CCG CTC GCG ACT ATT ACC GGA TGT CTT GAG CAG C-3' (negative strand, Y322A). Amplifications and rVV generation were as previously described (45, 46). The rVVs were thus named, VV-CD1d1WT (5), VV-CD1d1TD, and VV-CD1d1Y322A, respectively. The constructs were analyzed by dideoxynucleotide sequencing to ensure that the proper mutations were present before the rVVs were generated.

#### Cytofluorography

Staining for flow cytometry was performed as described previously (47). In brief, P13.9 cells were infected with the indicated rVV at a multiplicity of infection (MOI) of 5 for 8–12 h at 37°C in the presence or absence of inhibitors. The cells were then fixed in 0.05% paraformaldehyde and were used in the T cell assays (described below) or stained for 30 min on ice with the mouse CD1-specific PE-conjugated 1B1 mAb (43) (or isotype controls). After washing three times in HBSS containing 0.1% BSA and 0.02% azide (HBSS/BSA), the cells were resuspended in HBSS/BSA and analyzed using a FACScan cytofluorograph (BD Biosciences, Mountain View, CA).

#### T cell hybridoma assay

To measure endogenous Ag presentation by CD1d1 molecules, P13.9 fibroblasts were infected with rVV (MOI = 5)-expressing cDNAs for the WT CD1d1 molecule (5) or the mouse MHC class I molecule, K<sup>d</sup> (46), in the presence or absence of various concentrations of the lysosomotropic drugs chloroquine, bafilomycin A1, or an inhibitor of recycling (primaquine) for a total of 8–12 h at 37°C. The cells were then washed three times in cold PBS, fixed with 0.05% paraformaldehyde, and washed three additional times. The cells were then resuspended in IMDM at a cell density of  $5 \times 10^6$  cells/ml. The NKT cell hybridomas, DN32.D3 (42), N38-2H4 (23), N38-3C3 (23), or N37-1A12 (23) (all used at  $5 \times 10^4$  cells), were incubated with  $5 \times 10^5$  fixed target cells for 24 h at 37°C. Coculture supernatants were harvested, and IL-2 production was measured by ELISA.

#### Confocal microscopy

P13.9 cells were plated in sterile glass-bottom 35-mm dishes coated with poly(D-lysine) (MatTek, Ashland, MA) at a density of  $1 \times 10^7$  cells/dish. After overnight adherence, the cells were infected with VV-CD1d1WT, VV-CD1d1TD, or VV-CD1d1Y322A at an MOI of 5, as described above. Six hours postinfection, the cells were washed twice in ice-cold HBSS/BSA and fixed in 1% paraformaldehyde for 10 min at room temperature. Immunofluorescent localization of CD1d1 molecules was performed by incubating the cells with the anti-CD1 mouse mAb, 1H6 (this study), in permeabilizing buffer (HBSS/BSA with 0.1% saponin) supplemented with normal rat serum (Sigma-Aldrich). Cells were then washed three times in permeabilizing buffer and incubated with FITC-conjugated rabbit anti-

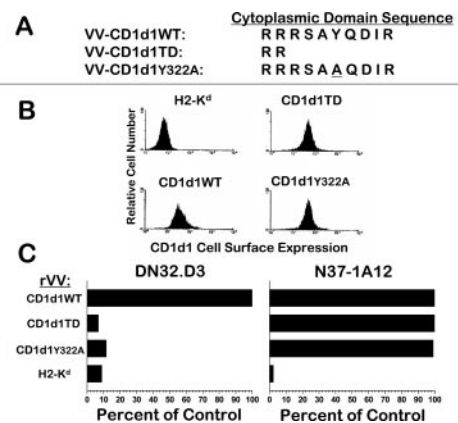
mouse Igs. Following three washes in permeabilizing buffer, the cells were incubated with anti-mouse LAMP-1 in permeabilizing buffer supplemented with donkey serum (Sigma-Aldrich). The cells were then washed three times in permeabilizing buffer, followed by staining with a Texas Red-labeled donkey anti-rat Ig antiserum. All Ab incubations were performed at room temperature for 1 h in the dark. The stained cells were stored in PBS containing 0.05% azide in the dark at 4°C until confocal analysis. The samples were viewed with a Bio-Rad MRC-1024 confocal laser-scanning microscope (Bio-Rad, Hercules, CA) equipped with a krypton-argon laser that has been modified for two-photon microscopy. The Texas Red and FITC emissions were recorded sequentially using a  $\times 60$  lens and pinhole aperture adjustment to obtain 0.3- to 0.5- $\mu$ m sections.

## Results

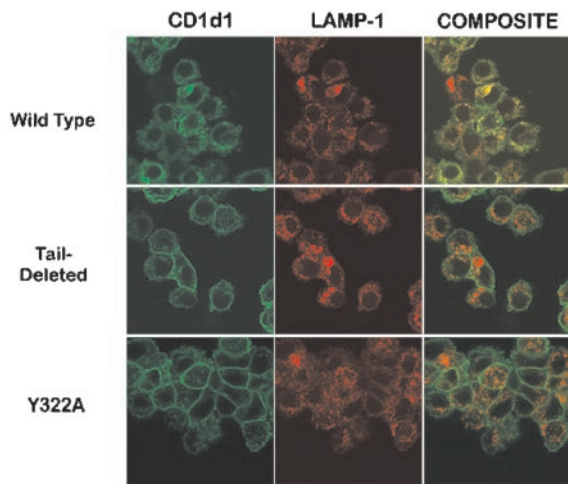
### WT (but not cytoplasmic tail mutant forms of) CD1d1 molecules are recognized by canonical (V $\alpha$ 14<sup>+</sup>) NKT cells

Alterations of the endosomal targeting motif of human or mouse CD1d1 molecules by either deleting the last several amino acids (including the Tyr) or substituting Ala for the Tyr have been shown to affect the intracellular trafficking of these molecules (7–9, 13) and, in the case of CD1d1, recognition by NKT cells (8, 9). All prior studies used permanently transfected cells. In the current study, we extended these findings to the VV expression system, which provides the significant advantage of transient expression. This allowed us to examine the effects of various inhibitors on the presentation of endogenous Ags without having to rid the cells of CD1d1 molecules loaded with Ags before the addition of inhibitors.

We generated rVVs expressing CD1d1 lacking the last eight residues of the cytoplasmic domain (including the Tyr; VV-CD1d1TD) or in which Tyr is substituted with Ala (VV-CD1d1Y322A; Fig. 1A). P13.9 fibroblasts, which do not express detectable amounts of CD1d1, were infected with rVV encoding the WT (5) or mutant CD1d1 molecules (or the mouse classical MHC class I molecule, K<sup>d</sup> (46) as a negative control), and cell surface expression of CD1d1 molecules was assessed by cytofluorography using the 1B1 mAb (43). Infection of P13.9 cells with

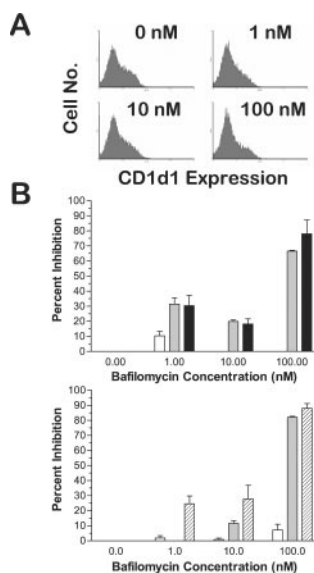


**FIGURE 1.** rVV-expressed mutant CD1d1 molecules are expressed on the surface of infected P13.9 cells and are not recognized by canonical NKT cells. *A*, Sequence of cytoplasmic tails expressed by rVV encoding WT, TD, and Y322A mutants. *B*, P13.9 cells were infected with rVV encoding K<sup>d</sup> or WT, TD, and Y322A CD1d1 molecules. Analysis was by cytofluorography using a direct FITC conjugate of the mCD1-specific 1B1 mAb. Results are representative of two experiments. *C*, P13.9 fibroblasts were infected with rVV encoding WT CD1d1, or the indicated mutant CD1d1 molecules. As a negative control, P13.9 cells were infected with a K<sup>d</sup>-expressing rVV. The infected cells were cocultured with the mouse CD1-specific, canonical (V $\alpha$ 14<sup>+</sup>) NKT cell hybridoma, DN32.D3 (*left*), or with the mouse CD1-specific, noncanonical (V $\alpha$ 5<sup>+</sup>) NKT cell hybridoma, N37-1A12 (*right*), for 24 h. IL-2 production into the supernatant was measured by ELISA. Results are representative of two experiments.



**FIGURE 2.** Intracellular trafficking of rVV-expressed WT and mutant CD1d1 molecules in P13.9 cells. P13.9 fibroblast cells were infected with rVV expressing WT or the indicated mutant CD1d1 molecules. CD1d1 was labeled using a mouse anti-mouse CD1 mAb (1H6), followed by a FITC-conjugated rabbit anti-mouse Ig antiserum, whereas rat anti-mouse LAMP-1 was detected using a Texas Red-labeled donkey anti-rat Ig antiserum. Analysis was by confocal microscopy. Note that in the composite images, only the WT CD1d1 molecules colocalize with LAMP-1, whereas the mutant CD1d1 molecules are mostly present on the cell surface and do not colocalize with LAMP-1.

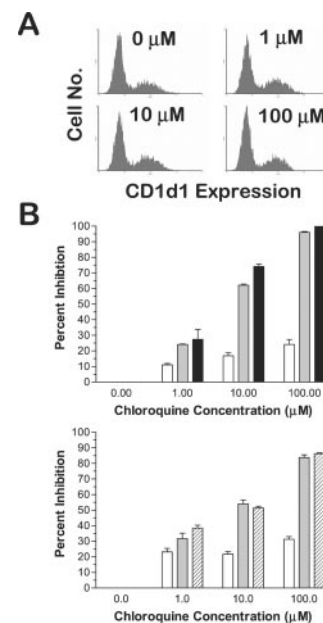
rVV encoding either the WT or mutant CD1d1 molecules resulted in comparable levels of cell surface expression well above control values observed with VV-K<sup>d</sup>-infected cells (Fig. 1B).



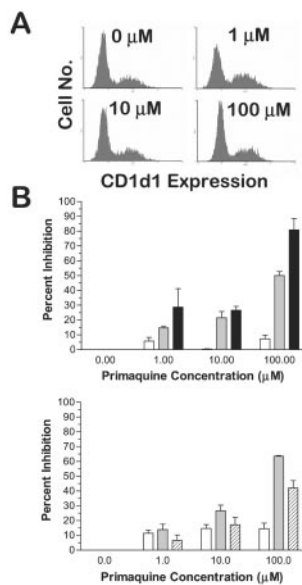
**FIGURE 3.** Bafilomycin A1 treatment of CD1<sup>+</sup> cells inhibits their recognition by NKT cells. P13.9 fibroblasts were infected with a rVV encoding WT CD1d1 molecules in the presence or absence of the indicated concentrations of bafilomycin A1. *A*, Cell surface expression of CD1d1 in bafilomycin A1-treated P13.9 cells. An aliquot of the P13.9 cells infected for the ELISA was stained with a PE-conjugated anti-mouse CD1 mAb, and cell surface CD1d1 expression was analyzed by cytofluorography. *B*, The rVV-infected P13.9 cells were cocultured with the V $\alpha$ 14<sup>+</sup> NKT cell hybridomas, DN32.D3 (▣), N38-3C3 (■), and N38-2H4 (▨), and the V $\alpha$ 14<sup>-</sup> NKT cell hybridoma, N37-1A12 (□). NKT cell recognition of CD1d1 was assessed by measuring IL-2 production in the supernatants by ELISA. The results are representative of five experiments.

To determine whether VV-CD1d1TD- and VV-CD1d1Y322A-expressed CD1d1 molecules could be recognized by NKT cells, rVV-infected P13.9 cells were cocultured with the canonical CD1d1-specific NKT cell hybridoma, DN32.D3 (5, 14) (Fig. 1C). As we previously reported (14), coculture of DN32.D3 with P13.9 cells infected with VV-CD1d1 (but not VV-K<sup>d</sup>) resulted in release of IL-2 from the hybridoma. By contrast, infection of cells with either of the rVVs expressing tail-mutated CD1d1 failed to stimulate IL-2 release. This could not be attributed to gross differences in CD1d1 conformation since the mutants induced similar levels of IL-2 release as WT CD1d1 from a CD1d1-specific NKT cell hybridoma, N37-1A12 (8), that does not use V $\alpha$ 14J $\alpha$ 281, and recognized both the WT and mutant CD1d1 molecules expressed by rVV at equivalent levels (Fig. 1C). Thus, these results using the rVV transient transfection system are in agreement with those observed using stably transfected cells (8, 9).

We confirmed that rVV-expressed WT and mutant CD1d1 molecules were localized in the expected intracellular compartments using confocal microscopic analysis of fixed and permeabilized cells stained with the 1H6 mAb (Fig. 2). The endosomal localization of CD1d1WT is clear from their colocalization with the late endosomal/lysosomal marker, LAMP-1. In contrast, the rVV-expressed CD1d1TD and CD1d1Y322A molecules were found mostly on the surface of the plasma membrane. Along with the functional data shown in Fig. 1, these findings validate the use of the rVV expression system because they recapitulate findings using transfected cells (8, 9).



**FIGURE 4.** NKT cell recognition of CD1d1 molecules is inhibited by chloroquine. P13.9 fibroblasts were infected with a rVV encoding WT CD1d1 molecules in the presence or absence of the indicated concentrations of chloroquine. *A*, Cell surface expression of CD1d1 in chloroquine-treated P13.9 cells. An aliquot of the P13.9 cells infected for the ELISA was stained with a PE-conjugated anti-mouse CD1 mAb, and cell surface CD1d1 expression was analyzed by cytofluorography. *B*, The rVV-infected P13.9 cells were cocultured with the V $\alpha$ 14<sup>+</sup> NKT cell hybridomas, DN32.D3 (▣), N38-3C3 (■), and N38-2H4 (▨), and the V $\alpha$ 14<sup>-</sup> NKT cell hybridoma, N37-1A12 (□). NKT cell recognition of CD1d1 was assessed by measuring IL-2 production in the supernatants by ELISA. The results are representative of five experiments.



**FIGURE 5.** NKT cell recognition of CD1d1 is inhibited by primaquine. P13.9 fibroblasts were infected with rVV encoding WT CD1d1 molecules in the presence or absence of the indicated concentrations of primaquine. *A*, Cell surface expression of CD1d1 in primaquine-treated P13.9 cells. An aliquot of the P13.9 cells infected for the ELISA was stained with a PE-conjugated anti-mouse CD1 mAb, and cell surface CD1d1 expression was analyzed by cytofluorography. *B*, The rVV-infected P13.9 cells were cocultured with the  $V\alpha 14^+$  NKT cell hybridomas, DN32.D3 (▨), N38-3C3 (■), and N38-2H4 (▩), and the  $V\alpha 14^-$  NKT cell hybridoma, N37-1A12 (□). NKT cell recognition of CD1d1 was assessed by measuring IL-2 production in the supernatants by ELISA. The results are representative of five experiments.

*Lysosomotropic agents inhibit CD1d1 recognition by canonical (but not noncanonical) NKT cells*

To examine the involvement of endosomes in CD1d1-mediated presentation to NKT cells, we treated cells with drugs that raise the pH of intracellular compartments and are known to interfere with the presentation of exogenous Ags by CD1 or MHC class II molecules (4, 10, 11, 26, 41). We used chemically dissimilar drugs with distinct modes of action, minimizing the chance that any effects are due to a common unexpected target. Bafilomycin A1 is a

specific inhibitor of the vacuolar  $H^+$  ATPase (48), whereas chloroquine acts as a classical lysosomotropic agent that neutralizes endosomal pH by accumulating in a charged membrane-impermeant form. P13.9 cells were infected with VV-CD1d1WT (5, 14) in the presence or absence of bafilomycin A1 or chloroquine, fixed in paraformaldehyde to prevent further CD1d1 trafficking, and cocultured with  $V\alpha 14^+$  or  $V\alpha 14^-$  NKT cell hybridomas. As shown in Figs. 3 and 4, increasing concentrations of bafilomycin A1 (Fig. 3*B*) or chloroquine (Fig. 4*B*) resulted in a concomitant inhibition of IL-2 release by the  $V\alpha 14^+$  NKT cell hybridomas (DN32.D3, N38-2H4, and N38-3C3). By contrast, neither drug affected activation of the CD1-restricted (but  $V\alpha 14^-$ ) N37-1A12 NKT cell hybridoma (Figs. 3 and 4). The effect of the drugs on  $V\alpha 14^+$  NKT cell activation cannot be attributed to a reduction in CD1d1 cell surface expression, as shown by cytofluorography (Figs. 3*A* and 4*A*). Therefore, these results strongly suggest that the processing of the endogenous Ag(s) recognized by canonical (i.e.,  $V\alpha 14^+$ ) NKT cells occurs in an endocytic compartment.

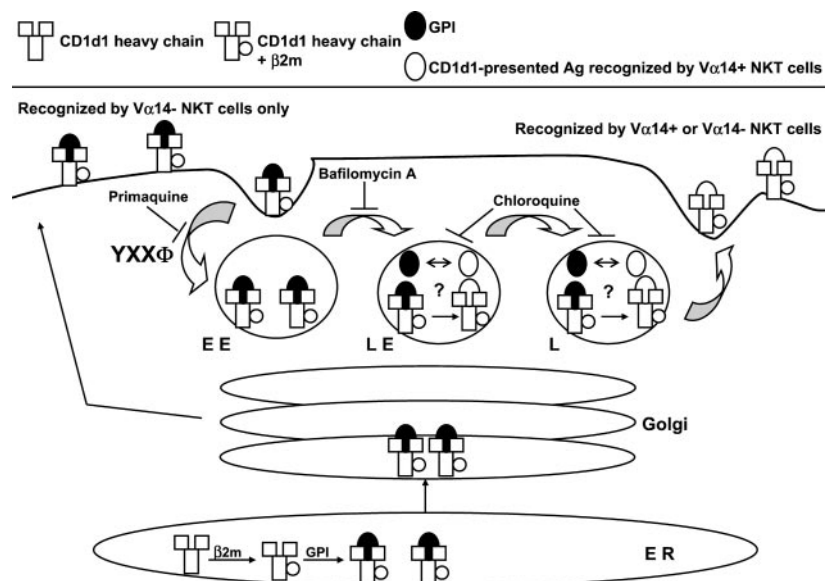
*Primaquine inhibits CD1d1 recognition by canonical (but not noncanonical) NKT cells*

Primaquine has been shown to inhibit the recycling through the endosomal/lysosomal compartment of cell surface molecules such as the transferrin and asialoglycoprotein receptors, as well as MHC class I and class II molecules (10, 49–54). This effect has been shown to be due to a direct effect on endosomes (55). As shown in Fig. 5*B*, primaquine treatment of VV-CD1d1WT-infected P13.9 cells resulted in the inhibition of canonical NKT cell (i.e., DN32.D3, N38-2H4, and N38-3C3 NKT hybridomas) recognition of CD1d1. Once again, there was no significant change in the activation of N37-1A12 cells (Fig. 5*B*) nor in levels of cell surface CD1d1, as detected by CD1-specific (1B1) staining (Fig. 5*A*). Therefore, the results suggest that the recycling of cell surface CD1d1 molecules to compartments of the endocytic pathway is required for the recognition of CD1 molecules by canonical (i.e.,  $V\alpha 14^+$ ) NKT cells.

## Discussion

Our findings strongly implicate loading of CD1d1 molecules with the endogenous Ag recognized by canonical NKT cells in an endosomal compartment. Like MHC class II molecules, CD1d1 molecules can potentially take two routes to the endocytic pathway,

**FIGURE 6.** Proposed mechanism of the processing of endogenous glycolipid Ags and their presentation by CD1d1 molecules. CD1d1 molecules are synthesized in the endoplasmic reticulum (ER) and form a complex with  $\beta_2m$ . It is within this compartment that CD1d1 also associates with an endogenous glycolipid, GPI. The complex then traverses through the Golgi complex and onto the cell surface. These GPI-loaded molecules can be recognized by noncanonical NKT cells (e.g., N37-1A12), but not by  $V\alpha 14^+$  NKT cells (e.g., DN32.D3). A Tyr-based endosomal targeting sequence (YXX $\Phi$ ) causes the CD1d1- $\beta_2m$ -GPI complex to reenter the cell and traffic to intracellular vesicular compartments in the endocytic pathway. It is there that GPI can be replaced by the appropriate endogenous Ag(s); this complex then returns to the cell surface, where it can be recognized by both canonical and noncanonical NKT cells. EE, early endosomes; LE, late endosomes or early lysosomes; L, lysosomes.



either directly from the Golgi complex or via the cell surface (1, 10). Our data are consistent with the latter pathway.

In collaboration with Joyce and colleagues (21), we reported that a major natural ligand of CD1d1 molecules is a normal cellular glycolipid, GPI. On repeated attempts we have failed to activate canonical NKT cells by the addition of GPI purified from WT CD1d1 molecules to cells expressing tail-modified forms. Notably, GPI was isolated from cells expressing either WT or soluble CD1d1, which is exceedingly unlikely to traffic to endosomes in sufficient quantities to enable recovery of endosome-loaded ligands. This implies, first, that GPI is not the ligand recognized by canonical NKT cells (although it may be recognized by non-canonical cells), and second, that GPI must be loaded elsewhere.

We propose that GPI functions in an analogous manner to the class II-associated invariant chain peptide portion of the invariant chain: it binds in the endoplasmic reticulum and serves to protect the binding site from inappropriate interactions or to stabilize the conformation of CD1d1. GPI would remain bound, while CD1d1 is initially expressed on the cell surface, but upon endocytosis into intracellular endosomal/lysosomal compartments, GPI is exchanged for other glycolipids, including the endogenous Ag(s) recognized by canonical NKT cells and other glycolipids obtained from exogenous sources (Fig. 6). This recycling activity for CD1d1 molecules is similar to that recently proposed by Brenner and colleagues (56) for the human CD1c molecule and is supported by recent work from the Bendelac laboratory (published while the current manuscript was being revised) using transgenic mice expressing a TD form of CD1d1 (57).

Control of NKT activation could be exerted at four levels in this model. First, the level of CD1d1 expression could be modulated. Second, the delivery of CD1d1 to loading compartments (endosomes/lysosomes) could be controlled. Third, GPI exchange could be controlled. Finally, the generation of the endogenous ligand (or its trafficking to endosomes/lysosomes) could be controlled. There is mounting evidence that NKT cells play an important role in initiating innate immunity (25, 58). Experiments designed to test this model should help to elucidate the role for CD1d1 molecules and NKT cells in immune defense against microbial invaders and neoplastic transformation.

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