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Distinct Endosomal Trafficking Requirements for Presentation of Autoantigens and Exogenous Lipids by Human CD1d Molecules

Xiu Xu Chen,* Xiaohua Wang,* Jason M. Keaton,* Faye Reddington,† Petr A. Illarionov,† Gurdyal S. Besra,‡ and Jenny E. Gumperz²*²

CD1d molecules present both self Ags and microbial lipids to NKT cells. Previous studies have established that CD1d lysosomal trafficking is required for presentation of autoantigens to murine invariant NKT cells. We show in this study that this is not necessary for autoantigen presentation by human CD1d, but significantly affects the presentation of exogenous Ags. Wild-type and tail-deleted CD1d molecules stimulated similar autoreactive responses by human NKT clones, whereas presentation of exogenous lipids by tail-deleted CD1d was highly inefficient. Chloroquine treatment markedly inhibited exogenous Ag presentation by wild-type CD1d transfectants, but did not affect NKT autoreactive responses. Conversely, APC expression of HLA-DRβ2 and the invariant chain (Ii) was associated with faster internalization of CD1d into the endocytic system and enhanced CD1d-mediated presentation of exogenous Ags, but did not appear to augment NKT autoreactivity. Knockdown of the Ii by small interfering RNA resulted in reduced CD1d surface expression and slower internalization in HLA-DR⁺ APCs, but not HLA-DR⁻ APCs, demonstrating a direct effect of MHC/Ii complexes on CD1d trafficking. CD1d-mediated presentation of exogenous Ags was much more efficient in immature dendritic cells, which actively recycle MHC class II molecules through the endocytic system, than in mature dendritic cells that have stabilized MHC class II expression at the cell surface, suggesting a physiological role for MHC/Ii complexes in modulating CD1d function. These results indicate that autoantigens and exogenous lipids are acquired by human CD1d at distinct cellular locations, and that Ii trafficking selectively regulates CD1d-mediated presentation of extracellular Ags. The Journal of Immunology, 2007, 178: 6181–6190.

The CD1d molecules bind lipids and glycolipids and present them at the cell surface to CD1d-restricted NKT cells (1). Whereas antigenic peptides from intracellular and extracellular proteins are largely segregated into separate MHC class I or class II presentation pathways, CD1d molecules present lipids derived from both intra- and extracellular sources (2). NKT cells respond functionally to self Ags and to foreign microbial lipids (1, 2). Self Ags recognized by NKT cells are thought to be lipids that are synthesized within the APC, whereas microbial Ags are taken up by APCs from the extracellular environment, or derive from phagocytosed bacteria (3). It is not clear whether there are mechanisms that differentially regulate CD1d-mediated presentation of Ags from intracellular and extracellular sources.

CD1d molecules contain a deep hydrophobic pocket that binds lipid alkyl chains, leaving the polar head group relatively exposed at the surface of the molecule (4, 5). Insertion of lipids into the CD1d binding site is facilitated by specific lipid transfer proteins such as the microsomal transfer protein in the endoplasmic reticulum (ER), and the saposins in the lysosome (6–8). The binding site of CD1d molecules accommodates a wide variety of different lipid structures, whereas lipid transfer proteins have specificity for different types of lipids (9). Therefore, the nature of the Ags presented by CD1d is probably significantly affected by the actions of lipid transfer proteins. Additionally, the lipid composition of different intracellular compartments varies, so access to different endosomal sites may allow binding of distinct lipids (10, 11). Finally, in some cases, T cell recognition cannot occur unless glycolipid Ags are processed by lysosomal glycosidases to remove interfering glycans (12). Thus, intracellular trafficking is likely to affect Ag presentation by CD1d by influencing access to specific lipids as well as to critical accessory proteins.

CD1d molecules are synthesized in the ER and then follow the secretory pathway through the Golgi to the cell surface (13). Subsequently, they are reinternalized from the cell surface and traffic through the endosomal vesicular system. This is accomplished by association with adaptor proteins (AP) that mediate internalization from the cell surface and direct localization to endosomal compartments. Four AP complexes (AP-1, 2, 3, 4) have been identified that bind to tyrosine or dileucine amino acid motifs in the

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*Department of Medical Microbiology and Immunology, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706; and †School of Biosciences, University of Birmingham, Birmingham, United Kingdom

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Address correspondence and reprint requests to Dr. Jenny E. Gumperz, Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, 405 Service Memorial Institute, 1300 University Avenue, Madison, WI 53706. E-mail address: jegumperz@wisc.edu

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cytoplasmic tails of transmembrane proteins (14). The cytoplasmic tail sequences of human and murine CD1d molecules both contain tyrosine-based motifs that mediate binding to the AP-2 complex, but the murine CD1d sequence also binds to the AP-3 complex, whereas the human CD1d sequence does not (15–17). The AP-2 complex mediates internalization from the cell surface into early endosomes, and the AP-3 complex directs trafficking from early endosomes to lysosomes (14). Thus, human and murine CD1d are expected to traffic differently due to their difference in AP-3 binding, and this probably explains the observation that murine CD1d molecules show steady-state intracellular localization almost exclusively in lysosomal compartments, whereas human CD1d is distributed in a variety of endosomal vesicles with only a fraction localizing to lysosomes (18).

Murine CD1d cytoplasmic tail deletion mutants do not undergo internalization from the cell surface into the endosomal system, and are deficient in the ability to stimulate the autoreactive responses of murine NKT cells that express a Vα14-invariant TCR (invariant NKT or iNKT cells), suggesting that the autoantigens recognized by these cells are derived from endosomal locations (19–21). Pharmacological disruption of lysosomal acidification also inhibited the autoreactive responses of murine iNKT cells, suggesting that lysosomal functions were important for the acquisition of autoantigens by CD1d (21). The basis for these effects was clarified recently by the observations that deletion of the saposins abrogates murine iNKT cell autoreactive responses, and that isoglobotrihexosyl ceramide (iGb3), a metabolic glycolipid that is a potent agonist for NKT cells (8, 24). Hence, these data suggest lysosomal functions may not be required for CD1d to present endogenous Ags, but that access to lysosomal lipid transfer proteins is important for the presentation of exogenous Ags.

Because human CD1d molecules do not appear to bind the AP-3 adaptor complex, the mechanisms by which they are directed to lysosomes remain poorly understood. One factor that can directly affect trafficking of CD1d molecules to lysosomes is association with MHC class II and invariant chain (Ii) complexes (26, 27). The complexing of MHC class II with Ii is responsible for its trafficking to lysosomes, whereupon Ii is cleaved and the MHC class II molecules are transported to the cell surface (28). A fraction of human CD1d molecules has been shown to physically associate with MHC class II and Ii in the ER, and to traffic to other sites, including the cell surface and lysosomes (26). Expression of Ii was sufficient to reconstitute lysosomal delivery of CD1d cytoplasmic tail deletion mutants, indicating that this association can provide signals for CD1d lysosomal trafficking (26, 27). However, the functional impact of the association of human CD1d with MHC class II and Ii remains unclear. In this study, we investigate the contributions of endosomal trafficking and association with MHC class II/Ii to the presentation of autoantigens and extracellular lipids by human CD1d.

**Materials and Methods**

*Cell lines and transfectants*

The 3023 and 2001 cell lines were a gift from R. DeMars (University of Wisconsin, Madison, WI) (29). Truncated human CD1d molecules lacking the last 10 C-terminal residues were generated by PCR amplification using the following primers: 5′-CCACGCGCCACCAT-3′ (forward) and 5′-TCAAAACCGGGAGTTAAGCCCACAAATG-3′ (reverse). Stable transfectants of wild-type or tail-deleted human CD1d were established by electroporation and drug selection, as described (30). Drug-resistant cells were flow cytometrically sorted for positive expression of cell surface CD1d, and maintained in culture medium (RPMI 1640 medium, 2 mM t-glutamine, 100 μg/ml each of penicillin and streptomycin from Mediatech, 5% FBS, 10% bovine calf serum from HyClone). The resulting CD1d-transfected 3023 and 2001 cell lines were designated “3023-d” and “2001-d”, respectively. For the 3023-d line, the culture medium was supplemented with 0.5 mg/ml G418 sulfate (Mediatech) and 0.5 μg/ml puromycin (Sigma-Aldrich), and for the 2001-d cells, 10 μg/ml mycophenolic acid and 7.6 μg/ml xanthine (both from Sigma-Aldrich) were also included. CD1d-transfected HeLa cells were grown in DMEM (Mediatech), supplemented with 5% FBS and 5% bovine calf serum (both from HyClone), 2 mM t-glutamine, and 100 μg/ml each of penicillin and streptomycin (all from Mediatech).

*T cell clones*

NKT cell clones were established, as previously described (31), and maintained in T cell medium (RPMI 1640 medium, 2 mM t-glutamine, 100 μg/ml each of penicillin and streptomycin, 10% FBS, 5% bovine calf serum, 5% human AB serum from Gemini Bio-Products, and 400 U/ml recombinant human IL-2 from Chiron), with periodic restimulation by irradiated allogeneic feeder PBMCs and PHA, as described (31).

*Flow cytometry*

Flow cytometric analysis was performed using Alexa-647-conjugated anti-human CD1d (clone CD1d42.1); Alexa-633-conjugated anti-human MHC class II (clone L243); Alexa-488-conjugated anti-human MHC class I (clone W6/32); or Alexa-647-, 633-, or 488-conjugated negative control mAbs UPC10 (IgG2a) or MOPC21 (IgG1), prepared according to the manufacturer’s protocol (Invitrogen Life Technologies/Molecular Probes). Samples were stained with the indicated specific mAbs or negative controls for 30 min on ice, then washed and resuspended in FACS buffer (1 mg/ml PBS/BSA containing 10 μg/ml 4′,6′-diamidino-2-phenylindole). Live cells were gated by forward and side scatter and exclusion of 4′,6′-diamidino-2-phenylindole.

*Preparation of lipid Ags*

The glycolipid Ags α-GalCer and Gal(α1-2)galactosylceramide (GalCerGalCer) were prepared from β-lyxose, as described (32), dissolved in DMSO at a concentration of 100 μg/ml, and stored frozen in glass vials at −20°C. Before use, the lipids were sonicated in a heated water bath for 15 min at 37°C.

*Ag presentation assays*

NKT cell clones (5 × 10^4/well) were incubated for 16 h with APCs (5 × 10^5/well) in 200 μl/well T cell medium lacking IL-2, at 37°C and 5% CO2. All assays were performed in triplicate. Supernatants were tested for GM-CSF concentration by a commercial ELISA (Biolegend), and quantitated by comparison with human rGM-CSF standards (PeproTech). Where indicated, the APCs were pretreated with the indicated concentration of α-GalCer or GalCerGalCer, or an equivalent volume of DMSO alone, for the indicated amount of time. Where noted, the APCs were pretreated with 40 μg/ml chloroquine dissolved in culture medium for 2 h; then GalCerGalCer lipid Ag was added for the indicated pulse period in presence of chloroquine.

*Immunofluorescence microscopy*

Logarithmically growing cells were incubated on lysine-coated slides for 10 min at 37°C, and then fixed in 2% paraformaldehyde in PBS for 30 min at room temperature. The cells were then permeabilized with Cytotox/Cytoperm buffer (BD Biosciences) for 30 min at room temperature, and stained at room temperature with 10 μg/ml each of Alexa-647-conjugated...
anti-CD1d (CD1d42.1) or mouse IgG1-negative control mAb, and FITC-conjugated anti-lysoosomal-associated membrane protein (LAMP)-1 (H4A3) or mouse IgG1-negative control mAbs (BD Biosciences). The cells were analyzed by confocal microscopy of 0.3-μm-thick sections, using a Carl Zeiss LSM 510 confocal fluorescence microscope. Z-stacks were created from the sections, color images were overlaid, and the fluorescence intensity was analyzed using LSM Zeiss software.

Determination of CD1d internalization by cell surface biotinylation
APCs (7 × 10^6 each) were labeled for 30 min on ice with 0.8 mM sulfo-NHS-SS-biotin (Pierce), then washed, resuspended in culture medium, and incubated for the indicated times at 37°C to allow protein internalization from the cell surface. The cells were then treated with 100 nM DTT (Sigma-Aldrich) for 20 min on ice to remove the remaining surface biotin. Residual surface biotin was blocked by addition of 0.5 mg/ml recombinant streptavidin (Pierce), followed by addition of 10 μg/ml free biotin to block the unbound streptavidin sites. The cells were then lysed in 1% Triton X-100, and lysates were tested by ELISA, using a CD1d-specific capture mAb (CD1d42.1), followed by direct detection with streptavidin-alkaline phosphatase (Zymed Laboratories) to detect biotinylated CD1d. Total CD1d was assessed by lysing the APCs in 1% Triton X-100 before surface biotinylation, capturing the CD1d by plate-bound CD1d42.1 mAb, and detecting bound CD1d using biotin-labeled rat anti-human β2-microglobulin Ab (2 μg/ml; DakoCytomation). Specificity of the ELISA was confirmed using a negative control mAb instead of the anti-CD1d capture mAb, and by testing lysates from CD1d-untransfected 30T3 and 2001 cells. All assays were performed in triplicate.

Determination of CD1d internalization or re-emergence by flow cytometry
APCs were incubated with 10 μg/ml unlabeled anti-CD1d mAb (clone CD1d42.1), then washed, resuspended in warm culture medium, and incubated at 37°C for the indicated times. The cells were then washed again, fixed in cold 2% paraformaldehyde in PBS, and labeled with 10 μg/ml FITC-conjugated goat anti-mouse Ab (BD Biosciences) before analysis by flow cytometry. For CD1d re-emergence assays, the cells were treated with 50 μg/ml cycloheximide (Sigma-Aldrich) for 30 min to prevent new protein synthesis, and then cell surface CD1d was blocked with unlabeled CD1d42.1 mAb, as described above. The cells were incubated for the indicated times at 37°C in culture medium, then washed and fixed with paraformaldehyde, and unlabeled CD1d on the cell surface was detected with PE-conjugated CD1d42.1 mAb (BD Biosciences).

Ii knockdown by small interfering RNA (siRNA)
Annealed siRNA constructs were obtained from Ambion. Sense strand sequences used were as follows: GGCUUUUCCAUCCUGGUGAtt, CCAAAGUCGGAACAGCAUtt, and CCCUAAUCUCUCAAAGUAGt; and GAPDH siRNA was used as a negative control. A total of 10–20 pmol of each siRNA was mixed with 100 μl of Nucleofector Solution V (Amaxa), and transfected into 5 × 10^6 cells using the A24 program of the Nucleofector electroporator device. The cells were then transferred into 5 ml of culture medium containing 20% FBS, and cultured for 3 days at 37°C and 5% CO₂ before use.

Generation of immature and mature human myeloid dendritic cells (DCs)
Human subject protocols were conducted according to the University of Wisconsin guidelines, and were approved by the University of Wisconsin Medical School’s Minimal Risk Institutional Review Board. All donors provided written informed consent before donating blood samples. PBMCs were purified from fresh blood obtained from healthy adult donors using Ficoll-Paque density gradient centrifugation (GE Health Sciences/Pharmacia). Monocytes were isolated by magnetic sorting using CD14 microbeads on an AutoMACS magnetic separation system (Miltenyi Biotec), according to the manufacturer’s guidelines. The purified monocytes were incubated in culture medium (RPMI 1640 culture medium supplemented with 2 mM L-glutamine; 100 μg/ml penicillin and streptomycin from Mediatech; and 10% FBS from HyClone) containing 300 U/ml human rGM-CSF (Berlex) and 200 U/ml human rIL-4 for 3 days. The cells were then split into two aliquots. One aliquot was maintained in this medium, and the other was exposed to 250 ng/ml of Salmonella typhimurium LPS (Sigma-Aldrich) for an additional 2 days. Differentiation of the monocytes into immature and mature DCs was confirmed by flow cytometric analysis of markers, including CD14, DC-specific ICAM-grabbing nonintegrin, CD86, and CD83.

Results
NKT cell autoreactive responses are independent of CD1d endosomal recycling
To compare the function of CD1d molecules that traffic normally with those that are transported from the ER to the cell surface, but do not recycle through the endosomal system, transfectants were generated that express wild-type human CD1d molecules or truncated tail-deleted CD1d molecules. The human lymphoblastoid cell line 30T3 (a derivative of LCL 721.174) was used to generate the transfectants (29). The tail-deleted CD1d molecules lack the final 10 aa residues of the cytoplasmic tail, which includes the AP-2 internalization motif and a lysine residue that is a target for ubiquitination (15, 17, 33). Hence, because they lack the known signals for internalization into the endosomal system, the tail-deleted CD1d molecules should undergo little or no endosomal recycling, whereas the wild-type should recycle normally.

The wild-type and tail-deleted CD1d transfectants were compared for the ability to stimulate CD1d-dependent autoreactive responses by a highly autoreactive human NKT cell clone (clone J24L.17) that expresses an invariantly rearranged Vα24+ TCR (34). There was no detectable response to untransfected 30T3 cells, whereas both the wild-type CD1d-transfected cells (30T3-d) and the tail-deleted CD1d transfectant (TD 3023-d) stimulated robust cytokine production (Fig. 1A). The response to the tail-deleted 30T3-d could be blocked by an anti-CD1d mAb, confirming that it was due to recognition of the transfected CD1d (Fig. 1A). Thus, in contrast to previous observations of murine NKT cells, the tail-deleted CD1d molecules did not seem diminished in the ability to stimulate autoreactive responses by a human iNKT cell clone.

To extend this analysis, we tested two other human NKT cell clones that express invariantly rearranged Vα24+ TCRs (J3N.4 and J3N.5) and two CD1d-restricted Vα24-negative clones (J24N.22 and J24N.70), for their autoreactive responses to three independent subclones of the TD 3023-d transfectant. All four of these CD1d-restricted NKT cell clones showed similar autoreactive responses to the tail-deleted and wild-type CD1d transfectants (Fig. 1C). Variation in the amount of autoreactive cytokine production in response to different TD 3023-d subclones appeared to correlate with differences in the level of CD1d cell surface expression (Fig. 1, B and C).

To confirm that CD1d endosomal recycling was affected by the tail-deletion mutation, the rate of CD1d internalization from the cell surface was assessed. The 30T3-d and TD 3023-d cells were labeled with an unconjugated anti-CD1d mAb, then washed and incubated for varying times at 37°C to allow internalization, and stained with a fluorescent second-step Ab to detect the remaining mAb-labeled CD1d molecules. Approximately 60% of the cell surface CD1d signal disappeared from the wild-type 30T3-d cells within 2 h, whereas only ~20% of the signal was lost from the TD 3023-d cells within this time (Fig. 1D, upper panel). In contrast, cell surface staining of MHC class I molecules diminished at the same rate for all the CD1d transfectants (Fig. 1D, lower panel), confirming that the tail-deletion mutation resulted in a specific defect in CD1d internalization from the cell surface.

Inefficient presentation of exogenous Ags by tail-deleted CD1d
The tail-deleted and wild-type CD1d transfectants were compared for the ability to present the synthetic glycolipid α-GalCer to NKT cells. Intracellular processing is not required for NKT cell recognition of α-GalCer, and this lipid can be loaded into human rCD1d molecules in a cell-free system, indicating that cellular accessory proteins are not necessary (34). However, the saposins have been
shown to significantly improve the efficiency of α-GalCer presentation, and the reduced pH of endosomal compartments may also facilitate lipid loading into CD1d (7, 8, 35). Therefore, we compared the amount of Ag exposure time required for presentation of α-GalCer by the tail-deleted and wild-type CD1d transfectants. After 4 h of pulsing with α-GalCer, the tail-deleted CD1d transfectants stimulated only modest increases in NKT cell cytokine production, whereas the responses to the wild-type CD1d transfectant were ~4-fold higher and appeared saturated after 1 h of Ag pulse (Fig. 2A). Thus, the tail-deleted CD1d transfectants were only inefficiently able to present exogenously delivered α-GalCer.

To confirm that the tail-deleted CD1d transfectants were unable to present lysosomal Ags, we tested their ability to present the GalGalCer glycolipid. This glycolipid is an analog of α-GalCer that requires cleavage of the terminal galactose by a lysosomal endoglycosidase before it can be recognized by NKT cells (12). Tail-deleted CD1d transfectants that were pulsed with GalGalCer over a 4-h time course stimulated no significant increase in NKT cell responses, whereas the wild-type CD1d transfectant presented GalGalCer in a pulse-time-dependent manner (Fig. 2B). Moreover, even after 16 h of pulsation, there was no significant NKT response to GalGalCer presented by the tail-deleted CD1d transfectants (data not shown). These results demonstrate that the tail-deleted CD1d transfectants are severely deficient in the ability to present lipid Ags from lysosomal compartments, consistent with their defect in endosomal recycling.

**CD1d autoantigen presentation does not require lysosomal acidification**

The finding that tail-deleted CD1d transfectants stimulated normal autoreactive responses from human CD1d-restricted T cell clones suggested that autoantigen presentation does not require access to lysosomes. To further test this requirement for lysosomal functions, the wild-type CD1d transfectants that were pulsed with GalGalCer over a 4-h time course stimulated no significant increase in NKT cell responses, whereas the wild-type CD1d transfectant presented GalGalCer in a pulse-time-dependent manner (Fig. 2B). Moreover, even after 16 h of pulsation, there was no significant NKT response to GalGalCer presented by the tail-deleted CD1d transfectants (data not shown). These results demonstrate that the tail-deleted CD1d transfectants are severely deficient in the ability to present lipid Ags from lysosomal compartments, consistent with their defect in endosomal recycling.
CD1d-restricted T cell clones. There was no significant difference in autoreactive cytokine production by any of the T cell clones in response to the chloroquine-treated APCs, compared with the mock-treated controls (Fig. 3A). In contrast, chloroquine treatment markedly reduced the NKT cell response to α-GalCer (Fig. 3B), and abrogated the presentation of GalGalCer (Fig. 3C). Thus, preventing APC lysosomal acidification selectively affected CD1d-mediated presentation of exogenous Ags.

Effect of MHC class II on CD1d-mediated Ag presentation

A fraction of human CD1d molecules physically associates with MHC class II molecules during cellular trafficking (26), but the functional impact of this association is not clear. To investigate the effect of MHC class II expression on Ag presentation by CD1d, we transfected wild-type CD1d into the 2001 cell line (2001-d). The 3023 and 2001 cell lines were both derived from CD1d, we transfected wild-type CD1d into the 2001 cell line (29). Thus, 3023 and 2001 are closely matched, but 2001 can produce functional MHC class II heterodimers, whereas 3023 lacks them. Flow cytometric analysis confirmed that the 3023-d transfectant was negative for cell surface HLA-DR, whereas the 2001-d cells were positive, and both were positive for CD1d (data not shown).

The 2001-d and 3023-d transfectants were pulsed with a saturating concentration of α-GalCer (50 ng/ml) for varying lengths of time, then washed and tested for the ability to stimulate cytokine secretion by NKT cell clones. This analysis revealed that the 2001-d transfectant was able to take up and present α-GalCer faster than the 3023-d cells (Fig. 4A). Notably, this was the case for both CD4+ and CD4- NKT cell clones, suggesting that the

FIGURE 4. Enhanced CD1d-mediated presentation of exogenous Ags in the presence of HLA-DRβ. The 3023-d transfectant (HLA-DRβ-) was compared with the closely related 2001-d transfectant (HLA-DRβ+) for efficiency of α-GalCer presentation to CD4+ and CD4- NKT cell clones. A, The APCs were pulsed with 50 ng/ml α-GalCer for the indicated times, then washed and exposed to NKT cell clones. The plots show the fold increase over the following autoreactive responses: CD4+ clone, 261 pg/ml (2001-d) and 323 pg/ml (3023-d); CD4- clone, 46 pg/ml (2001-d) and 111 pg/ml (3023-d). B, The APCs were pulsed for 16 h with 50 ng/ml α-GalCer (a saturating concentration), then washed and exposed to NKT cell clones. The plots show the fold increase over the following autoreactive responses: CD4+ clone, 73 pg/ml (2001-d) and 221 pg/ml (3023-d); CD4- clone, 447 pg/ml (2001-d) and 599 pg/ml (3023-d). C, The APCs were pulsed for 16 h with the indicated concentrations of α-GalCer (limiting doses). The data are plotted as fold increase over the following autoreactive responses: CD4+ clone, 602 pg/ml (2001-d) and 727 pg/ml (3023-d); CD4- clone, 1018 pg/ml (2001-d) and 1418 pg/ml (3023-d). D, NKT cell clones were tested for autoreactive cytokine production in response to three subclones each of 2001-d (Δ) and 3023-d (△) with differing CD1d expression levels. The x-axis shows the cell surface staining level of each transfectant as determined by flow cytometric analysis, and the y-axis shows the autoreactive cytokine secretion by the indicated NKT cell clone.
difference was not due to a direct costimulatory effect of MHC class II expression in 2001-d (Fig. 4A). When the APCs were pulsed with a saturating concentration of α-GalCer overnight (16–18 h), the 3023-d transfectant approached or equaled the 2001-d transfectant in presentation of α-GalCer (Fig. 4B), suggesting that 3023-d was not intrinsically deficient in exogenous Ag presentation, but was slower than 2001-d. When nonsaturating amounts of α-GalCer were used to pulse the APCs overnight, the concentrations of lipid Ag required to stimulate α-GalCer-dependent NKT cell responses were ~10-fold lower for the 2001-d line than the 3023-d line, suggesting that the 2001-d line was more efficiently able to load limiting doses of exogenous Ags than 3023-d (Fig. 4C).

In contrast, the autoreactive responses of NKT cell clones to the 3023-d and 2001-d cell lines appeared similar. Three subclones each of 2001-d and 3023-d that expressed different levels of cell surface CD1d were tested for the ability to stimulate autoreactive cytokine secretion by NKT cell clones in the absence of added Ags (Fig. 4D). As we had observed previously for the tail-deleted CD1d transfectants, the autoreactive responses of NKT cell clones appeared very sensitive to the level of CD1d cell surface expression, but there did not seem to be a significant difference between the responses stimulated by 3023-d and 2001-d cells (Fig. 4D). Hence, the presence of HLA-DRαβ appeared to selectively affect the rate and efficiency of exogenous Ag presentation by CD1d, without substantially enhancing autoantigen presentation.

**CD1d intracellular localization**

We used confocal microscopy to investigate whether there were detectable differences in lysosomal localization of CD1d in the 2001-d and 3023-d lines. The permeabilized cells were stained for CD1d, and LAMP-1 as a marker of lysosomes. In both cell lines, a fraction of the intracellular CD1d signal overlapped with that of LAMP-1, and a fraction appeared not to colocalize with LAMP-1 (Fig. 5, A and B). The percentage of the CD1d signal that overlapped with LAMP-1 did not appear significantly different for a sampling of 66 2001-d cells compared with 77 3023-d cells (Fig. 5E). The transfectants were also analyzed for colocalization of HLA-DR and LAMP-1, and HLA-DR and CD1d. As expected, the HLA-DR signal for the 3023-d cells was no greater than that of the isotype control (data not shown). In the 2001-d cells, the intracellular HLA-DR signal overlapped almost completely with that of LAMP-1, and the CD1d signal partially overlapped with that of HLA-DR (Fig. 5, C and D). Hence, these results are consistent with previous findings that intracellular human CD1d is localized partially within lysosomal vesicles and partially in other compartments (37), and suggest that expression of MHC class II molecules does not greatly affect the steady-state fraction of CD1d that is present in lysosomes.

**HLA-DR promotes rapid CD1d internalization**

To further investigate the basis for the exogenous Ag presentation by the 2001-d and 3023-d transfectants, we tested whether there was a difference in the rate of CD1d internalization from the cell surface. The 2001-d and 3023-d cells were labeled with an unconjugated anti-CD1d mAb, then washed and incubated for varying times at 37°C, and stained with a fluorescent second-step Ab to detect the remaining labeled CD1d molecules. The cell surface CD1d signal disappeared more rapidly from the 2001-d than the 3023-d cells (Fig. 6A), suggesting faster internalization. To confirm this, cell surface proteins were biotinylated on CD1d-transfected and mock-transfected 3023 and 2001 cells using a cleavable cross-linking reagent, and then the cells were incubated at 37°C for varying times to allow CD1d internalization, and the remaining surface biotin was cleaved. The cells were then lysed, and the amount of the biotinylated CD1d (representing the molecules that were protected from biotin cleavage by internalization) was determined by a specific capture ELISA. A higher fraction of the total CD1d consistently appeared biotinylated in the 2001-d than in the 3023-d cells (Fig. 6B), suggesting that the rate of CD1d internalization was faster in these cells. The mock-transfected 3023 and 2001 cells gave little or no detectable signal in this assay, confirming that the assay was specific for biotinylated CD1d (Fig. 6B).

In contrast, CD1d emerged from the endosomal system at the same rate in the 3023-d and 2001-d cells (Fig. 6C). The two cell lines were treated with cycloheximide to prevent new protein synthesis, and the CD1d present at the cell surface was blocked with

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/content/jimmunol/168/12/6186.full.png)
an unconjugated anti-CD1d mAb (clone CD1d42.1). The cells were then incubated at 37°C, and aliquots were removed at varying times and stained with fluorescently labeled anti-CD1d (also clone CD1d42.1) to detect unblocked CD1d molecules that had newly emerged from the endosomal system. Because the rate of CD1d exit from the endosomal system appeared similar in the two cell lines, but internalization appeared faster in the HLA-DR2001-d cells, these results suggested that the presence of MHC class II molecules specifically facilitates CD1d entry into the endosomal system. This result is consistent with a role for the Ii in promoting CD1d endosomal trafficking, because the Ii is known to facilitate MHC class II transport into the endosomal system, but it becomes degraded within lysosomes and thus does not contribute to egress from lysosomes to the cell surface.

Role of the Ii

Both the 3023-d and 2001-d cells express the Ii, and therefore, to investigate the effect of MHC class II expression on CD1d function in the absence of Ii, we tested CD1d-transfected HeLa cells. HeLa cells are ordinarily negative for MHC class II and Ii, but MHC class II expression is induced by exposure to IFN-γ, whereas the Ii is not (26, 38). CD1d-transfected HeLa cells were treated with IFN-γ for 96 h, resulting in >10-fold elevation of MHC class II expression, but little or no increase in Ii expression (Fig. 7A). The IFN-γ-treated and untreated control CD1d-HeLa cells were pulsed with α-GalCer or GalGalCer for varying lengths of time, and

![FIGURE 6. Rate of CD1d cell surface trafficking in 3023-d and 2001-d cells. A, Internalization of CD1d from the cell surface was assessed by flow cytometry. The plots show the amount of cell surface staining as a percentage of the starting level. B, CD1d internalization assessed by biochemical analysis. The cell surface proteins of CD1d-transfected or mock-transfected 2001 and 3023 cells were biotinylated, and then the biotin was cleaved after the indicated incubation times. Cell lysates were prepared, and biotinylated and total CD1d were assayed by specific capture ELISA. Results are shown as the biotinylated CD1d signal normalized by the total CD1d signal. C, Emergence of CD1d molecules from the endosomal system was analyzed by flow cytometric analysis of cycloheximide-treated cells. Results are shown as the emerged CD1d signal normalized by the total CD1d expression.]

![FIGURE 7. Effect of Ii. A, Induction of MHC class II expression in CD1d-transfected HeLa cells (HeLa-d) by IFN-γ treatment. HeLa-d cells were treated for the indicated times with 200 U/ml IFN-γ, and analyzed by flow cytometry for cell surface expression of MHC class II, or fixed, permeabilized, and analyzed for expression of the Ii. The results are shown as the fold change compared with untreated HeLa-d cells. B, Ag presentation by IFN-γ-treated and untreated HeLa-d cells. The HeLa-d cells were pulsed with 50 ng/ml α-GalCer or GalGalCer for the indicated times, and tested for stimulation of cytokine production by an NKT cell clone. C, Knockdown of Ii protein expression by siRNA treatment affects CD1d cell surface expression in HLA-DR2001-d cells. CD1d expression was analyzed by flow cytometry in unpermeabilized (■) and permeabilized (□) 3023-d and 2001-d cells. The results are shown as the CD1d signal of the siRNA-treated cells, normalized by the CD1d expression of mock-treated cells. The 100% mark is shown as a dashed line. D, Internalization of CD1d (left plot) and MHC class I (right plot) in negative control and Ii siRNA-treated 3023-d and 2001-d cells. Internalization was assessed by flow cytometric analysis. The 2001-d cells are represented by squares, and the 3023-d cells by triangles; filled symbols show cells that were transfected with negative control siRNA, and open symbols show those that were transfected with siRNA specific for the Ii.]}
tested for the ability to stimulate cytokine secretion by NKT cells. There was no significant difference in the NKT cell responses to the treated and untreated CD1d-HeLa cells (Fig. 7B), suggesting that MHC class II expression in the absence of Ii was not sufficient to promote CD1d-mediated presentation of exogenous Ags.

Therefore, to further investigate the requirement for Ii expression, transient siRNA transfection was used to knockdown the protein levels of Ii in the 3023-d and 2001-d lines. From 48–96 h after transfection, the total amount of Ii protein in the siRNA-treated 3023-d and 2001-d cells showed ~50% reduction compared with the amount detectable in cells that were transfected with negative control siRNA, or in untransfected cells (data not shown). The siRNA-transfected and control cells were tested for cell surface and total (cell surface plus intracellular) CD1d. The level of cell surface CD1d expression was diminished by a mean of 31% (±5.9% SD) in four experiments in the 2001-d cells that were transfected with Ii siRNA, compared with untreated 2001-d cells (Fig. 7C). There was no significant reduction in the total CD1d expression in the Ii siRNA-treated 2001-d cells, and negative control siRNA-treated cells appeared similar to untreated control cells, suggesting that the reduction of CD1d surface expression was a specific effect of the Ii knockdown. In contrast, Ii knockdown in the 3023-d cells resulted in CD1d cell surface staining that was reduced by a mean of 5% (±7.4%, n = 4) compared with the untreated and control siRNA-treated cells (Fig. 7D). Thus, in the presence of HLA-DRαβ, a fraction of the CD1d molecules depends on access to Ii for cell surface expression.

To confirm the role of Ii in enhancing CD1d internalization from the cell surface, we investigated whether the rate of internalization of the remaining cell surface CD1d molecules was altered in the Ii knocked-down cells. CD1d staining disappeared from the cell surface at a slower rate in 2001-d cells that were treated with Ii siRNA, than in those that were treated with negative control siRNA (Fig. 7D, left panel). In contrast, the CD1d signal diminished at an identical rate for 3023-d cells that were treated with Ii siRNA or negative control siRNA (Fig. 7D, left panel). The rate of MHC class I internalization appeared similar for both the 3023-d and 2001-d cell lines, regardless of whether they were transfected with Ii or negative control siRNA (Fig. 7D, right panel). Hence, in the presence of HLA-DRαβ, the Ii specifically influences the kinetics of CD1d cell surface expression. Thus, association with HLA-DR/Ii complexes appears to promote rapid internalization of CD1d into the endosomal system.

Ag presentation differences between immature and mature DCs

In immature myeloid DCs, MHC class II molecules are prominently localized to intracellular Ag-processing vesicles, and rapidly recycle from the cell surface into the endosomal system (39). Upon DC maturation, MHC class II molecules are transported to the cell surface, and expression there is stabilized (39). Immature myeloid DCs were generated by culturing freshly isolated monocytes with GM-CSF and IL-4 for 3 days, and one-half were then further matured by exposure to LPS for an additional 2 days. The resulting immature and mature DCs were tested for the ability to present α-GalCer and GalGalCer to NKT cells. Both immature and mature DCs presented α-GalCer, although the immature DCs appeared to be more efficient (Fig. 8A). The immature DCs also presented GalGalCer, but the mature DCs showed little or no ability to present this Ag (Fig. 8B). To confirm that MHC class II trafficking was altered in the two DC populations, the rate of internalization from the cell surface was assessed by flow cytometric analysis. Consistent with previously reported results, the MHC class II staining on immature DCs diminished by ~70% within 2 h, whereas it was only reduced by ~20% on mature DCs (Fig. 8C).

These results demonstrate that CD1d-mediated Ag presentation differs significantly in immature and mature DCs. Mature DCs presented exogenously added α-GalCer, which does not require intracellular processing, but were not able to present GalGalCer, which requires glycosidic trimming in lysosomes before it can be recognized by NKT cells. In contrast, immature DCs efficiently presented both lipids. Hence, mature DCs appear to have a selective defect in presenting lysosomally dependent lipid Ags. Whether this is due to differences in lipid uptake, transport, or processing by lipid transfer proteins or glycosidic enzymes remains unclear. However, these results are consistent with the possibility that the reduction in endosomal recycling of MHC class II and Ii complexes upon DC maturation affects CD1d-mediated presentation of exogenous Ags.

Discussion

The results presented in this study demonstrate that the autoreactive responses of a panel of human CD1d-restricted T cells do not depend on CD1d endosomal recycling or lysosomal acidification. This points to a surprising difference between human and murine NKT cells, because it has been clearly established by a number of previous studies that the autoreactive responses of murine iNKT cells are dependent on CD1d trafficking to lysosomes and require unimpaired lysosomal function (19–21). Moreover, recent studies
The Journal of Immunology

have demonstrated specific iNKT cell recognition of iGb3, a mammalian glycolipid that is thought to accumulate in lysosomes through the metabolic activity of the Hex b enzyme (22). We have previously analyzed recognition of iGb3 by the human NKT cell clones tested in this study, and found that one iNKT cell clone (J3N-4) demonstrated enhanced cytokine secretion in response to APCs that were pulsed with iGb3, but increased responses were not observed for two other iNKT cell clones (J3N.5 and J24L.17), or for three Vα24-negative CD1d-restricted clones (34). However, because the two human iNKT cell clones that did not respond to iGb3 had very high autoreactive responses to unpulsed APCs, it is unclear whether or not they could recognize iGb3. Our current findings suggest that iGb3 is not responsible for the autoreactive responses of the NKT cell clones to the APCs tested in this study, because this Ag requires endosomal trafficking for loading into CD1d molecules.

The Ags responsible for stimulating the autoreactive responses of human NKT cells remain unknown. It is possible that the autoreactivity of our NKT cell clones does not require presentation of a specific Ag, and is dependent simply on the intrinsic affinity of their TCRs for the CD1d molecule. However, we have observed previously that the clones show no detectable response to secreted human CD1d molecules, but are able to respond if the CD1d molecules are pulsed with α-GalCer, or in one case, weaker Ags such as phospholipids (34). Thus, the secreted CD1d molecules can be recognized by the NKT clones, but they appear not to contain an appropriate endogenous Ag. Hence, it seems likely that similar to murine NKT cells, the autoreactivity of human NKT cells requires presentation of specific Ags by CD1d, but that the autoantigens recognized by these clones are loaded before CD1d entry into the endosomal system.

Interestingly, the autoreactive responses of noncanonical murine NKT cells (i.e., CD1d-restricted T cells that use diversely rearranged TCRs) also do not depend on CD1d endosomal trafficking (19, 20). These diverse NKT cells differ from iNKT cells in that they do not recognize α-GalCer (35, 40). Moreover, their autoreactive responses were not affected by the Hex b mutation, which prevents the presentation of iGb3 by CD1d (22). Thus, noncanonical murine NKT cells appear to recognize different cellular autoantigens than murine iNKT cells. Although the human Vα24+ and Vα24− clones that we have used in this study differ from diverse murine NKT cells in that they all recognize α-GalCer, it is notable that they share the lack of a requirement for CD1d endosomal trafficking. Thus, it is possible that the cellular Ag specificities of human NKT cells may be more similar to those of the noncanonical murine NKT cell subset than to murine iNKT cells.

Our results are consistent with previous findings indicating that lysosomal functions facilitate the presentation of exogenous lipids by human CD1d (8, 24). The signals that control the lysosomal trafficking of human CD1d molecules remain poorly understood. We show in this study for the first time that cotrafficking with MHC class II molecules and the Ii selectively enhances CD1d-mediated presentation of exogenous Ags.

There are several mechanisms by which this could occur. One possibility is that CD1d binding to MHC II/Ii complexes results in altered intracellular trafficking, such that loading of exogenous Ags is favored. The Ii contains cytoplasmic dileucine- and tyrosine-based trafficking motifs that direct MHC class II molecules to lysosomes (14). However, this can occur by two distinct trafficking routes, as follows: MHC/Ii complexes can be transported intracellularly directly from the Golgi complex to lysosomes, or they can follow the secretory pathway to the cell surface and then become rapidly internalized into the endosomal system (28). Our data are more consistent with this second route of trafficking, because knockdown of Ii expression resulted in diminished cell surface expression of CD1d in the MHC class II αβ+ 2001-d cell line, but not in the MHC class II β− 3023-d line, suggesting that CD1d is complexed with both MHC class II and Ii at the cell surface. Because the Ii is cleaved by proteases after entry into the lysosome, it should not affect trafficking after lysosomal delivery, and thus, should only affect CD1d cell surface expression if this occurs before entry into the lysosome. However, the presence of additional trafficking signals (e.g., an AP-2-binding motif) conferred by the Ii might explain our observation that CD1d molecules were internalized into the endosomal system more rapidly in the 2001-d cells. The increased rate of entry into the endosomal system could explain the enhanced efficiency of exogenous Ag presentation in the 2001-d cells, because this might allow for more rapid uptake of Ags that are internalized into intracellular compartments.

Additionally, association with MHC/Ii complexes might prevent CD1d from binding intracellular lipids until the Ii is cleaved in the lysosome, which could specifically facilitate uptake of lysosomal lipids. The observation that Ii knockdown resulted in reduced CD1d surface expression in the 2001-d cells suggests that binding to MHC class II and Ii complexes rescues a pool of CD1d molecules that otherwise are not able to traffic to the cell surface, perhaps because they did not fold stably or did not associate with an ER lipid. Thus, the Ii might stabilize a fraction of the CD1d molecules that have not bound a lipid in the ER, until lipids are loaded in the endosomal system. Finally, it is also possible that association with MHC class II molecules recruits CD1d molecules into lipid raft microdomains that are enriched for costimulatory molecules, and that this makes the MHC class II-associated CD1d molecules more potently stimulatory to NKT cells.

The effect of MHC/Ii cotrafficking on CD1d-mediated presentation of extracellular Ags has important implications for the Ag-presenting function of myeloid DCs. MHC class II and Ii trafficking are highly regulated in DCs, such that uptake, processing, and loading of exogenous Ags into MHC class II molecules are very efficient in immature DCs, whereas mature DCs no longer perform these functions effectively, but have stabilized the expression of MHC class II/peptide complexes at the cell surface, which facilitates their ability to promote Ag-specific T cell responses (39). Our results suggested that the differences in MHC/Ii trafficking between mature and immature DCs might contribute to more efficient uptake of exogenous Ags by CD1d molecules in immature DCs. However, we also observed that 3023-d cells were able to present GalGalCer, which demonstrates that cotrafficking with MHC/Ii complexes is not required for this function. Thus, there may be additional mechanisms that block lysosomal CD1d lipid uptake or glycosidic processing in mature DCs. Understanding the distinct factors that affect the presentation of intracellular and extracellular Ags by human CD1d molecules will provide important insights into how the complex functions of NKT cells are regulated.

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Disclosures
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