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Mutation of a Positively Charged Cytoplasmic Motif within CD1d Results in Multiple Defects in Antigen Presentation to NKT Cells

Jung Hoon Shin, Ji-Young Park, Young Hyun Shin, Hyunji Lee, Yoon-Kyung Park, Sundo Jung, and Se-Ho Park

CD1d is an MHC class I-like molecule that presents glycolipid Ags to types I and II NKT cells. The YxxI motif in the cytoplasmic tail of CD1d contributes to its intracellular localization to the endolysosomal compartment and is important for Ag presentation to type I NKT cells. In this study, we identified the ^{327–329}RRR motif in CD1d and showed that it is critical for the control of CD1d intracellular trafficking and Ag presentation. The replacement of the arginines in this motif with alanines resulted in the extensive accumulation of CD1d in lysosomes but did not affect the cell surface expression. The defect in its cellular localization was accompanied by defects in Ag presentation to both type I and type II NKT cells. These results demonstrated that the ^{327–329}RRR motif of CD1d is required for proper cellular distribution of CD1d and optimal Ag presentation to both type I and type II NKT cells. *The Journal of Immunology*, 2012, 188: 2235–2243.

The mammalian CD1 family consists of five isotypes that can be classified into two groups based on sequence homology. Group 1 is composed of CD1a, CD1b, CD1c, and CD1e, whereas group 2 consists of only CD1d (1). The structure of CD1d is similar to that of MHC class I, which forms noncovalent heterodimers with β 2-microglobulin (2); however, unlike MHC class I molecules, which are polymorphic and present self- and foreign-peptide Ags to conventional CD8⁺ T cells, CD1d is nonpolymorphic and presents endogenous and foreign glycolipid Ags to NKT cells (3–5).

NKT cells can be divided into two types based on their TCR α -chain. Type I NKT cells (V α 14⁺ invariant NKT [iNKT] cells) use the V α 14-J α 18 invariant chain, and type II NKT cells use other various V α -chains (6). Both type I and type II NKT cells are CD1d-autoreactive T cells and can recognize endogenous Ags presented by mouse CD1d (mCD1d) in mice (7–9). To activate type I NKT cells, mCD1d must traffic through the endolysosomal pathway, where it is loaded with glycolipid Ags; in contrast, the activation of type II NKT cells does not require endolysosomal trafficking of mCD1d (10–13).

Isoglobotrihexosylceramide (iGb3) was recently identified as an endogenous self-Ag that can stimulate type I NKT cells (14). In addition to endolysosomal trafficking of CD1d (14), the presentation of iGb3 by CD1d requires lysosomal saposin, which trans-

fers lipids from the lysosomal membrane compartments to the CD1d-binding groove (15). Furthermore, the development of type I NKT cells is impaired in iGb3-deficient mice (14).

The tyrosine-based YxxI (where x is any amino acid) motif located in the cytoplasmic tail of mCD1d (YQDIR) is involved in the regulation of Ag loading and the intracellular trafficking of CD1d to the endolysosomal compartment; thus, this motif is critical for Ag presentation and the development of type I NKT cells. Deletion of the YxxI motif by the deletion of the CD1d cytoplasmic tail (CD1d-TD) resulted in a defect in type I NKT cell development and activation (11–13, 16). The tyrosine-based motif in the cytoplasmic tail of mCD1d or human CD1b interacts with the μ subunit of adaptor protein-3 (AP-3), which aids in the transport of CD1d to endosomal or lysosomal compartments (17–20). A deficiency in AP-3 also inhibits the development of iNKT cells (18). Despite the critical roles of the cytoplasmic endosomal targeting motif in the cellular distribution of CD1d and type I NKT cell development, CD1d-TD is capable of activating type II NKT cells (11–13). These results suggested that distinct intracellular compartments are involved in CD1d Ag processing for Ag presentation to type I and type II NKT cells; therefore, intracellular trafficking of CD1d must be differentially regulated (10). In contrast to the extensive evidence supporting the endolysosomal compartment as the Ag-loading site for the stimulation of type I NKT cells, the Ag-loading mechanism for stimulation of type II NKT cells is poorly understood. It has been proposed that stimulation of type II NKT cells requires that glycolipid Ags are loaded on CD1d as it is transported to the cell surface via the *trans*-Golgi network (TGN); however, there is no conclusive evidence supporting this mechanism (12).

In this study, we demonstrated that the three consecutive arginine residues (^{327–329}RRR) between the transmembrane region and cytoplasmic tail of mCD1d are required for presentation of glycolipid Ags to both type I and type II NKT cells. Furthermore, we demonstrated that the ^{327–329}RRR motif of mCD1d plays an essential role in the intracellular trafficking of CD1d to the lysosomal compartment; in fact, this trafficking is required for the stimulation of both types I and II NKT cells.

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Abbreviations used in this article: AP-3, adaptor protein-3; DC, dendritic cell; α -GalCer, α -galactosylceramide; iGb3, isoglobotrihexosylceramide; iNKT, invariant NKT; mCD1d, mouse CD1d; RA, ArgArgArg^{327–329}→AlaAlaAla; TD, tail deletion; TGN, *trans*-Golgi network; WT, wild-type.

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Materials and Methods

Mice, cell lines, and reagents

C57BL/6 mice were purchased from the Jackson Laboratory, and V α 14 transgenic mice were provided by Dr. Albert Bendelac (University of Chicago, Chicago, IL). All mice used in the current study were on the C57BL/6 background and were housed in a specific pathogen-free environment at Korea University. The experimental protocols of this study were approved by the Laboratory Animal Care and Use Committee of Korea University. Murine type I NKT hybridomas (DN32.D3), murine type II NKT hybridomas (TCB11 and 1C8.DC1), and RBL cells were as described (8, 21). The murine B cell lymphoma line A20 and the CTLL-2 cell line were purchased from American Type Culture Collection. Cell cultures were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, 10 μ g/ml gentamicin sulfate, 50 μ M 2-ME (all from Life Technologies, Grand Island, NY), and 10% heat-inactivated FBS (Hyclone).

A mAb specific for mouse CD1d (20H2) was provided by Dr. Albert Bendelac. mAbs specific for mouse TCR β (H57), IFN- γ (XMG-1), IL-4 (11B11), CD1d (1B1), NK1.1 (PK136), clathrin H chain (23), flotillin (18), and mouse IgG (A85-1) were purchased from BD Biosciences. A soluble mouse CD1d-mouse IgG1 Fc fusion protein was purchased from BD Biosciences. Mouse anti-rat LAMP-1 mAb (LY1C6), HRP-conjugated goat anti-rat IgG(H+L) Ab, and HRP-conjugated goat anti-mouse IgG(H+L) Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 633-conjugated goat anti-mouse IgG(H+L) Ab, PE-conjugated anti-mouse CD45.2 mAb, and CFSE were purchased from Molecular Probes. α -galactosylceramide (α -GalCer) and iGb3 were purchased from Alexis Biochemicals.

mCD1d transfectants

The cloning and expression of wild-type (WT) and cytoplasmic tail-deleted mutant mCD1d were described previously (7, 11). The ArgArgArg³²⁷⁻³²⁹ \rightarrow AlaAlaAla (CD1d-RA) and ArgArgArg³²⁷⁻³²⁹ \rightarrow AlaAlaAla tail-deleted (CD1d-RATD) constructs were generated using oligonucleotide-directed mutagenesis. The final PCR products were cloned into the PCR3 expression vector. The transfection was performed using 10–20 μ g linearized expression vectors, stable transfectants were selected via G418 treatment, and subclones with CD1d expression levels similar to those observed in CD1d-WT cells were selected.

Flow cytometry

Cells were washed and blocked with an anti-Fc γ R2/3 mAb (2.4G2) for 15 min and then labeled for 30 min on ice with the appropriate mAbs. For detection of surface and intracellular CD1d, we examined CD1d-transfected cell lines, as described previously (22). For intracellular cytokine staining, cells were fixed with a Cytotfix/Cytoperm kit (BD Biosciences), according to the manufacturer's protocol. The stained cells were analyzed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Cell enrichment via MACS

Dendritic cells (DCs) were enriched from splenocytes of naive mice via MACS using anti-CD11c-conjugated magnetic beads (Miltenyi Biotec). V α 14⁺ iNKT cells and whole NKT cells were sorted positively or negatively from the spleens of V α 14 transgenic mice via MACS (Miltenyi Biotec) using PE-conjugated CD1d/ α -GalCer dimer and anti-PE mAb-conjugated magnetic beads or a mixture of anti-MHC II, anti-CD8 α and anti-B220-conjugated magnetic beads, respectively. The purity of the cells sorted using the MACS system was >85%.

Biotin-based internalization and recycling assay

The biotin-based internalization assays were performed as previously described (11, 12). A cleavable biotin reagent, Sulfo-NHS-S-S-Biotin (Pierce), was dissolved in HBSS (Sigma) to a concentration of 10 mg/ml and added to 1×10^7 cells at a final concentration of 10 mM in a final volume of 300 μ l. The cells were rotated for 15 min at 4°C and washed three times with cold HBSS to quench any excess NHS. The biotinylated cells were incubated in culture medium at 37°C for the indicated periods to allow internalization of biotinylated cell surface proteins. The control cells were kept in cold HBSS until ELISA analysis. After the internalization period, the cells were chilled to 4°C with an excess of cold medium to stop internalization. Biotinylated proteins that remained on the cell surface were stripped of their biotin by rocking cells in a glutathione solution (50 μ M glutathione, 75 μ M NaCl, 1 μ M EDTA, and 75 μ M NaOH in 10% FBS) for two cycles of 20 min at 4°C. The cells were washed three times

with HBSS and lysed in 1% Triton X-100. To determine the amount of biotinylated CD1d protein, the cell lysates were added to ELISA plates coated with anti-mCD1d Ab (20H2). HRP-conjugated streptavidin (R&D Systems) was used to detect biotin, and the amount of captured biotin in the lysates of stripped cells was compared with that of nonstripped control cells. For the recycling assay, the surface proteins were biotinylated at 4°C, and the cells were incubated for 30 min at 37°C. Residual surface biotin was stripped, as described above, and the cells were incubated for the indicated periods to allow the recycling of internalized biotinylated CD1d to the surface. Surface biotin was stripped again, and the residual biotinylated CD1d molecules were measured using an ELISA, as described above. The amount of captured biotin in the lysates of the cells that were stripped twice was compared with that of control cells that were stripped only once.

Ab-based internalization assay

The Ab-based internalization assays were performed following a previously described protocol (19), with some modifications. RBL transfectants were washed and blocked in staining buffer containing anti-Fc γ R2/3 mAb (2.4G2) for 30 min on ice. The cells were then incubated with biotin-conjugated anti-CD1d mAb (20H2) for 30 min on ice and shifted to 37°C for different periods to allow internalization. Control cells were kept on ice. The cells were then surface labeled with streptavidin-PE and analyzed using flow cytometry.

Conjugation assay

Conjugation of DN32.D3 cells and RBL cells was analyzed following a previously described protocol (23), with some modifications. Briefly, α -GalCer-pulsed RBL cells were labeled with CFSE. DN32.D3 cells were labeled with PE-conjugated anti-mCD45.2 Ab. Equal numbers of CFSE-labeled RBL cells and PE-labeled DN32.D3 cells were mixed and incubated for 30 min at 37°C. The cells were collected, fixed with 1% paraformaldehyde for 10 min at room temperature, washed, and resuspended in PBS for flow cytometry analysis.

In vitro activation of NKT cells

For stimulation of type I NKT cells by exogenous Ags, 5×10^4 APCs (7, 11) were pulsed with the indicated concentrations of α -GalCer or iGb3 for the indicated periods and cocultured with 5×10^4 NKT cells or hybridoma cells for 18 h. The concentrations of cytokines in the culture supernatants were measured using an ELISA or a CTLL-2 assay. For stimulation of type I or type II NKT cells with endogenous Ags, experiments were performed similarly to those described above but without exogenous Ag treatment.

Treatment with lysosomotropic agent and fixation of APCs

Before the stimulation of NKT cells, 5×10^4 APCs were treated with 50 μ M chloroquine (Sigma) or 10 nM concanamycin A (Sigma) for 6 h. After treatment, excess lysosomotropic agents were removed by washing with PBS. APCs were fixed with 0.05% glutaraldehyde and then washed and quenched with excess fixation solution of 0.2 M glycine in PBS. For pulse experiments with exogenous α -GalCer, APCs were pulsed with exogenous Ags for 4 h in the presence of lysosomotropic agents without fixation. The APCs pretreated with lysosomotropic agents were cocultured with 5×10^4 NKT hybridoma cells for 18 h.

Confocal microscopy

To study the colocalization of mCD1d with LAMP-1, PE-conjugated rat anti-mouse CD1d (1B1) and mouse anti-rat LAMP-1 (LY1C6) were used as primary Abs. Alexa Fluor 633-conjugated goat anti-mouse IgG(H+L) was used as a secondary Ab. Cells were incubated on coverslips for 18 h at 37°C to allow for adherence. The coverslips were washed with PBS, and the cells were fixed with 4% paraformaldehyde for 15 min, quenched with 100 mM glycine in PBS for 10 min, permeabilized with saponin solution (0.2% BSA and 0.05% saponin in PBS) for 20 min, and blocked with 2.4G2 in saponin solution for 30 min. Coverslips were then incubated in primary Abs diluted in saponin solution for 1 h, washed three times with PBS, and incubated in secondary Ab for 1 h. To detect the colocalization of α -GalCer/mCD1d complexes with LAMP-1 and free mCD1d, cells were incubated with α -GalCer for 24 h on coverslips and were fixed and permeabilized as described above. The cells were then stained with mouse anti- α -GalCer/mCD1d complex mAb (L363; eBioscience), biotin-conjugated rat anti-mCD1d mAb (20H2), and rabbit polyclonal anti-LAMP-1 (Abcam) Ab at room temperature. Tetramethylrhodamine isothiocyanate-conjugated donkey anti-mouse IgG(H+L) Ab, Alexa Fluor 488-conjugated streptavidin (Invitrogen), and Alexa Fluor 633-conjugated goat anti-rabbit IgG(H+L) Ab

were used as secondary Abs. The coverslips were then washed and mounted on slides. An LSM 510 Meta confocal microscope (Carl Zeiss) was used to obtain all confocal images.

Isolation and analysis of lipid rafts

Lipid rafts were isolated through sucrose density-gradient centrifugation, as previously described (21). Subconfluent cells from three 150-mm culture dishes, or three homogenized spleen tissues, were lysed on ice for 30 min in 1 ml lysis buffer (1% Brij35 in 25 mM HEPES, 1 mM EDTA, and 150 mM NaCl [pH 6.5]) supplemented with protease inhibitor mixture (Complete tablets; Roche) and homogenized (10 strokes) with a loose-fitting glass Dounce homogenizer. The homogenates were mixed with 1 ml 80% sucrose, made with HEPES buffer (25 mM HEPES, 1 mM EDTA, and 150 mM NaCl [pH 6.5]), and placed at the bottom of a centrifuge tube. The samples were then overlaid with 6.5 ml 30% sucrose and 3 ml 5% sucrose and centrifuged at $188,000 \times g$ for 16 h at 4°C. One-milliliter fractions were collected from the bottom to the top of the gradient and subsequently analyzed with an immunoblot. The pellet present at the bottom of the gradient was sonicated in 1 ml 1% SDS lysis buffer before analysis. Rat anti-mouse CD1d (20H2), mouse anti-mouse flotillin, and mouse anti-mouse clathrin H chain were used as primary Abs. HRP-conjugated goat anti-rat IgG(H+L) and anti-mouse IgG(H+L) were used as secondary Abs for immunoblot analysis.

Statistical analysis

Unless stated otherwise, Student *t* tests were used to determine the statistical significance of differences between the comparison groups; $p < 0.05$ was considered significant.

Results

Construction of CD1d mutant cell lines

The comparison of the amino acid sequences of CD1d molecules among mammalian species resulted in the discovery of a conserved positively charged motif at the cytoplasmic end of the transmembrane region (Supplemental Fig. 1A). A stretch of three consecutive arginines (^{327–329}RRR) found in mCD1d was conserved among other species, although in some cases a histidine or lysine was substituted for the arginine. The position of the RRR motif between the transmembrane and cytoplasmic regions, as well as the strong conservation of the motif among mammalian species, led to the examination of the role of this motif in CD1d metabolism. To analyze the function of this positively charged motif of mCD1d, we constructed a mutant CD1d in which the arginine motif (^{327–329}RRR) was replaced with three nonpolar alanines (CD1d-RA) (Fig. 1A). RBL cells were transfected with the arginine mutant construct, and a monoclonal CD1d-RA-transfected cell line was generated. The overall surface expression level of CD1d in the CD1d-RA transfectants was comparable to those in WT CD1d (CD1d-WT) transfectants and tail-deleted CD1d (CD1d-TD, which had the ^{332–336}YQDIR motif of WT CD1d deleted) transfectants (Fig. 1A, Supplemental Fig. 1B).

Defects in α -GalCer presentation to type I NKT cells by CD1d-RA

We analyzed the ability of α -GalCer-pulsed RBL cells to stimulate DN32.D3 cells, a type I NKT hybridoma. We compared the stimulation by α -GalCer-pulsed RBL cells transfected with CD1d-WT, CD1d-TD, and CD1d-RA. Similar to previous reports (12), cells transfected with CD1d-TD were not able to stimulate DN32.D3 as strongly as were cells transfected with CD1d-WT (Fig. 1B). Interestingly, cells transfected with CD1d-RA showed a defect in DN32.D3 stimulation that was similar to that observed in cells transfected with CD1d-TD (Fig. 1B). The inability of CD1d-RA expression to stimulate NKT cells was confirmed using primary splenic V α 14⁺ iNKT cells. Intracellular staining and ELISA analysis demonstrated that NKT cells stimulated with CD1d-RA produced substantially lower levels of IFN- γ and IL-4 than did NKT cells stimulated with CD1d-WT (Fig. 1C, 1D). In addition, we

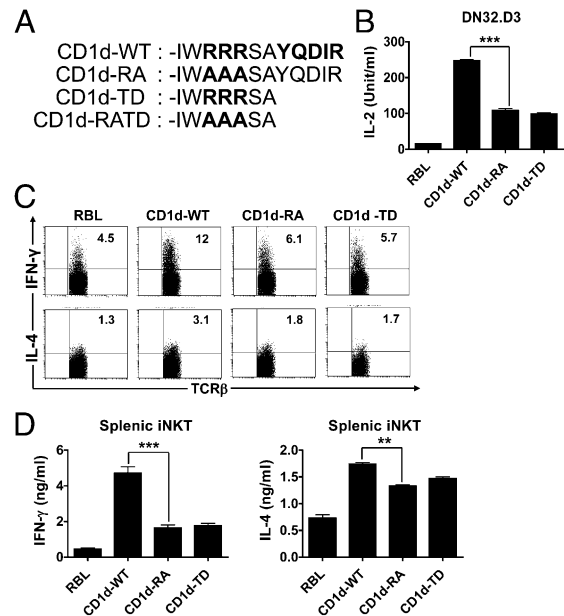


FIGURE 1. Impaired stimulation of type I NKT cells by CD1d-RA. **(A)** The amino acid sequences localized at the cytoplasmic tail of mouse CD1d mutants. **(B)** APCs were pulsed for 4 h with 100 ng/ml α -GalCer, and the excess α -GalCer was removed by washing. Type I NKT hybridomas (DN32.D3) were cultured for 18 h with α -GalCer-pulsed APCs expressing the indicated forms of CD1d. The concentration of IL-2 released by DN32.D3 cells was determined using CTLL-2 assays. The data are shown as the mean \pm SD of triplicate measurements and are representative of five independent experiments. Sorted splenic type I NKT cells were stimulated as described in **(B)**, and the production of IFN- γ and IL-4 was determined after 6 h **(C)** or 18 h **(D)** using intracellular staining **(C)** and ELISA **(D)**. The results shown in **(C)** are representative of CD1d dimer⁺ TCR β ⁺ cells, and the results shown in **(D)** were expressed as the mean \pm SD of triplicate measurements. The data are representative of three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

identified that the overall pattern of autoreactivity of iNKT cells and that of the NKT hybridoma DN32.D3 were also similar to the activity of iNKT cells with α -GalCer-pulsed APCs, although the magnitude is low (data not shown). These findings indicated that the presence of ^{327–329}RRR in mCD1d is important for the presentation of α -GalCer to type I NKT cells.

Defects in endogenous Ag presentation by CD1d-RA to type II NKT cells

Next, we determined whether mCD1d-dependent Ag presentation to type II NKT cells also requires ^{327–329}RRR. To assess the ability of CD1d-RA to present endogenous Ags to type II NKT cells, we used the autoreactive NKT cell hybridomas TCB11 and 1C8DC1, which express non-V α 14 TCRs (24). The hybridomas were cocultured with APCs expressing CD1d-RA or CD1d-TD that were not treated with exogenous glycolipid Ags, and the amount of IL-2 secreted by the hybridomas was measured. As previously reported, CD1d-TD stimulated type II NKT cells to similar levels as observed with CD1d-WT (7, 11–13, 24). Surprisingly, the stimulation of type II NKT cells by CD1d-RA was substantially weaker than the levels observed using either CD1d-WT or CD1d-TD (Fig. 2A). To exclude the possibility that this defect in CD1d-RA-mediated Ag presentation to type II NKT cells was specific to RBL cells, we confirmed these results using the APC cell line A20. Similar to RBL cells, A20 cells expressing CD1d-RA also failed to stimulate type II NKT cell hybridomas (Supplemental Fig. 2C). Furthermore, similar results were obtained when primary splenic NKT cells were activated with APCs expressing CD1d-TD

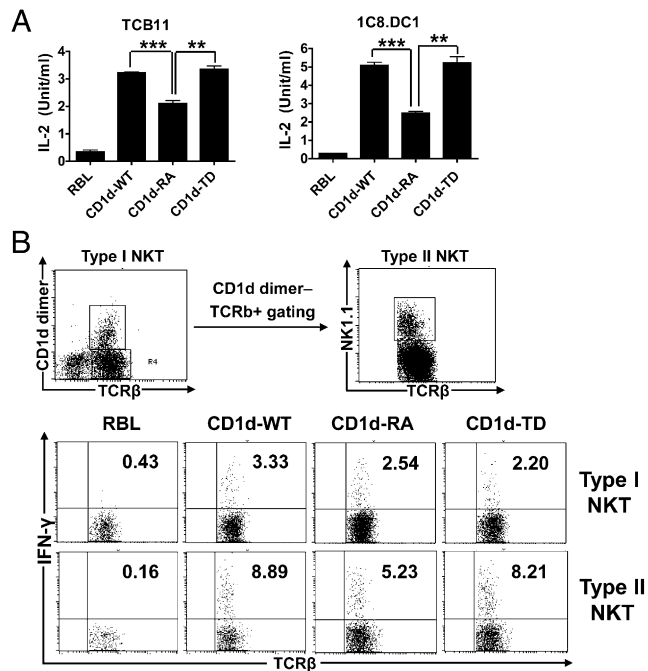


FIGURE 2. Impaired presentation of endogenous Ags to type II NKT cells by CD1d-RA. Autoreactive mouse type II NKT hybridomas TCB11 and 1C8.DC1 (**A**) and splenic type I and type II NKT cells (**B**) were stimulated with RBL cells expressing the indicated mCD1d molecules. The concentrations of IL-2 in culture supernatants were determined as described in Fig. 1. IFN- γ production by type I NKT cells (CD1d dimer⁺ TCR β ⁺) and type II NKT cells (CD1d dimer⁻ NK1.1⁺ TCR β ⁺) was determined using intracellular staining after a 6-h incubation. The results are representative of three independent experiments. ** p < 0.01, *** p < 0.001.

or CD1d-RA. Primary type I NKT cells, identified as CD1d/ α -GalCer dimer⁺ T cells, were stimulated at similar levels by cells expressing CD1d-TD or CD1d-RA; however, primary type II NKT cells (CD1d/ α -GalCer dimer⁻ NK1.1⁺ T cells, whose population includes type II NKT cells, as well as CD1d nonrestricted NKT cells) responded weakly to cells expressing CD1d-RA compared with cells expressing CD1d-WT or CD1d-TD (Fig. 2B, Supplemental Fig. 2). These results clearly demonstrated that the ³²⁷⁻³²⁹RRR motif has an important function in the presentation of endogenous Ags by mCD1d to both types I and II NKT cells.

Internalization and recycling of CD1d-RA

The defects of CD1d-RA in NKT cell stimulation prompted us to investigate whether the internalization of surface-expressed CD1d-RA is impaired in a manner similar to that of CD1d-TD, which had the endosomal-targeting motif in the cytoplasmic tail removed. To determine the rate of internalization of CD1d-RA, we used an Ab-based assay (19) and a biotin-based endocytosis assay (11). CD1d-RA was internalized more slowly than CD1d-WT but substantially more rapidly than CD1d-TD (Fig. 3A, data not shown). The recycling rates of CD1d-TD and CD1d-RA were similar (Fig. 3B,

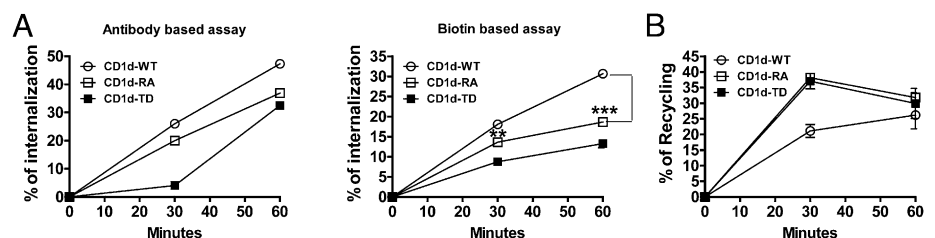
data not shown). The reduced internalization speed of CD1d-RA compared with CD1d-WT might contribute to the defect observed in type I NKT cell stimulation with CD1d-RA; however, it is possible that there is an additional defect inherent to CD1d-RA because it showed similar levels of defective type I NKT cell stimulation, despite its faster internalization compared with CD1d-TD. A recent study suggested that the optimal presentation of α -GalCer to type I NKT cells requires endolysosomal trafficking, although α -GalCer can be directly loaded onto CD1d at the cell surface (16). Because the result in Fig. 1 was obtained after a short 4-h α -GalCer pulse, we extended the α -GalCer treatment up to 16 h prior to DN32.D3 stimulation to test the effects of α -GalCer endolysosomal processing. Unlike shorter α -GalCer pulses (4 h), the degree of type I NKT cell activation by CD1d-RA was higher than that of CD1d-TD when sufficient time for intracellular Ag processing was allowed (>8 h) (Fig. 4A). Because intracellular Ag processing appeared important for Ag presentation by CD1d-RA, we subsequently analyzed the Ag-presenting ability of CD1d-RA using endogenous Ags and iGb3, which has an absolute requirement for endolysosomal processing prior to CD1d loading (14), unlike α -GalCer.

Although the presentation of exogenous iGb3 and endogenous Ags was severely defective in cells expressing CD1d-TD (Fig. 4B, 4C, data not shown), CD1d-RA loaded with exogenous iGb3 or endogenous Ags stimulated type I NKT cells more potently than did CD1d-TD. The degree of type I NKT cell activation by exogenous iGb3 in the context of CD1d-RA, CD1d-TD, and CD1d-WT correlated exactly with the internalization rate for each form of CD1d (Fig. 4A, 4C, data not shown). These results clearly showed that cells expressing CD1d-RA have a defect in the internalization of cell surface molecules, and this defect is responsible for impaired NKT cell activation by the glycolipid Ags, which require endolysosomal processing.

Impairment of α -GalCer presentation by cell surface CD1d-RA

The cells expressing CD1d-RA and presenting exogenous iGb3, endogenous Ags, or exogenous α -GalCer following a long-term incubation (>8 h) activated type I NKT cells more efficiently than did CD1d-TD (Fig. 4), whereas the cells expressing CD1d-RA showed a similar defect to cells expressing CD1d-TD when presenting exogenous α -GalCer that was pulsed for a shorter amount of time. Because exogenous α -GalCer can be directly loaded onto CD1d at the extracellular surface in addition to being loaded in the endolysosomal compartment (16), we hypothesized that the loading of exogenous α -GalCer onto CD1d-RA may be defective not only in the endolysosome but also at the extracellular surface. To examine the direct exogenous α -GalCer loading onto CD1d-RA at the extracellular surface, we fixed α -GalCer-pulsed APCs to prevent intracellular Ag processing before stimulation of DN32.D3. Surprisingly, APCs expressing CD1d-RA showed severe defects in DN32.D3 stimulation compared with APCs expressing CD1d-TD (Fig. 5A). To confirm our results using RBL cells as APCs, we also tested the APC line A20, in which α -GalCer-loaded CD1d-TD was shown to activate type I NKT cells as ef-

FIGURE 3. Defects in the internalization rate of CD1d molecules in cells expressing CD1d-RA. (**A**) The rates of internalization of surface CD1d-WT, CD1d-RA, and CD1d-TD were measured using an Ab-based assay (left panel) and a biotin-based assay (right panel). (**B**) Recycling rates were measured using a biotin-based assay. ** p < 0.01, *** p < 0.001.



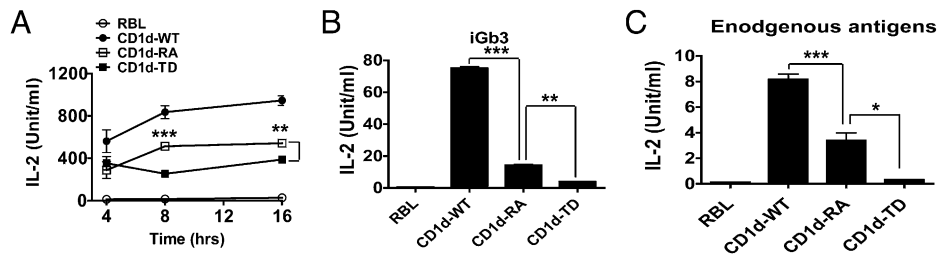


FIGURE 4. Defects in the endolysosomal pathway in cells expressing CD1d-RA. Type I NKT hybridomas (DN32.D3) were cultured for 18 h with Ag-pulsed APCs expressing the indicated forms of CD1d. (A) APCs were pulsed for 4, 8, or 16 h with 100 ng/ml α -GalCer, and the excess α -GalCer was removed by washing. (B) APCs were pulsed for 16 h with 1 μ g/ml iGb3, and the excess iGb3 was removed by washing. (C) APCs were loaded only with self-endogenous Ags without any exogenous Ags. Concentrations of IL-2 in culture supernatants were determined as described in Fig. 1. The data are presented as the mean \pm SD of triplicate measurements and are representative of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.

ficiently as CD1d-WT (16). Similar to the results obtained with the RBL cells, A20 cells expressing CD1d-RA were not able to stimulate DN32.D3 as strongly as A20 cells expressing CD1d-WT or CD1d-TD (Fig. 5B). These results suggested that extracellular loading of α -GalCer onto CD1d-RA was less efficient than that of CD1d-WT or CD1d-TD. Thus, we reasoned that if extracellular α -GalCer loading onto CD1d-RA is defective, then the formation of immunological synapses between type I NKT cells and APCs expressing CD1d-RA and presenting exogenous α -GalCer should be impaired. To test this hypothesis, we examined the formation of immune synapses between type I NKT hybridoma cells and APCs expressing different forms of CD1d using a conjugation assay. As we hypothesized, the formation of immune synapses between DN32.D3 and APCs expressing CD1d-RA was weaker than those between DN32.D3 and APCs expressing CD1d-WT or CD1d-TD (Fig. 5C). Collectively, these results indicated that the ³²⁷⁻³²⁹RRR motif plays a critical role in exogenous α -GalCer loading onto mCD1d both inside the cell and at the cell surface.

Defects in endolysosomal trafficking of CD1d-RA

The defect in presentation of exogenous α -GalCer by CD1d-RA could be attributed to structural modifications caused by the amino acid replacement in the ³²⁷⁻³²⁹RRR motif, which could render CD1d-RA unable to present exogenous α -GalCer; however, this is unlikely because the Ag binding site is located across the plasma membrane from the ³²⁷⁻³²⁹RRR motif, and CD1d-RA can present

exogenous α -GalCer to type I NKT cells as well as CD1d-TD if the APCs are not fixed.

It is possible that the loss of the ³²⁷⁻³²⁹RRR motif in CD1d-RA influences the distribution of CD1d on the plasma membrane and disturbs the formation of immunological synapses. It was shown that CD1d is localized to lipid rafts on the cell surface and that the disruption of lipid rafts impairs both exogenous and endogenous α -GalCer presentation by mCD1d to iNKT cells (21, 25). We found that the lipid raft localization of CD1d-RA was not affected (Fig. 6A), which suggests that the ³²⁷⁻³²⁹RRR motif is not required for the lipid raft localization of mCD1d.

Another possibility is that Ags preloaded onto CD1d-RA at the cell surface may differ from those preloaded onto CD1d-WT and CD1d-TD, resulting in a reduction in the rate of the replacement of exogenous Ags (α -GalCer) onto CD1d-RA relative to that of CD1d-WT and CD1d-TD. If this hypothesis is true, it may be caused by the trafficking of CD1d-RA to a distinct intracellular compartment where it is loaded with different endogenous Ags than CD1d-TD and CD1d-WT. Conversely, the hypothesis indicates that the ³²⁷⁻³²⁹RRR motif is required for the normal intracellular trafficking of mCD1d. Importantly, CD1d-RA, unlike CD1d-TD, had partial defects in presenting endogenous Ags to type II NKT cells, as shown in Fig. 2. To explore the endolysosomal localization of CD1d-RA, we used confocal microscopy to visualize mCD1d. Surprisingly, we found that CD1d-RA (red) colocalized more strongly with LAMP-1 (lysosomal marker; green)

FIGURE 5. Defective α -GalCer presentation by CD1d-RA on the extracellular surface. (A) APCs were pulsed for 4 h with 100 ng/ml α -GalCer, and the excess α -GalCer was removed by washing. α -GalCer-pulsed RBL cells (A) and A20 cells (B) expressing the indicated CD1d molecules were either fixed (right panel, A) or not fixed (left panel, A, B), and these cells were used as APCs to stimulate DN32.D3 cells. IL-2 concentrations in culture supernatants were determined as described in Fig. 1. The data are presented as the mean \pm SD of triplicate measurements and are representative of three to five independent experiments. (C) CFSE-labeled RBL cells expressing the indicated CD1d molecules were pulsed with α -GalCer, cocultured with CD45.2-PE-labeled DN32.D3 cells for 20 min, and fixed. The frequency of cell-cell conjugation between DN32.D3 and RBL cells was assessed using flow cytometry. The data are representative of four independent experiments. ** p < 0.01.

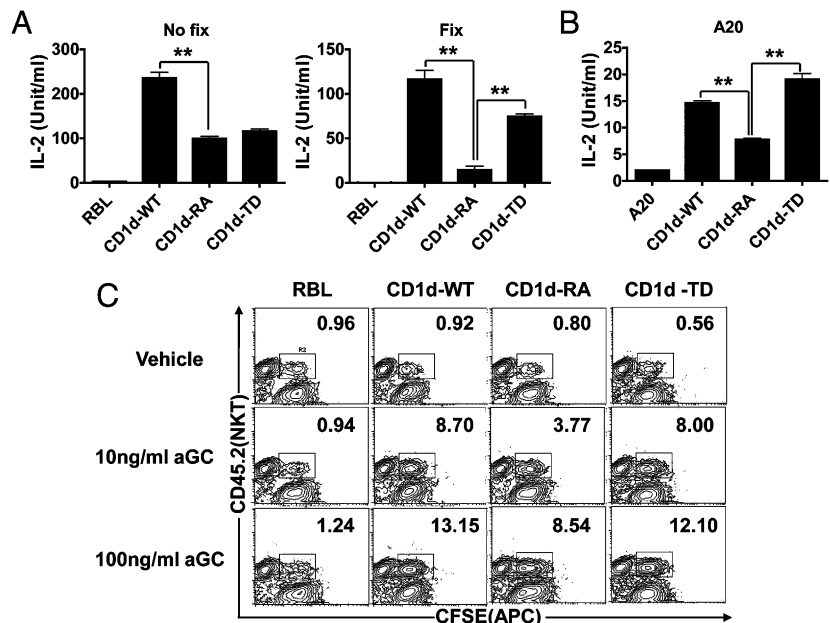
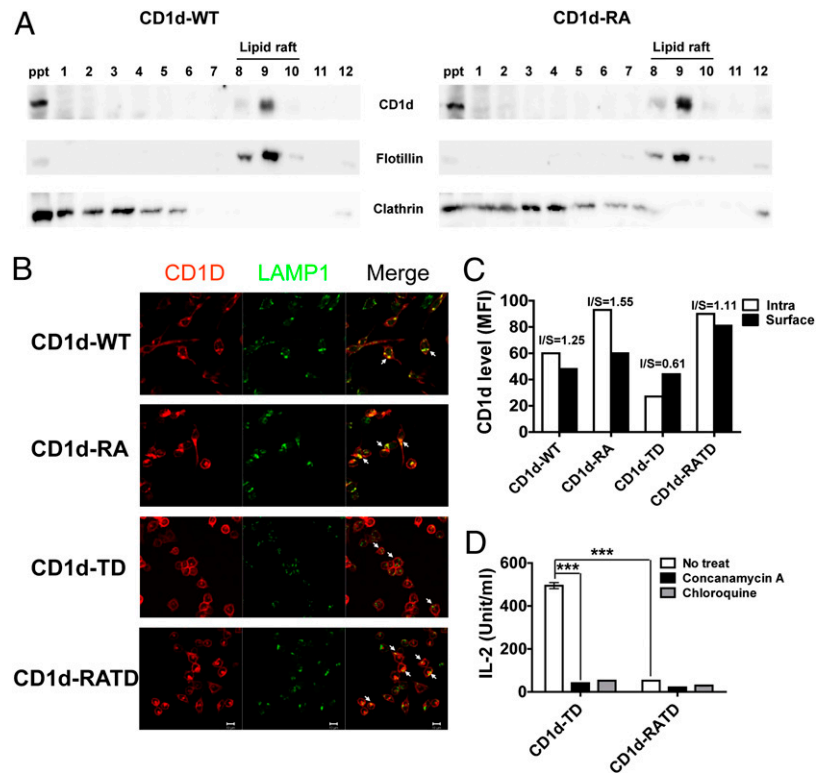


FIGURE 6. Defects in the internalization and abnormal intracellular distribution of CD1d-RA. **(A)** Lipid raft distribution of CD1d-WT and CD1d-RA was analyzed with immunoblot analysis with Abs against mCD1d (upper row), flotillin (lipid raft marker; middle row), and clathrin (nonlipid raft marker; lower row). The data are representative of three independent experiments. **(B)** The colocalization of LAMP-1 (lysosomal marker; green) and mCD1d (red) in RBL cells expressing CD1d-WT, CD1d-RA, CD1d-TD, and CD1d-RATD was analyzed with confocal microscopy. The results are representative of three independent experiments. Scale bars, 10 μ m. **(C)** The expression levels of intracellular versus surface mCD1d were measured using flow cytometry using FITC- and PE-conjugated anti-mCD1d Abs before and after permeabilization. The ratio of intracellular/surface mCD1d (I/S) is indicated for each clone. The data are representative of three independent experiments. **(D)** α -GalCer-pulsed cells expressing CD1d-TD or CD1d-RATD were pretreated with concanamycin A or chloroquine and then cocultured with DN32.D3 cells. The concentrations of IL-2 in culture supernatants were determined as described in Fig. 1. The data are the mean \pm SD of triplicate measurements and are representative of three independent experiments. *** $p < 0.001$.



than did CD1d-WT (Fig. 6B). In addition, the intracellular/surface ratio of CD1d-RA (1.55) was substantially higher than that of CD1d-WT (1.25) (Fig. 6C). These results were unexpected, because the rate of internalization of CD1d-RA was slower than that of CD1d-WT, whereas the surface expression of CD1d-RA is comparable to that of CD1d-WT and CD1d-TD (Fig. 3A).

To analyze the role of the ³²⁷⁻³²⁹RRR motif in the intracellular trafficking of mCD1d further, we removed the cytoplasmic tail in addition to the ³²⁷⁻³²⁹RRR motif, thereby creating CD1d-RATD, as described in *Materials and Methods*. Newly synthesized mCD1d is expressed first at the surface by trafficking through the TGN; subsequently, it recycles to the lysosomal compartment where it is loaded with glycolipid Ags (12). The role of the mCD1d cytoplasmic tail in the recycling of surface mCD1d was shown by experiments with cells expressing CD1d-TD. CD1d-TD showed severely impaired trafficking to the late endosomal and lysosomal compartments during steady-state conditions (7, 11–13, 16, 24).

Our results obtained from cells expressing CD1d-TD were similar to those of previous reports. Surprisingly, however, we observed that CD1d-RATD, which also lacks the endosomal targeting motif and, therefore, should have internalization defects similar to those of CD1d-TD, was densely localized to the lysosome (Fig. 5B). Moreover, whereas the intracellular/surface ratio of CD1d-TD was low (0.61), the intracellular/surface ratio of CD1d-RATD (1.11) was similar to that of CD1d-WT (Fig. 6C). These results suggested that, in contrast to the current working model, CD1d-TD may be able to traffic to the lysosomal compartment, and the ³²⁷⁻³²⁹RRR motif may be involved in the trafficking of CD1d-TD from the lysosome to the plasma membrane.

To investigate the consequences of abnormal lysosomal retention of CD1d-RATD on α -GalCer presentation to type I NKT cells, APCs were treated with the lysosomotropic agents concanamycin A or chloroquine before α -GalCer pulsing. The concentration of lysosomotropic agents used in this study to modify the endolysosomal compartment was adapted from previous studies (26–28).

The concentrations of the reagents used in these studies should not influence cell viability and CD1d expression, except in relation to the endolysosomal compartment; thus, we tested the expression of WT CD1d and the viability of RBL in the presence of the agents. There was no noticeable change in the cell viability or the CD1d expression level of RBL-WT cells when treated with up to 2–10 times of the concentrated reagents used in this study (Supplemental Fig. 3). When pretreated with lysosomotropic agents, APCs expressing CD1d-TD displayed a profound decrease in α -GalCer presentation to type I NKT cells compared with untreated control APCs. However, the degree of inhibition of α -GalCer presentation upon disturbance of lysosomal function was less profound in APCs expressing CD1d-RATD, although these cells had a lower stimulatory capacity than did APCs expressing CD1d-TD when the cells were not treated with lysosomotropic agents (Fig. 6D). Taken together, our findings indicated that the lysosomal machinery is required for the optimal stimulation of type I NKT cells, even when α -GalCer is directly loaded onto the extracellular surface CD1d-TD; furthermore, the ³²⁷⁻³²⁹RRR motif plays an essential role in the re-emergence of mCD1d from the lysosome as it traffics to the plasma membrane.

Requirement of lysosomes for presentation of endogenous Ags to type II NKT cells

According to previous reports, treatment with lysosomotropic agents, which alter the normal lysosomal environment, can impair the presentation of exogenous Ags to both type I and type II NKT cells by CD1d (26, 28). Furthermore, lysosomotropic agents can impair the presentation of endogenous Ags by CD1d to type I NKT cells (13). Thus, we wondered whether the lysosomal pathway is required for presentation of endogenous Ags to not only type I NKT cells but also to type II NKT cells. To test this possibility, type II NKT hybridomas were stimulated with concanamycin A- or chloroquine-pretreated RBL cells expressing CD1d-WT or CD1d-TD or splenic DCs expressing CD1d-WT. Regardless of the type of

APC used and the presence of the cytoplasmic tail of mCD1d, treatment with lysosomotropic agents resulted in partial defects in the presentation of endogenous Ag to type II NKT cells (Fig. 7). In contrast to current hypotheses regarding mCD1d, these results suggested that optimal activation of type II NKT cells requires the trafficking of mCD1d to the lysosome and the presence of functional lysosomal Ag-loading machinery.

Distinct Ag-loading compartments in the endolysosome for type I NKT cells

The striking accumulation of CD1d-RATD (Fig. 6B), which lacks the endosomal-targeting motif, in the endolysosomal compartments and the requirement for recycling of surface-expressed CD1d for the stimulation of type I NKT cells (as shown by the inability of CD1d-TD in activation of type I NKT cells), led us to hypothesize that CD1d can traffic to distinct endolysosomal compartments via two independent routes. The first route involves the recycling of cell surface CD1d to the endolysosome, where α -GalCer loading takes place. The second route entails direct trafficking from the endoplasmic reticulum (ER) to the lysosome, where the ^{327–329}RRR motif is necessary for further migration of CD1d to the cell surface. In the second route, α -GalCer would not easily reach the lysosomal compartment because CD1d-TD showed a severe defect in type I NKT cell stimulation. To explore this possibility, we monitored the intracellular distribution of CD1d before and after α -GalCer treatment with an anti-free CD1d Ab, 20H2 (29), and an anti- α -GalCer/CD1d complex Ab, L363 (30), respectively. In CD1d-WT expressing cells, the CD1d/ α -GalCer complex (blue) and free CD1d (red) both colocalized with LAMP-1 (green), as shown by strong white staining; however, in CD1d-RA-expressing cells, yellow spots (free CD1d + LAMP-1) were observed in addition to white patches (Fig. 8A). As expected, CD1d-TD- or CD1d-RATD-expressing cells were not stained with the L363 anti-CD1d/ α -GalCer complex Ab, although CD1d-RATD strongly accumulated in the lysosomes (Fig. 8B). In addition, a flow cytometry analysis revealed via L363 staining that neither CD1d-TD nor CD1d-RATD was detected on the extracellular surface of the corresponding transfectants (Fig.

8C). These results suggested that CD1d traffics to functionally separated lysosomal compartments through two independent pathways and that α -GalCer presentation to type I NKT cells mainly requires recycling of CD1d from the extracellular surface to the lysosome.

Discussion

In this study, we identified the ^{327–329}RRR motif, which controls the intracellular trafficking of mCD1d. The ^{327–329}RRR motif was required for Ag presentation to both type I and type II NKT cells and for the trafficking of mCD1d from the lysosome to the cell surface.

To stimulate type I NKT cells, CD1d expressed on the cell surface is recycled to the endolysosomal compartment, where it is loaded with endogenous Ag. This recycling is mediated by AP-3, which interacts with a tyrosine-based sorting motif (YxxI) in the cytoplasmic tail of CD1d (18). Previous studies clearly demonstrated that deletion of the YxxI motif impairs the ability of CD1d to present endogenous Ags to type I NKT cells but not to type II NKT cells (7, 11–13, 24). Based on these results, it was reasonable to hypothesize that although endogenous Ag loading onto CD1d for stimulation of type I NKT cells takes place in the endolysosomal compartment, endogenous Ag loading for stimulation of type II NKT cells must occur in a nonendolysosomal compartment. Tail-deleted CD1d-TD is expressed at normal levels on the cell surface after trafficking through the ER–TGN pathway, and it can stimulate type II NKT cells, which suggests that CD1d Ag loading for stimulation of type II NKT cells occurs in the ER–TGN pathway (12).

In contrast to previously published reports, we used a CD1d-RATD mutant to demonstrate that tail-deleted CD1d can be trafficked to the lysosomal compartment. Because the internalization of cell surface CD1d-TD was severely impaired, the accumulation of CD1d-RATD in the lysosome suggests that CD1d-RATD must be delivered to the lysosomal compartment by a pathway that does not involve its recycling from the cell surface. It is highly likely that it involves trafficking via the secretory pathway from the TGN directly to the lysosome. The presence of an independent delivery route of tail-deleted CD1d to the lysosome raises the question of why colocalization of CD1d-TD with lysosomal markers, such as LAMP-1, was not observed in the previous studies. According to our results, it is highly likely that CD1d-TD is delivered to the lysosome through a nonrecycling pathway and is quickly rerouted to the surface. The accumulation of CD1d-RA and CD1d-RATD in the lysosome clearly suggests that the ^{327–329}RRR motif of mCD1d is involved in the rerouting of lysosomal CD1d to the cell surface.

Our model suggests that direct surface expression of mCD1d through the ER–TGN pathway does not function optimally as an Ag-loading pathway for stimulation of type II NKT cells because, unlike CD1d-TD, neither CD1d-RA nor CD1d-RATD (both of which are expressed at normal levels on the cell surface through the ER–TGN pathway) is able to stimulate type II NKT cells (Fig. 2). Recently, it was reported that mutations in the Thr³²² at the cytoplasmic tail of human CD1d directly targeted CD1d from the TGN to lysosome in the absence of a tyrosine-based endosomal-targeting motif (31). The investigators of that study also suggested that there might be targeting motifs other than the tyrosine-based endosomal-targeting motif. However, the relevance of this residue is unclear, because the threonine residue is not conserved among mammalian CD1ds. In contrast, the positively charged ^{327–329}RRR motif is well conserved among mammalian CD1ds (Supplemental Fig. 1).

Because the accumulation of CD1d-RA and CD1d-RATD in the lysosome is evident (Fig. 6), the near-normal rates of internali-

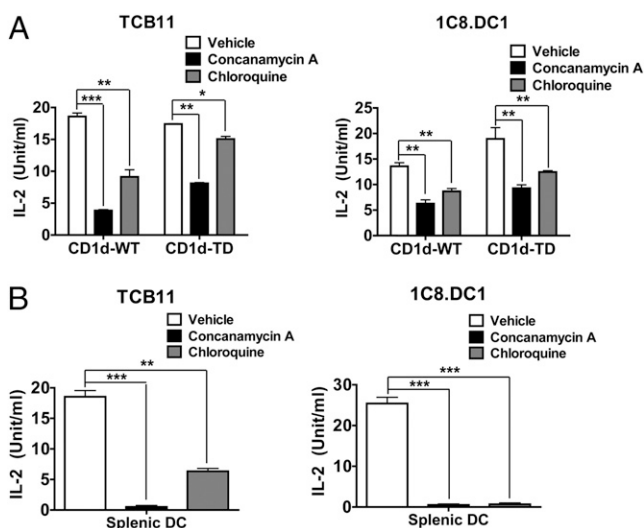


FIGURE 7. Stimulation of type II NKT cells was impaired by treatment with lysosomotropic agents. RBL cells expressing CD1d-WT or CD1d-TD (**A**) and splenic CD11c⁺ DCs (**B**) were pretreated with concanamycin A or chloroquine before coculture with TCB11 or 1C8.DC1 cells. The concentrations of IL-2 in culture supernatants were determined as described in Fig. 1. The data are the mean \pm SD of triplicate measurements and are representative of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.

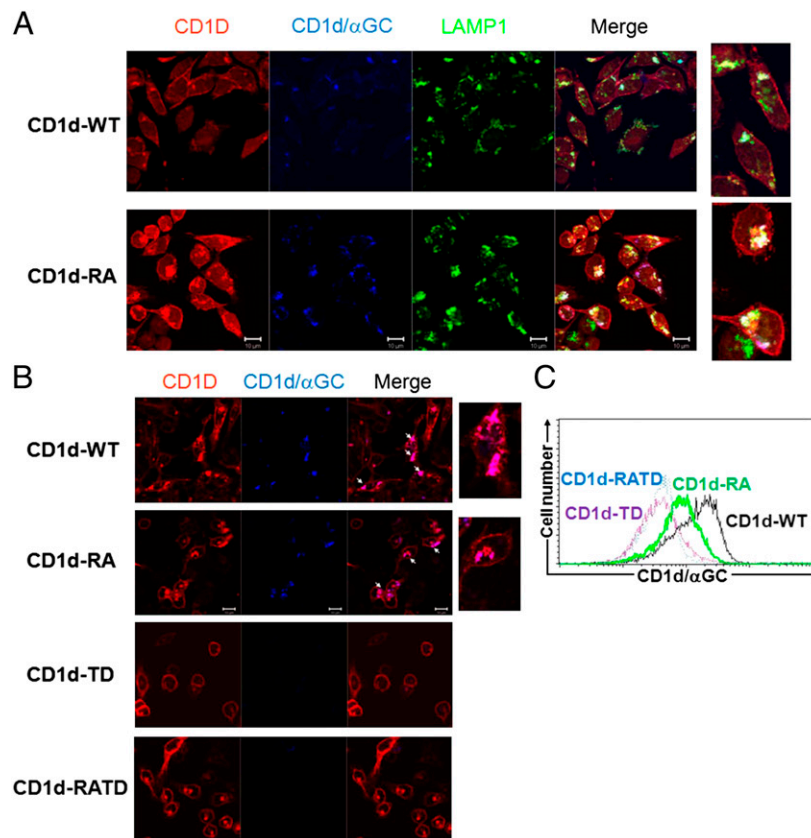


FIGURE 8. Distinct distribution of CD1d for the α -GalCer presentation. Each RBL cell line was pulsed for 16 h with 100 ng/ml α -GalCer. **(A)** The colocalization of LAMP-1 (lysosomal marker; green), mCD1d/ α -GalCer (blue), and mCD1d (red) in RBL cells expressing CD1d-WT and CD1d-RA was analyzed using confocal microscopy. The results are representative of three independent experiments. Scale bars, 10 μ m. **(B)** The colocalization of mCD1d/ α -GalCer (blue) and mCD1d (red) in RBL cells expressing CD1d-WT, CD1d-RA, CD1d-TD, and CD1d-RATD was analyzed using confocal microscopy. The results are representative of three independent experiments. Scale bars, 10 μ m. **(C)** The surface expression levels for each cell line were determined via flow cytometry using an anti-mCD1d/ α -GalCer mAb (L363). The results are representative of three independent experiments.

zation (Fig. 3A) and recycling (Fig. 3B) of CD1d-RA are unexpected. We hypothesized that these contradicting results suggest that mCD1d can be trafficked to two independent intracellular compartments. We propose that the first compartment, which we tentatively refer to as the type I lysosomal compartment, contains mCD1d internalized from the cell surface and allows the recycling of CD1d-RA to the cell surface; the second compartment, which we refer to as the type II lysosomal compartment, contains mCD1d that might traffic directly or indirectly from the ER-TGN and does not permit the recycling of CD1d-RA to the surface (Supplemental Fig. 4). However, we were unable to distinguish these compartments clearly as physically separate compartments via a specific marker or distribution. Thus, we cannot rule out the possibility that these compartments are one site. Despite the undefined physical distribution of these compartments, the type I and type II lysosomal compartments have separate functional characteristics. The ³²⁷⁻³²⁹RRR motif may be involved in the internalization of mCD1d from the cell surface to the type I lysosomal compartment, but this motif is more critical for the trafficking of mCD1d from the type II lysosomal compartment to the cell surface. We also reasoned that these compartments most likely reflect distinct Ag-loading compartments for type I and type II NKT cells. Supporting this hypothesis, CD1d-TD stimulated type II NKT cells normally (both type II NKT cell hybridomas and CD1d/ α -GalCer dimer⁻ primary NKT cells), but CD1d-RA lacked the ability to activate type II NKT cells (Fig. 2). Because CD1d-TD can traffic to the type II lysosomal compartment, it is able to activate type II NKT cells as strongly as CD1d-WT (Fig. 2); however, the mutation in the ³²⁷⁻³²⁹RRR motif prevented the trafficking of CD1d-RA from the type II lysosomal compartment to the surface and the subsequent activation of type II NKT cells by CD1d-RA. In contrast, the mutation had no effect on the trafficking of CD1d-RA to the type II lysosomal compartment via

a yet-unknown endocytic route from the ER-TGN (Fig. 6B, Supplemental Fig. 4).

The fact that CD1d-RATD was not stained by L363 (anti- α -GalCer/CD1d complex mAb), despite the obvious colocalization of CD1d-RATD with LAMP-1 (Fig. 8B), suggested that the type II lysosomal compartment is not the location of α -GalCer presentation; in addition, these results suggested that the ³²⁷⁻³²⁹RRR motif probably controls the trafficking of CD1d through the type II lysosomal compartment for the purpose of lipid Ag presentation to type II NKT cells. However, the direct data that the ³²⁷⁻³²⁹RRR motif impairs the loading of endogenous Ag in endolysosome were not determined; thus, we are unable to rule out other possibilities, such as a defect in Ag presentation at nonendolysosomal sites. Furthermore, we plan to study in more detail the ability of the ³²⁷⁻³²⁹RRR motif to control Ag loading in endolysosomal or nonendolysosomal sites.

Taken together, these findings suggested that endogenous glycolipid Ags loaded on CD1d through the ER-TGN pathway may not serve optimally as Ags for type II NKT cells. Rather, our results argue that there must be a distinct lysosomal compartment for the processing of endogenous glycolipid Ags for optimal stimulation of type II NKT cells; furthermore, the ³²⁷⁻³²⁹RRR motif is critical for the trafficking of mCD1d to the cell surface from this compartment.

Our results are in contrast to the current belief that Ag loading onto CD1d while it is trafficked from the ER-TGN to the cell surface may serve for the stabilization of this molecule until recycling to the endolysosomal compartment. Moreover, the glycolipid Ags loaded in this pathway appear to bind onto CD1d more tightly than do the Ags loaded in either type I or II endolysosomal compartments. In support of this hypothesis, CD1d-RA showed very poor type I NKT cell stimulation after a short α -GalCer pulse (Fig. 1), although it could activate type I

NKT cells more strongly compared with CD1d-TD when a sufficiently long α -GalCer pulse was allowed (Fig. 4A). In addition, α -GalCer-pulsed CD1d-RA formed poor immunological synapses with NKT cells compared with CD1d-WT and CD1d-TD (Fig. 5C). Moreover, formaldehyde-fixed CD1d-RA or CD1d-RATD showed a substantially reduced α -GalCer presentation compared with CD1d-WT or CD1d-TD (Figs. 5A, 6D). Thus, these results imply that glycolipid Ags that occupy the groove of CD1d-RA or CD1d-RATD are different from those of CD1d-WT and CD1d-TD. Additionally, it is difficult to replace these glycolipids with exogenous α -GalCer compared with the glycolipid Ags on CD1d-WT and CD1d-TD, whose trafficking to the type I or II endolysosomal compartments was not impaired.

In conclusion, our study demonstrated a novel pathway of CD1d trafficking and Ag presentation to type I and type II NKT cells that is controlled by the ^{327–329}RRR motif in the cytoplasmic tail of mCD1d.

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Disclosures

The authors have no financial conflicts of interest.

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