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Expression of CD1d2 on Thymocytes Is Not Sufficient for the Development of NK T Cells in CD1d1-Deficient Mice

Yi-Hua Chen,* Bin Wang,* Taehoon Chun,* Lillian Zhao,* Susanna Cardell,† Samuel M. Behar,‡ Michael B. Brenner,§ and Chyung-Ru Wang*‡

CD1 is an MHC class I-like molecule that has been conserved throughout mammalian evolution. Unlike MHC class I molecules, CD1 can present unique nonprotein antigens to T cells. The murine CD1 locus contains two highly homologous genes, CD1d1 and CD1d2. CD1d1 is essential for the development of a major subset of NK T cells that promptly secrete IL-4 following activation. However, the function of CD1d2 has not yet been demonstrated. In the present study, we examined the expression of CD1d2 in CD1d1-deficient (CD1d1°) mice with the anti-CD1 Ab 3H3. Unlike CD1d1, which is expressed by all lymphocytes, CD1d2 can be detected only on the surface of thymocytes. To determine whether CD1d2 can select a unique subset of NK T cells, we compared the remnant population of NK T cells in CD1d1° and CD1d1, CD1d2-double deficient (CD1d1°CD1d2°) mice. No significant difference in the number of NK T cells and cytokine secretion capacity can be detected between CD1d1° and CD1d1°CD1d2° mice, indicating that CD1d2 cannot substitute for CD1d1 in NK T cell development. The inability of CD1d2 to select NK T cells is not due to the structural constraints of CD1d2 since CD1d2-transfected cells can be recognized by both NK T cell hybridomas and freshly isolated NK T cells. Given the structural similarities, it is possible that the low levels of surface expression and limited tissue distribution of CD1d2 may prevent it from functioning in the selection and expansion of NK T cells. The Journal of Immunology, 1999, 162: 4560–4566.

The CD1 genes encode a family of nonpolymorphic cell surface glycoproteins that are structurally related to MHC class I and class II molecules. Recent studies have shown that the CD1 group represents a distinct lineage of Ag-presenting molecules that present lipid/glycolipid Ags, as well as peptide Ags to T cells (1–6). Based on sequence homology and tissue distribution, CD1 genes have been classified into two distinct groups: group one, including human CD1A, -B, and -C, and group two, consisting of human CD1D and both of its murine homologues, mCD1d1 (CD1d1) and mCD1d2 (CD1d2) (7, 8). The two mouse CD1 genes share 95% sequence homology to each other, and are likely to be the product of a gene duplication. Mouse CD1 molecules are expressed primarily by cells in the hemopoietic lineage, including B and T cells, macrophages, and dendritic cells (9–11). Since no serological reagents can distinguish CD1d1 from CD1d2, the relative contribution of CD1d1 and CD1d2 in the surface expression levels of CD1 has not been assessed. At the transcriptional level, CD1d1 is found to express at higher levels than CD1d2 in all tissues tested, except the thymus (12).

CD1d1 was shown to be the ligand for a unique subset of T cells, the NK T cells (13). Most of the NK T cells used an invariant α-chain, with Vα14Jα281 rearrangement, paired with a limited number of Vβ-chains (Vβ8, Vβ7, or Vβ2) (14, 15). Upon activation, NK T cells rapidly produce significant amounts of cytokines, particularly IL-4 (16–21), which may influence the overall dynamics of the immune response to both infections and autoimmunity. In addition to their cytokine production ability, a recent study has shown that NK T cells can mediate tumor rejection through an NK-like effector mechanism after activation with IL-12 (22). We and others have shown that the development of NK T cells is impaired in mice lacking either CD1d1 alone (23) or both CD1d1 and CD1d2 (24, 25). These mutant mice are functionally incapable of producing IL-4 after systemic T cell activation with anti-CD3 Ab. The induction of other cytokines, such as IFN-γ, was not significantly affected in CD1d1, CD1d2-double deficient (CD1d1°CD1d2°) mice (24, 25), but appeared to be reduced in CD1d1° mice (23). It is unclear whether this result is due simply to the heterogeneous genetic backgrounds of mice used in the study, to the different parameters (mRNA vs protein) used to measure the cytokine induction, or to the qualitative differences in the remnant population of NK T cells between CD1d1° and CD1d1°CD1d2° mice.

In this study, we have investigated the expression of CD1d2 in CD1d1° mice and assessed the functional role of CD1d2 by comparing T cell development in CD1d1° and CD1d1°CD1d2° mice. To ascertain whether CD1d2 can act as a restriction element for T cell responses, we also analyzed the reactivity of a panel of CD1-specific T cell hybridomas and T cell clones to CD1d2-expressing cells. Our data demonstrated that the surface expression patterns of CD1d1 and CD1d2 are differentially regulated. In contrast to the broad distribution of mouse CD1d1, CD1d2 surface expression can be detected in substantial quantities only on thymocytes, similar to the pattern found for human CD1a and CD1b (26). Although...
CD1d2 protein can be recognized by NK T cells, the expression of CD1d1 alone is not sufficient for the development and function of NK T cells in CD1d1° mice.

Materials and Methods

Generation of CD1d1° mice

A DNA fragment containing the CD1d1 gene was isolated from a 129/sv genomic DNA phage library (Stratagene, La Jolla, CA). The targeting construct was designed to delete a 1.6-kb BamHI fragment containing exons 1 to 3 of the CD1d1 gene and replace these coding regions with the neomycin resistance gene. The linearized targeting construct was transfected into J-1 embryonic stem (ES) cells by electroporation, and G418 and gancyclovir-resistant clones were screened for homologous recombination by Southern blot analysis, using probes both outside and within the target region. ES clones with the mutant CD1d1 gene were injected into C57BL/6 blastocysts. The resulting chimeras were crossed to B6 mice. Mice heterozygous for the mutation were intercrossed to produce homozygous CD1d1° offspring, screened by Southern blot analysis. CD1d1°CD1d2° mice were generated in our laboratory as previously described (25). All animals were housed under specific pathogen-free conditions.

CD1 mRNA expression

RNA was extracted from various tissues of WT, CD1d1°, and CD1d1°CD1d2° mice with TRIzol Reagent (Life Technologies, Grand Island, NY). cDNA was prepared using random hexamer primers and amplified by PCR using primer sets specific for CD1d1 or CD1d2. The sequences of primers were 5′-ACGTCCTGGCAGACAGTCCCAGG-3′ and 5′-TTAATGTTGAAAAGAGCGTACTGGC-3′ for CD1d1, and 5′-ACAATCCTTGCCAGAGGTGTCTTGAAGG-3′ and 5′-TCTTGGCAGAGGGTCCTAGG-3′ (in exon 1) and 5′-ATACTGGT-3′ for CD1d2. The amount of template cDNA used in each reaction was normalized to the amount of Hprt mRNA amplified with primers 5′-GGTGGGATACAGGCCAGACTTTGTTG-3′ and 5′-GAGGGTAGGCTGGCCTATTTGTCCG-3′. The specificity of amplified products was confirmed by Southern blot hybridization using 32P-labeled oligonucleotide probes specific for CD1d1 (5′-ACGGGCGCCAATCTTGTCGG-3′) or CD1d2 (5′-ACCTGGGCCCAGTTTGGCCG-3′).

Flow cytometric analysis

mAbs specific for CD3 (FITC-2C11), TCRαβ (FITC-H57-597), CD8a (FITC-53-67), NK1.1 (PE-53-58), CD4 (Cy-chrome-RM4-5), and hamster IgG (FITC-G70-204 and G94-56) were obtained from PharMingen (San Diego, CA). 3H3, a hamster mAb specific to CD1, was developed in our laboratory (11). Single cell suspensions from most tissues of designated mice were prepared by standard procedures. Liver, kidney, and spleens were removed after 90 min for RNA preparation.

Cytokine mRNA analysis

CD1d1°, CD1d1°CD1d2°, and WT controls were injected i.v. with 2 μg of purified anti-CD3 Ab (kindly provided by Dr. Jeffrey Bluestone, University of Chicago), and spleens were removed after 90 min for RNA preparation. RNA extraction and reverse transcription were conducted as described above. Competitive PCR was used to evaluate the relative amounts of cytokine mRNA. Briefly, a polycolorimetric construct (kindly provided by Dr. Steve Reiner, University of Chicago) containing addition-mutations of authentic cDNA was amplified in the same reaction as the experimental cDNA. The ratio of the larger m.w. products (due to the amplification of the competitors) and the lower m.w. products (due to the amplification of authentic cytokines) was used to assay the relative amount of cytokine mRNA production in vivo. Amplification of Hprt was used to equalize the amount of template cDNA used in each reaction.

Generation of RMA-S and L929 cells stably transfected with CD1d2.

CD1d2 cDNA was obtained by PCR amplification of a B6/CBAF1 thymus cDNA library (Stratagene, La Jolla, CA) with primers 5′-ACAGATTAACATGGCGGTACTCCTACATTATGAGGCATGGTGTGGA-3′ (in exon 1) and 5′-CATCATCCAGTAGAGGATGATATC-3′ (in exon 4). The resulting PCR products were cloned into PCrScript (Stratagene). cdNA sequencing was performed to identify clones containing the CD1d2 gene. The insert was then shuffled into a pcDNA-3 expression vector (Invitrogen, San Diego, CA) that already contained the DNA sequence encoding the transmembrane, cytoplasmic tail, and 3′-untranslated regions from CD1d1. The resulting chimeric molecule was expected to have an identical amino acid sequence to native CD1d2 molecules since there are no amino acid differences between CD1d1 and CD1d2 beyond exon 4. Twenty micrograms of plasmid DNA were electroporated into RMA-S and L929 cells. Stable transfectants were first selected for G418 resistance, and then for high surface expression of CD1 by staining with the anti-CD1 Ab 3H3.

Activation of T cell hybridomas and NK T cells

Hybridomas (5 × 104) were cultured in 200 μl of RPMI 1640 medium supplemented with 10% FCS, 2 mM t-glutamine, 20 μg/ml 2-ME, and 100 U/ml penicillin/streptomycin together with irradiated thymocytes or splenocytes (5 × 105 cells per well) or cell lines (5 × 105 cells per well). After 24–48 h, culture supernatants were harvested, and IL-2 release was quantitated by ELISA (PharMingen). CD4+ NK1.1+ T cells were purified from the livers of C57BL/6 mice by FACS. Sorted CD4+ NK1.1+ T cells (1–2 × 105 cells) were stimulated with irradiated CD1d2-transfected RMA-S cells (5 × 105 cells per well) or parental RMA-S cells in the presence of 10 U/ml of IL-2. After 2 to 3 days, supernatants were collected, and IL-4 levels were determined by ELISA.

Cytotoxicity assay

Target cells (5 × 106 cells) were labeled with 100 μCi [35S]sodium chromate for 1 h at 37°C. A total of 1 × 104 cells were added to round-bottom microtiter wells containing variable numbers of effector cells. After 4 h incubation at 37°C, 100 μl of supernatant from each well was assayed for 35S release. Results are given as follows: percentage of specific lysis = (experimental release – spontaneous release) × 100/(maximal release – spontaneous release).

Results

Generation of CD1d1° mice

ES cells lacking a functional CD1d1 gene were generated by homologous recombination, as outlined in Fig. 1A. The disruption of the CD1d1 gene was confirmed by Southern blot analysis. The targeted embryonic stem cells were used to derive homozygous mice in which both of the CD1d1 alleles were disrupted (Fig. 1B). The background for both CD1d1° and CD1d1°CD1d2° mice used in this study was a mixed Sv129/C57BL/6 background.
**CD1d2 expression in WT and CD1d1° mice**

Although CD1d1 and CD1d2 are 95% homologous, we have created two primer sets that specifically amplify these isotypes based on nucleotide differences in exon three and the 3’ untranslated regions. RT-PCR was used to examine the expression of CD1d1 and CD1d2 in different tissues from WT and CD1d1° mice. In WT mice, CD1d1 mRNA can be detected in all tissues tested, including thymus, spleen, lymph node, liver, and intestine, with expression highest in liver (Fig. 2A). CD1d2 appears to be expressed at lower levels than CD1d1, with expression highest in the thymus and barely detectable in the intestine. In CD1d1° mice, the CD1d1 mRNA is undetectable, but the expression pattern of CD1d2 is similar to the WT controls except that the levels of CD1d2 mRNA are slightly higher in the thymus and liver of CD1d1° mice. Both CD1d1 and CD1d2 mRNA are undetectable in CD1d1°CD1d2° mice. The specificity of amplified products was further confirmed by Southern blot hybridization with oligonucleotide probes specific to CD1d1 and CD1d2 respectively (Fig. 2B).

The expression of CD1 at the protein level was analyzed by immunofluorescence staining and flow cytometry with anti-CD1 mAb 3H3. FITC-anti-mouse CD1 mAb 3H3. FITC-anti-TNP was used as a negative control (dashed line).

**CD1d2 does not compensate for the loss of CD1d1 function in NK T cell development**

Our previous results and those of Smiley et al. have shown that the development of NK T cells is impaired in CD1d1°CD1d2° mice (24, 25). Mendiratta et al. have also shown that the number of NK T cells was dramatically reduced in the mice lacking only CD1d1 (23). However, it was unclear whether CD1d2 is completely dispensable or whether it can perform at a lower level the same function as CD1d1 in NK T cell development. To address this issue, we compared the development of NK T cells in CD1d1°, CD1d1°CD1d2°, and WT mice by flow cytometry and analyzed their capacity for cytokine production upon primary T cell stimulation.

Lymphocytes from thymus, spleen, lymph node, and liver were stained with various combinations of fluorochrome-labeled mAbs and analyzed by flow cytometry to analyze T cell populations in CD1d1°, CD1d1°CD1d2°, and WT mice. The number of lymphocytes and the proportion of CD4+, CD8+, CD4+CD8+, and CD4−CD8+ cells did not differ significantly between groups (data not shown) by staining with anti-TCRαβ, anti-CD4, and anti-CD8 Abs. However, the percentages of NK1.1+ T cells were reduced by 70–75% in the liver, where NK1.1+ T cells are normally prevalent, and 60–75% in other organs tested in both CD1d1° and
CD1d1°CD1d2° mice (Fig. 3A), compared with the control mice. There was no significant difference in the levels of reduction between the two knockout mouse lines.

Using CD1-deficient mice, we and others have demonstrated that CD1 mutant mice are defective in producing an early burst of IL-4 upon stimulation with anti-CD3 Ab. To determine whether there is residual NK T cell activity in CD1d1° mice, we injected anti-CD3 Ab into CD1d1°, CD1d1°CD1d2°, and normal mice and compared cytokine gene expression using competitive RT-PCR (Fig. 3B). Both CD1d1° and CD1d1°CD1d2° mice failed to produce a significant amount of IL-4 mRNA 90 min after injection of anti-CD3 mAb, while heterozygous littermate controls expressed high levels of IL-4 mRNA at this time point. The levels of IFN-γ, IL-2, IL-10, and IL-12 were not diminished in either CD1d1° or CD1d1°CD1d2° mice compared with controls. Furthermore, the expression levels of various cytokines in response to anti-CD3 are comparable in CD1d1° and CD1d1°CD1d2° mice.

We therefore, observe no quantitative or qualitative differences in NK T cell development or function between CD1d1° and CD1d1°CD1d2° mice. Our results suggest that CD1d2 is unable to compensate for the loss of CD1d1 in NK T cell development, despite being expressed on the surface of thymocytes.

**The expression of CD1d2 during embryonic development**

Several possibilities may account for the inability of CD1d2 to select NK T cells. One possible explanation is that CD1d2 may not be expressed during early embryonic development, which could prevent it from positively selecting NK T cells. We therefore examined cell surface expression of CD1 on thymocytes of CD1d1° and WT mice during embryonic development by surface staining with mAb 3H3. As depicted in Fig. 4, a substantial amount of CD1 can be detected on cells isolated from day 14.5 fetal thymus from both CD1d1° and WT mice. The cell surface levels of CD1 remained relatively constant throughout development in CD1d1° and WT mice, with cells from CD1d1° mice expressing 25 to 35% of the WT levels of CD1. The expression of CD1d2 before TCR rearrangement suggests that CD1d2 could be active in thymic selection of CD1-restricted T cells.

**CD1d2 can be recognized by NK T cell hybridomas**

Although CD1d1 and CD1d2 are highly homologous to each other, they differ in 17 amino acids in the coding region, including a cysteine (residue 168) to tryptophan substitution in the α2 domain of CD1d2. This cysteine participates in disulfide bond formation and is conserved in most MHC class I molecules. Lack of this intradomain disulfide bond may cause CD1d2 to adopt a different conformation from that of CD1d1 and may prevent CD1d2 from interacting with NK T cells. To investigate whether CD1d2 can be recognized by NK T cells, we measured the cytokine production of NK T cell hybridomas (D32.D3, and DN3A4) in response to CD1d2-transfected cells and lymphocytes isolated from CD1d1° and control mice. As shown in Fig. 5A, NK T cell hybridomas secreted high levels of IL-2 in response to CD1d2-transfected RMA-S and L929 cells but not in response to untransfected cells. However, NK T cell hybridomas did not secrete IL-2 in response to thymocytes from CD1d1° mice, yet secreted substantial amounts of IL-2 in response to thymocytes from WT controls (Fig. 5B). Additionally, purified liver NK T cells also can secrete IL-4 in response to CD1d2-transfected RMA-S cells (Fig. 5C) but not when stimulated by cells isolated from CD1d1° mice (data not shown). Thus, our results suggest that, although the CD1d2 molecule can be recognized by NK T cells, the amount of CD1d2 expressed on lymphocytes may be too low to trigger the T cell response.

**Recognition of CD1d2 by other CD1d1-specific T cells**

In addition to NK T cells, CD1-restricted CD4+, CD8+, and DN T cells have been isolated (5, 6, 28). Unlike NK T cells, these CD1-restricted T cells have diverse TCR repertoires. To analyze the extent of cross-reactivity between CD1d1 and CD1d2, we also examined the reactivity of the CD1d2 transfectant with several CD1d1-specific T cell hybridomas (VII49.1, VII68.1, VIII24.1, and XV19.2) derived from CD4+ splenocytes from MHC II° mice and CD1d1-specific DN CTLs (14S.7, 14S.10, and 24S.7) from B6 mice (29). In contrast to the two NK T cell hybridomas, most of the CD4+ T cell hybridomas respond only to CD1d1 transfectants and do not respond to CD1d2 transfectants (Fig. 5D). Only one CD4+ T cell hybridoma responded weakly to CD1d2 transfectants (Fig. 5D) but not to thymocytes from CD1d1° mice (data not shown). The specific recognition of CD1d2 by CD1d1-specific DN CTLs was determined by the cytolytic assay. All three CTLs could recognize CD1d1-transfected RMA-S cells but not untransfected RMA-S cells, and two of them (14S.7 and 24S.7) also recognized CD1d2 transfectant (Fig. 5E). Thus, the amino acid substitutions in CD1d2 can affect the recognition by some CD1d1-specific T cells.

**Discussion**

The role of CD1 molecules in the development of NK T cells has been examined in CD1d1°CD1d2° and CD1d1° mice by several laboratories (23–25). Due to the lack of direct comparison, it was still unclear whether CD1d2 might function similarly to CD1d1 in positive selection of NK T cells, but to a lesser extent. In this study, we demonstrated that CD1d2 can be expressed at the protein level and can be recognized both by Ab and by a panel of T cells, indicating that CD1d2 is not a pseudogene in 129/sv strain. Failure
to demonstrate CD1d2 expression with 3C11 in the CD1d1 knock-out mouse model reported by Mendiratta et al. (23) may be due to the low affinity of this Ab for CD1d2. In contrast to CD1d1, a substantial amount of CD1d2 can be detected only on the cell surface in thymocytes. Previous work has shown that the positive selection of NK T cells requires the expression of CD1 on cortical thymocytes (30). Thus, the presence of cell surface CD1d2 in the thymus suggested that CD1d2 might also contribute to the positive selection of NK T cells. However, in comparing the number of residual NK T cells and the cytokine secretion capacities of these NK T cells in response to anti-CD3, we did not observe any significant differences between CD1d1° and CD1d1°CD1d2° mice. Our results indicate that, although CD1d2 is expressed in the thymus of CD1d1° mice, it cannot compensate for the lack of CD1d1 in NK T cell development.

We have investigated several possible explanations that could account for the inefficacy of CD1d2 in the selection and/or expansion of NK T cells. First, we examined the expression of CD1d2 during embryonic development and showed that CD1d2 is already expressed on thymocytes on gestational day 14.5, before TCR rearrangement. This ontogeny eliminates the possibility that lack of expression of CD1d2 during early embryonic development precludes it from functioning as a restriction element for NK T cell development. Second, we examined the reactivity of NK T cells to CD1d2 transfectants. Cells of CD4° T cell hybridomas (VII49.1, VII68.1, VIII24.1, and XV19.2) were stimulated with RMA-S, L929, or the two cell lines transfected with CD1d1 or CD1d2, and IL-2 production was determined by ELISA. Results are representative of three experiments. E. Reactivity of CD1-specific DN cytotoxic T cell clones to CD1 transfectants. The T cell clones (14S.7, 14S.10, and 24S.7) were incubated with 1 × 10⁶ ⁵¹Cr-labeled RMA-S (○), RMA-S CD1d1 (●), or RMA-S CD1d2 (■). The E:T ratios were indicated in the figure.

FIGURE 5. Recognition of CD1d2 by CD1-specific T cell hybridomas and freshly isolated NK T cells. A, Recognition of CD1d2 transfectants by NK T cell-derived hybridomas. An amount equal to 5 × 10⁴ cells of CD1-specific NK T cell hybridomas (DN3A4 and D32.D3) was cultured with 5 × 10⁴ RMA-S cells, RMA-S cells transfected with CD1d1 or CD1d2, or L929 cells or L929 cells transfected with CD1d2. IL-2 levels in the supernatant were detected by ELISA. Bars represent means and SDs of duplicate determinations. Results are representative of three experiments. Comparable amounts of CD1 were detected on all transfectants. B, Response of NK T cell-derived hybridomas to thymocytes and splenocytes from CD1d1° mice. An amount equal to 5 × 10⁴ cells of D32.D3 or DN3A4 was cultured with 5 × 10⁵ thymocytes or splenocytes from CD1d1° (−/−) and control mice (+/+). IL-2 levels in the supernatant were detected by ELISA. Results are representative of three experiments. C, Response of freshly isolated NK T cells to CD1d2 transfectant. An amount equal to 1 × 10⁵ sorted NK T cells was cultured with 5 × 10⁵ CD1d2 transfectant (RMA-S CD1d2), and IL-4 production was assayed by ELISA. Results are representative of two experiments. D, Response of CD1-specific CD4° T cell hybridomas to CD1 transfectants. Cells of CD4° T cell hybridomas (VII49.1, VII68.1, VIII24.1, and XV19.2) were stimulated with RMA-S, L929, or the two cell lines transfected with CD1d1 or CD1d2, and IL-2 production was determined by ELISA. Results are representative of three experiments. E, Reactivity of CD1-specific DN cytotoxic T cell clones to CD1 transfectants. The T cell clones (14S.7, 14S.10, and 24S.7) were incubated with 1 × 10⁶ ⁵¹Cr-labeled RMA-S (○), RMA-S CD1d1 (●), or RMA-S CD1d2 (■). The E:T ratios were indicated in the figure.
freshly isolated NK T cells. However, these NK T cells cannot secrete cytokines in response to the CD1d2-expressing thymocytes isolated from CD1d1° mice. These results suggested that the epitopes recognized by NK T cells are preserved in the CD1d2 molecule; however, the amount of CD1d2 expressed on thymocytes may not be sufficient to promote the development of NK T cells. Although CD1d2 can be recognized by NK T cells, we do not know whether NK T cells can interact with CD1d2 with the same affinity as with CD1d1. Our FACS analysis showed that the expression level of CD1d1 on thymocytes is 3- to 4-fold higher than that of CD1d2. Therefore, it is likely that the inability of CD1d2 to select NK T cells may result from its low abundance and low affinity for NK T cells. It is also possible that the presence of CD1 molecules in lymphoid organs is essential for the expansion of NK T cells and that lack of expression of CD1d2 in peripheral lymphoid organs precludes the expansion and functional development of NK T cells. If this were the case, we would expect that the development of NK T cells would be partially restored in the thymus of CD1d1° mice. However, both surface phenotype analysis and in vitro cytokine secretion ability of thymocytes in response to plate-bound anti-CD3 (data not shown) showed lack of functional NK T cells in the thymus of CD1d1° mice.

The expression pattern of CD1d2 appears to be controlled at the transcriptional level since the surface expression level of CD1d2 is consistent with its mRNA expression level. To elucidate the mechanisms regulating the expression of the murine CD1 genes, we have cloned and sequenced the 5' flanking region of the murine CD1d1 and CD1d2 genes (Y.-H. Chen and C.-R. Wang, unpublished results). CD1d1 and CD1d2 share greater than 95% sequence homology in the coding exons and intervening introns. The homology extends about 350 bp 5' of the translational start site with marked upstream divergence. There is an L1 repeat in the upstream region of CD1d2, which may explain the lower expression of CD1d2 relative to CD1d1.

Of the 17 amino acid changes between CD1d1 and CD1d2, 16 substitutions occurred in the α1 and α2 domains (Fig. 6). Six of these substituted amino acids extend into the Ag-binding groove, which might affect Ag binding. If the binding mode of CD1 with the TCR is similar to that between other MHC molecules and their receptors (32, 33), then two substituted residues (162M→Q) protruding up out of the groove might affect the interaction with the Vα-chain of the TCR. Nine CD1d1-specific T cell hybridomas or T cell lines, including two NK T cell hybridomas, four CD4⁺ T cell hybridomas, and three DN cytotoxic T cell clones, were tested for their reactivity with CD1d2 in this study. Three of these T cells (D32.D3, DN3A4, and 24S.7) use a Vα-chain with Vα14Ja281 rearrangement, while the other six use different Vα-chains. Four of these T cell lines showed differential recognition of CD1d1 vs CD1d2, suggesting that the structural differences between CD1d1 and CD1d2 can be distinguished by some T cells. It is worth mentioning that all three Vα14Ja281-expressing T cells reacted with both CD1d1 and CD1d2. Thus, it is likely that the epitopes recognized by the invariant TCR α-chain of NK⁺ T or NK⁻ T cells are preserved in CD1d2 but that the epitopes recognized by heterogenous TCR used by other subsets of CD1-autoreactive T cells may be mostly unique to CD1d1.

Tissue-specific recognition of mouse CD1 by CD1-autoreactive T cells has been reported recently (34, 35). In agreement with these studies, tissue-specific recognition of CD1 was also observed in our study. Two thymus-derived NK T cell hybridomas could respond to thymocytes but not splenocytes from WT mice (Fig. 5B), and three of four spleen-derived hybridomas secreted more IL-2 when stimulated with splenocytes than with thymocytes (data not shown). Although our results showed that the responses to CD1d2 by these CD1-specific T cells are heterogeneous, it is unlikely that the differential ability of these T cells to recognize CD1d2 would be the explanation for tissue-specific CD1-recognition since CD1d2 is not expressed on splenocytes. It is more likely that CD1-specific autoreactive T cells can distinguish a set of cell type-specific ligands bound to CD1, as suggested by Brossay et al. (34) and Park et al. (35).

The CD1d2 gene is present in all mice strains studied so far, including many inbred strains, and in wild mice of the subspecies castaneus and spretus (B. Wang et al., unpublished results). However, rats have only one CD1 gene, which appears to be related more to CD1d1 than to CD1d2 (36). Thus, the gene duplication event of mouse CD1 occurred less than 10 million years ago. Has CD1d2 acquired a unique function in the mouse or is it simply a by-product of gene duplication that will eventually become a pseudogene by accumulating mutations in both
the coding and promoter regions? Although CD1d2 cannot substitute for CD1d1 in positive selection of NK T cells, we cannot eliminate the potential role of CD1d2 in shaping the repertoire of other CD1-restricted T cells. The fact that CD1d2 is capable of encoding a functional protein and being recognized by NK T cells and other autoreactive CD1-specific T cells raises the possibility that induced expression of CD1d2 in the periphery may lead to activation of some CD1-specific T cells. Control of the tissue distribution and expression level of CD1d2 may therefore be necessary in the mouse to prevent autoimmune responses.

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