Structural requirements for antigen presentation by mouse CD1

Nicolas Burdin*, Laurent Brosset*, Massimo Degano†, Hiroshi Iijima§, Ming Gui†, Ian A. Wilson‡, and Mitchell Kronenberg*‡

*La Jolla Institute of Allergy and Immunology, 10355 Science Center Drive, San Diego CA 92121; †Department of Molecular Biology and Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037; ‡Pharmaceutical Research Laboratory, Kirin Brewery Co., Ltd., 3 Miyahara-cho, Takasaki-shi, Gunma 370-1295, Japan; §Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111; and ¶Structural Biology Laboratory, Sincitore Trieste Elettra, Basovizza (Ts), Italy

Communicated by Howard M. Grey, La Jolla Institute for Allergy and Immunology, San Diego, CA, July 3, 2000 (received for review March 22, 2000)

The structural basis for the T cell response to glycolipid antigens (Aggs) remains poorly understood. T lymphocytes autoreactive for mouse CD1 (mCD1.1) or reactive for the glycosphingolipid α-galactosylceramide (α-GalCer) presented by mCD1.1 have been described previously. In this paper it is shown that mutations at the top of the α helices and in the bottom of the Ag-binding groove can disrupt both mCD1.1 autoreactivity and α-GalCer recognition. The locations of the positions that affect T cell responses indicate that recognition of mCD1.1 is not likely to be unconventional or superantigen-like. Furthermore, the effects of the bottom of the pocket mutation suggest that the autoreactive response could require an autologous ligand, and they indicate that α-GalCer binds to the groove of mCD1.1, most likely with the shorter 18-carbon hydrophobic chain in the A’ pocket. Natural killer T cell hybridomas with identical T cell antigen receptor (TCR) α chains and different β chains respond differently to α-GalCer presented by mCD1.1 mutants. This finding indicates a role for TCR β in defining natural killer T cell specificity, despite the more restricted diversity of the β chains in these cells. Overall, the data are consistent with a mode of lipoglycan recognition similar to that proposed for glycopeptides, in which the TCR α and β chains survey a surface composed of both mCD1.1 and the carbohydrate portion of α-GalCer.

CD1 molecules are cell surface proteins expressed as a 45- to 55-kDa heavy chain noncovalently associated with β2-microglobulin (1). Several features discriminate CD1 from the major histocompatibility complex (MHC)-encoded class I and class II molecules, most notably the ability of CD1 molecules to present lipid antigens (Aggs) (1, 2). The mouse CD1 (mCD1.1) molecule has a groove typical of the MHC-encoded Ag-presenting molecules (3), but this groove is very hydrophobic, it is deeper than those in class I and class II proteins, and it contains only two pockets, termed A’ and F’.

Two subgroups of CD1 molecules have been defined on the basis of their amino acid sequence (1, 4). Considering mice and humans, group 2 molecules include the closely related mCD1.1 and mCD1.2 glycoproteins, as well as human CD1d (hCD1d). The majority of T cells reactive to group 2 CD1 molecules are natural killer (NK) T cells. NK T cells are a specialized subset of NK cells; CDR, complementarity-determining region; h, human; m, mouse; mfi, mean fluorescence intensity; NK, natural killer; TCR, T cell antigen receptor.

Abbreviations: Ag, antigen; α-GalCer, α-galactosylceramide; APCs, antigen-presenting cells; CDR, complementarity-determining region; h, human; m, mouse; mfi, mean fluorescence intensity; NK, natural killer; TCR, T cell antigen receptor.

1To whom reprint requests should be addressed. E-mail: mitch@lai.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.
Site-Directed Mutagenesis. Wild-type mCD1.1 transfected A20 cells and A20 transfected controls, which have been obtained by transfection with the wild-type mCD1.1 cDNA in the wrong orientation, were obtained and cultured as previously described (20). The mCD1.1 mutant constructs have been generated by using the altered sites II in vitro mutagenesis system, according to the manufacturer’s protocol (Promega, Madison, WI). For each mutation reaction, five to eight clones of JM109 bacteria, which were ampicillin resistant but tetracycline sensitive, were sequenced by using the dye primer cycle sequencing kit with the 14 Taq DNA polymerase FS. The sequences of the mutagenic primers are as follows (5’ → 3’, with mutated bases in boldface): F10A, CAAAAGATTACACGCCGCCCGCTGCTGCA-TGATG; C12G, TACACCTTCCGGCCTGCGAGATGTC; T3F, CAGCATATTTCAAGTCTTTCCGAAGCAGC-TTTACAGG; S76G, CAAGTCTATCGAGTCGATG; GC; G78G, CCAGTCTATCGAGGCTTTTAC- CAGGGACATAAC; R79E, CGAGTCTATTTACGAGG- GACATACAGGAATTAGTC; and LD150 y C57BL Francisco), and hybridomas 2C12, 3C3, 2D5, 2H4 and 1A12 generously provided by M. Bix (University of California, San Francisco). Gene Cloning and Transfection. The derivation and characterization of the mCD1.1 mutants have been described previously (9). Hybridomas 1.4 and 1.2, previously (9). Hybridomas 68 and 24 were kindly provided by Burdin T Cell Hybridomas. The derivation and characterization of the mCD1.1-autoreactive T cell hybridomas have been described previously (9). Hybridomas 1.4 and 1.2, generically provided by M. Bix (University of California, San Francisco), and hybridomas 2C12, 3C3, 2D5, 2H4 and 1A12 (M.G., unpublished results) were all derived from sorted C57BL/6 NK T cell hybridomas. T cell hybridomas were cultured in the presence of antigen-presenting cells (APCs) pulsed with glycolipids or their respective controls as indicated in the figure legends. The cytokine levels in cell culture supernatants were assayed after 16 h of stimulation by using standard sandwich ELISAs. Cytokine levels are expressed as mean ± SD of culture triplicates.

Results and Discussion

Diverse β Chain CDR3s of α14+ T Cell Hybridomas. The properties of the thymus-derived α14+ NK T cell hybridomas that were used in this study are presented in Table 1. Four of the six hybrids show some degree of reactivity for mCD1.1 transfectedants of A20 cells in the absence of exogenous Ag. Two of the hybridomas, 2D5 and 2H4, were not autoreactive to mCD1.1 autoactive APCs, whereas the responses of the autoreactive ones ranged from 5 to 80 units/ml IL-2. Three of these four autoreactive T cells express Vβ8.2. All six α14+ T cells are reactive to α-GalCer presented by mCD1.1, with a moderate (1–1.5 times) to strong (>50 times) enhancement of the mCD1.1 response by the four α14+ T cells that showed autoreactivity.

Nucleotide sequence analysis demonstrated that all six α14+ hybridomas express the invariant Vα14Jα281 rearrangement that is typical of NK T cells (data not shown). The sequences of the TCR β chains from these cells also are shown in Table 1. We do not find evidence for a conserved length or sequence motif in the CDR3β regions of α-GalCer-reactive T cells. In agreement with the results from previous studies of Vα14+ T cells (19, 21), however, there is an over-representation of Jb2 segments. Furthermore, the CDR3s are enriched for the presence of amino acids that are negatively charged, polar, or have small side chains. Collectively, there are a total of eight negative charges and only one positive charge in the CDR3βs, with five of the six hybridomas having at least one negatively charged amino acid. Polarity amino acids serine, threonine, glutamine, and asparagine also are highly represented. Likewise, the number of glycines is greater than expected (13 of 56 amino acids). The majority of these are in the N-D-N region of CDR3, however, and a glycine bias may be introduced by the guanosine-rich sequences of the germ-line Dβ segments. Interestingly, the Vβ1 sequences of human T cells that were expanded in α-GalCer share some of these sequence features (22).

X-ray crystallographic analyses of several carbohydrate-binding proteins have revealed that the most important interactions with sugars involve hydrogen bonds, with side chains having acidic (aspartate or glutamate) or amide (asparagine or glutamine) groups prevalent in forming these interactions with carbohydrate (23). Five of the hybridomas have three amino acids in their CDR3β that fit into these two categories, whereas 2H4 has two amino acids with amide side chains (Table 1). On the basis of these sequence patterns, it is possible that CDR3β amino acids of the NK T cells could be contacting the carbohydrate of α-GalCer. The data from the TCR β chains from mouse and human NK T cells are in marked contrast with the data obtained for a set of five human T cells reactive to diverse lipid Ags and presented by group 1 CD1 molecules, which contain many basic amino acids (16). The difference in CDR3β sequence patterns could reflect differences in the types of Ags recognized; several of the human T cells analyzed recognize Ags that do not contain carbohydrate.

Expression of mCD1.1 Mutants. We have generated stable transfectedants expressing one of eight single or two different double mCD1.1 mutations (summarized in Table 2). These a1 and α2

Table 1. Properties of α14+ NK T cell hybridomas

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>mCD1.1 autoactivity</th>
<th>α-GalCer reactivity, SI</th>
<th>Vβ</th>
<th>CDR3</th>
<th>Jβ start</th>
<th>Jβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>Intermediate</td>
<td>9–73</td>
<td>8.2</td>
<td>GAPSGGYDAEQ</td>
<td>FFG</td>
<td>2.1</td>
</tr>
<tr>
<td>2C12</td>
<td>High</td>
<td>1.5–3</td>
<td>8.2</td>
<td>GDEGYTQ</td>
<td>YFG</td>
<td>2.5</td>
</tr>
<tr>
<td>3C3</td>
<td>Intermediate</td>
<td>12–55</td>
<td>8.2</td>
<td>GDQGITGQL</td>
<td>YFG</td>
<td>2.2</td>
</tr>
<tr>
<td>2D5</td>
<td>None</td>
<td>∞</td>
<td>8.3</td>
<td>SEPGTGAHYEQ</td>
<td>FFG</td>
<td>2.1</td>
</tr>
<tr>
<td>1.4</td>
<td>Intermediate</td>
<td>10–42</td>
<td>10</td>
<td>SPGLTNGGQ</td>
<td>YFG</td>
<td>2.2</td>
</tr>
<tr>
<td>2H4</td>
<td>None</td>
<td>∞</td>
<td>7</td>
<td>LRGQUNT</td>
<td>YFG</td>
<td>2.4</td>
</tr>
</tbody>
</table>

The levels of mCD1.1 autoactivity are indicated (intermediate = 5–50 units/ml; high = 50 units/mL). The α-GalCer reactivity is expressed as a stimulation index (SI), comparing IL-2 release induced by α-GalCer-pulsed APC versus the vehicle-pulsed APCs. ∞ = mCD1.1SI of hybridomas that are not mCD1.1 autoactive. CDR3 is defined as beginning after the conserved serine at the end of Vβ and ending just before the conserved FγF motif in Jβ. N–D–N region amino acids are in boldface.
Table 2. Summary of responses to mCD1.1 mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Expression, mfi</th>
<th>Location</th>
<th>Orientation</th>
<th>Reduced autoreactivity</th>
<th>Reduced α-GalCer</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD150-153IY</td>
<td>264*</td>
<td>a2 helix</td>
<td>F, 153 → F’</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>F171Y</td>
<td>1,086</td>
<td>a2 helix</td>
<td>A’</td>
<td>All</td>
<td>None</td>
</tr>
<tr>
<td>T156I</td>
<td>416</td>
<td>a2 helix</td>
<td>F</td>
<td>2 (1A12, 24)</td>
<td>All</td>
</tr>
<tr>
<td>D153Y</td>
<td>899</td>
<td>a2 helix</td>
<td>F’</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>RD79-80EA</td>
<td>508</td>
<td>a1 helix</td>
<td>F’</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>R79E</td>
<td>1,917</td>
<td>a1 helix</td>
<td>F’</td>
<td>All</td>
<td>3 (1, 2, 3C3, 2D5)</td>
</tr>
<tr>
<td>S76G</td>
<td>1,612</td>
<td>a1 helix</td>
<td>A’</td>
<td>1 (24)</td>
<td>1 (2D5)</td>
</tr>
<tr>
<td>Y73F</td>
<td>1,630</td>
<td>a1 helix</td>
<td>F’</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>C12G</td>
<td>398</td>
<td></td>
<td>A’</td>
<td>1 (1A12)</td>
<td>None</td>
</tr>
<tr>
<td>F10A</td>
<td>488</td>
<td></td>
<td>A’</td>
<td>2 (1A12, 68)</td>
<td>None</td>
</tr>
</tbody>
</table>

Substitutions are shown in the left column with CD1b replacements underlined. The cell surface expression in mean fluorescence intensity (mfi) when the 1B1 anti-mCD1.1 mAb is used is shown. The location and orientation of the mutated side chains are shown: ↓, into the groove; →, across the groove; ↑, up towards the TCR. Autoreactive responses of the Vα14+ cells (24, 68, 1A12) and Vα14+ hybridomas (1.2, 2C12, 3C3, 1,4), and Vα14+ α-GalCer responses (1.2, 3C3, 2D5, 1,4, 2HS) are tabulated separately; the number of hybridomas tested in each category is shown in parentheses. “None” indicates that no hybridoma was affected by the mutation, whereas “All” indicates that every tested hybridoma did not react to the mutant. Otherwise, the numbers of affected hybridomas (names in parentheses) are indicated. Effected responses were either undetectable or, in few cases, were at least 10-fold reduced. Data from at least three experiments in every case were used.

*No reactivity with 1B1, mfi is for 5C6 mAb.

domain mutations have been designed on the basis of several criteria, including visual inspection of the three-dimensional structure of the mCD1.1 Ag binding groove. We also have relied on a preliminary theoretical model of the interaction between mCD1.1 and α-GalCer (H.I., unpublished results). Because of the conserved T cell recognition of mCD1.1 and hCD1d (11), positions conserved between these molecules were targeted as potentially important, while avoiding drastic changes in charge or hydrophobicity that might interfere with surface expression. In 5/10 positions, 76, 150, 153, 156, and 171, mCD1.1 amino acids were replaced with amino acids found in the same positions in human CD1b. Human CD1b can bind to α-GalCer (M.K., unpublished data), but it cannot present α-GalCer to NK T cells (12). At three other positions, we replaced mCD1.1 amino acids having smaller side chains (F10A, C12G, and D80A). Finally, we reasoned that multiple hydrophobic interactions in the groove probably stabilize ligand binding, and that this would be difficult to disrupt, short of introducing potentially disruptive charged amino acids. We therefore focused attention on the few conserved hydrophobic amino acids, which could be important for interaction with the TCR or the hydrophilic portion of the Ag. The location of the mutated amino acid side chains is shown in a supplementary figure (www.pnas.org). These mutations fall into four categories: (i) F10A, C12G, and F171Y are located either in (positions 10 and 12) or close to (position 171) the A’ pocket. (ii) Y73F and L150F point into the F’ pocket. (iii) S76G, R79E, and D80A alter hydrophilic amino acids pointing upward near the end of the long H2 α helix encoded by the a1 domain. Amino acid positions in this group could be interacting with the TCR, and possibly also with the more external, hydrophilic portions of the mCD1.1-bound ligand. (iv) The D153Y and T156I mutations affect side chains pointing horizontally across the F’ pocket of the groove. These changes affect the character of the F’ pocket, and the side chains of these amino acids could be involved in binding to the more exposed portions of the Ag or to the TCR.

Two wild-type mCD1.1 transfectants were selected for this study, clone 1 with an mfi with the 1B1 anti-mCD1.1 mAb of 1,903, and clone 8 with an mfi of 444. Untransfected A20 cells do not express detectable mCD1.1 (mfi = 6). Stable transfectants that express each of the mCD1.1 mutants were generated, and those that express surface mCD1.1 levels that fell close to or between those of the two wild-type mCD1.1 transfectants were used. The mfi for the mutants ranged from 398 to 1,917 (Table 2) except for the L150I D153Y double mutant, which was not recognized by 1B1 (Table 2). 5C6, a second anti-mCD1 mAb, revealed surface expression of L150I D153Y mCD1.1. The mfi of 264 for the L150I D153Y mutant was slightly below that of clone 8 with this mAb (mfi 345).

**Fine Specificity of Autoreactive T Lymphocytes for mCD1.1 Mutants.** Transfectants expressing the mutants were tested, in the absence of exogenous Ag, for their ability to stimulate seven mCD1.1-autoreactive hybridomas (Table 2, Fig. 1A). In addition to the four Vα14+ autoreactive hybridomas described above, three mCD1.1 autoreactive hybridomas that do not express Vα14 were tested. Hybridomas 24 and 68 are derived from the remaining CD4+ T cells in the spleen of MHC class II−/− mice (20), and 1A12 expresses a Vα5/Vβ14 TCR that is atypical for the NK1+ thymocyte population it originated from. Representative response data from a Vα14− and a Vα14+ hybridomas are shown in Fig. 1A. Six mutations gave similar effects on at least 6/7 hybridomas (Table 2). The F171Y and Y73F mutations generally did not reduce autoreactivity. The C12G mutation located on a β strand on the floor of the A’ pocket affected the response of 1A12 only, decreasing it to the background level. Note that the relevant comparison is with the response to the clone 8 wild-type mCD1.1 transfected, as the expression level of the C12G mutant is only slightly less than the clone 8 transfected (Table 2). By contrast, the D153Y mutant reduced autoreactivity to the undetectable level for every hybridoma except 68, and the two double mutations reduced it to an undetectable level in every case. The effect of the remaining mutations varied more, depending upon the cell tested. The responses to the T156I mutation, which points across the F’ pocket from the a2 helix, and the R79E mutation, pointing upward from the a1 helix above the F’ pocket, were similar. The reactivity of the 2C12 and 1.4 Vα14+ hybridomas was unaffected by these mutations, while the mCD1.1 autoreactivity of the remaining five hybridomas was eliminated by either change. The S76G mutant, which like R79E alters an amino acid pointing upward from the top of the α helix, showed a different pattern. This change completely eliminated the autoreactivity of hybridoma 24 and the Vα14+ hybridoma 3C3. It had little effect on the remaining cells, including those shown in Fig. 1A. The F10A mutation on the floor of the A’ pocket abrogated the responses of 1A12 and 68 only, with the
other cells showing little or no effect compared with the relevant clone 8 wild-type control.

Several inferences can be drawn from this analysis of mCD1.1 autoreactivity. First, the mutant mCD1.1 molecules are not likely to have a grossly altered conformation. Outside of the two double mutants, in every case there was at least one hybridoma that had an undiminished response to a particular mCD1.1 mutant. This is consistent with the ability of most of the mutants to have a grossly altered conformation. Outside of the two double mutants, in every case there was at least one hybridoma that expressed this mCD1.1 mutation also could stimulate autoreactivity. The V\text{\textsubscript{b}} chain is important for autoreactivity to wild-type CD1.1 transfectants (\(>200\) units/ml of IL-2 in each case). Second, the mCD1.1 autoreactive T cells are heterogeneous in their response to different mutants. This heterogeneity may reflect the different TCRs expressed by the autoreactive hybridomas, and the importance of particular contacts between these TCRs and mutated mCD1.1 amino acids. Alternatively, this could reflect the recognition of different autologous ligands by the autoreactive T cells, as has been suggested previously (20, 24). Third, the CDR3 region of the \(\beta\) chain is important for autoreactivity. The V\text{\textsubscript{e}}14\textsuperscript{+} hybridomas 1.2, 3C3, and 2C12 all express V\text{\textsubscript{\beta}}2, but only 3C3 is incapable of responding to the S76G mutant, while both 1.2 and 3C3 cannot respond to R79E. Fourth, the amino acid substitutions that affect mCD1.1-autoreactive T cells imply that the interaction with the TCR is conventional, as opposed to superantigen-like. Hydrophilic amino acids at the top of the groove, such as those at positions 76, 79, 80, 153, and 156, participate in T cell stimulation. The important hydrophilic amino acids are good candidates for those that make contacts with the TCR, although interactions with hydrophilic portions of mCD1.1-bound autologous ligands also are possible. Furthermore, amino acids at the bottom of the A' pocket also participate in stimulating autoreactivity. The most straightforward interpretation of the effect of the A' pocket mutations is that the autoreactive hybridomas respond to an autologous ligand(s) bound to the mCD1 groove. The alternative explanation, which is not ruled out, is that the A' pocket mutations cause significant changes in the \(\alpha\) helices that are detected by some of the autoreactive TCRs.

**Presentation of \(\alpha\)-GalCer by Mutant mCD1.1 Molecules.** We tested the ability of the mCD1.1 mutants to present \(\alpha\)-GalCer to five V\text{\textsubscript{\textalpha}}14\textsuperscript{+} hybridomas. Data from hybridoma 2C12 were not included in the analysis, because this highly autoreactive T cell exhibits a weak (approximately 2-fold) and variable stimulation with \(\alpha\)-GalCer (Table 1). The other T cells exhibited optimal \(\alpha\)-GalCer responses of 60–400 units/ml of IL-2 release, well above the level of mCD1.1 autoreactivity. The responses of two V\text{\textsubscript{\textalpha}}14\textsuperscript{+} hybridomas to the mutants are presented in Fig. 1B, and the results are summarized in Table 2.

There was less diversity in the responses of various V\text{\textsubscript{\textalpha}}14\textsuperscript{+} T cells to a defined Ag, compared with the mCD1.1-autoreactive response. A similar pattern of \(\alpha\)-GalCer responses for all five T cells was obtained with 8 of the 10 mCD1.1 mutants. There were no \(\alpha\)-GalCer responses to the 2 double mutants or to either the D153Y or T156I single mCD1.1 mutants, whereas all 5 V\text{\textsubscript{\textalpha}}14\textsuperscript{+} cells responded to the 2 bottom of the A' pocket mutants, F10A and C12G, as well as to the Y73F and F171Y mutants. Two mutations differentially affected the \(\alpha\)-GalCer responses by V\text{\textsubscript{\textalpha}}14\textsuperscript{+} T cells. S76G eliminated the \(\alpha\)-GalCer reactivity of 2D5, decreasing the response from more than 100 units/ml of IL-2 release to less than 1 unit/ml. The R79E mCD1.1 mutation completely eliminated the reactivity of 1.2 and 3C3 as well as 2D5 (Fig. 1B and Table 2), whereas the other V\text{\textalpha}14\textsuperscript{+} cells were not affected. It is noteworthy that the three cells unresponsive to \(\alpha\)-GalCer presented by the R79E mCD1.1 mutant did not express a V\text{\textsubscript{\text\beta}}2 TCR, whereas those expressing V\text{\textsubscript{\text\beta}}7 and V\text{\textsubscript{\text\beta}}10 can respond. This finding demonstrates that the V\text{\textscript{\textbeta}} region is important for the \(\alpha\)-GalCer response. Because the mutated mCD1.1 amino acid side chains for both positions 76 and 79 are pointing upwards, these positions represent possible mCD1.1 contact points for TCR 8.

There is a high degree of similarity in the pattern of responses of the V\text{\textalpha}14\textsuperscript{+} hybridomas to the mCD1.1 mutants in the presence or in the absence of \(\alpha\)-GalCer, suggesting that the mode of recognition of autologous Ags and \(\alpha\)-GalCer could be similar. There are two exceptions to this highly similar response pattern. First, the mutation S76G abolished the mCD1.1 autoreactivity of 2D5–GalCer, suggesting that the mode of \(\alpha\)-GalCer presentation. Because the mutated mCD1.1 amino acid side chains for both positions 76 and 79 are pointing upwards, these positions represent possible mCD1.1 contact points for TCR 8.

![Fig. 1. T cell response to mCD1.1 mutants. (A) Diverse patterns of mCD1.1 autoreactivity. The responses of representative V\textsubscript{\textalpha}14\textsuperscript{+} T cells and V\textsubscript{\textalpha}14\textsuperscript{+} NK T cells are shown. The TCR V regions expressed by these cells are indicated. (B) Pattern of \(\alpha\)-GalCer reactivity of V\textsubscript{\textalpha}14\textsuperscript{+} NK T cells. NK T cell hybridomas were cultured as described with the indicated A20 APCs. APCs were prepulsed with 100 ng/ml of \(\alpha\)-GalCer. Cell-free supernatants were tested by ELISA for IL-2 content as described. One representative experiment of three is shown.](image-url)
mutant mCD1.1 molecules (data not shown). We also used an
hybridomas when tested with the panel of APCs expressing
ide (8, 12, 15), likewise were unable to stimulate any of the T cell
587(data not shown). By contrast, the responses to the bottom of
sistent with a previous report (15), the remaining four V
stimulated by AGL 587 presented by either wild-type mCD1.1 or
 generally were 2- to 10-fold weaker than the responses to
160
u
[52x197]their effects upon the responses to both
[52x56]10160
[52x126]hybridomas could recognize AGL 587. The magnitude of the
responses to wild-type mCD1.1 induced by 100 ng
hybridomas were pulsed with 100 ng
A20 wt CD1
y
8) or A
9
ml AGL 587
A
a
[77x32]www.pnas.org Burdin
[93x187]a
[98x187]-GalCer, AGL 587, whose acyl chain is reduced to
[121x247]-GalCer Analogs by Mutant mCD1.1 Molecules.
[129x157]14
[129x227]-galactosylceramide, and
[138x160]hybridomas, 2C12 and 2D5, were not
[140x329]A
a
[145x329]) Structure of
[196x329]a
[200x302]a
[200x302]ml either
[220x66]a
[225x311]y
[229x311]8) or A
[237x302]-GalCer or AGL 587
[247x106]a
[249x311]9
[251x106]ml AGL 587
[276x329]-GalCer
[276x320]B
[280x320]) APCs
[286x126]14
[315x96]bottom of the A
[315x106]a
[315x126]type interactions. We also provide evidence that mCD1.1 auto-
[315x56]sphingosine in the larger A
[315x66]the groove of mCD1.1, and that it is most likely to do so with the
[315x226]-GalCer. Possible contact points identified for
galactose of
a
[315x226]-GalCer. Although the
[315x236]a
[315x246]TCR is similar to that for glycopeptides, involving inter-
[315x256]molecule. We propose that the mechanism of contact by the NK
[315x266]helical amino acids of the Ag-presenting
[315x266]a
[338x726]pocket mutations F10A and C12G exhibit striking dif-
[349x696]a
[355x696]-GalCer stimulation of V
[361x526]a
[367x586]-GalCer presented by the F10A mCD1.1 molecule
[370x486]a
[367x586]-GalCer, however, presumably because the inter-
[373x486]a
[373x586]pocket mutation reduces the ability of the mCD1.1
[376x486]a
[376x586]molecule to interact with the sphingosine of AGL 587, placing
[379x486]a
[379x586]the likelihood of the sphingosine binding to the A’ pocket.
The F10A mutation causes a more modest reduction in the
reactivity to α-GalCer, however, presumably because the inter-
action of the mCD1.1 F’ pocket with the acyl chain partially
compensates for the loss of binding energy in sphingosine
binding to the mutant A’ pocket.

Conclusions. The presentation of glycosphingolipids by mCD1.1
to NK T cells is a useful model for studying the molecular basis
for lipid Ag recognition by T lymphocytes, because of the
availability of both analogs of α-GalCer and mCD1.1-reactive
cells with very closely related TCRs, and because the structure
of mCD1.1 is known. The data presented here provide an outline
of how the trimolecular complex of TCR, lipid, and mCD1.1 is
formed. Regarding the TCR, we find preferential use of acidic
and polar amino acids in the CDR3 region of the β chain of
α-GalCer-reactive Vα14+ T cells, suggesting that some of these
amino acids could be involved in hydrogen bond formation with
the galactose moiety of the Ag. Although the α chain is most
highly conserved, we find evidence for the involvement of TCR
β in both mCD1.1 autoreactivity and α-GalCer plus mCD1.1
responses. Recent studies (25, 26) demonstrate that glycopept-
ides with mono- or disaccharides attached to the central region
of the peptide allow for a conventional type of T cell recognition
of carbohydrate Ags, but the α helical amino acids of the α helix
are at least consistent with the diagonal orientation
found for peptide plus MHC class I reactive TCRs (27). It
remains to be determined, however, whether the strong conser-
vation of the Vα-Jα junction in α-GalCer-reactive NK T cells is
due to selection for interaction with a bound Ag.

Because mutations along the top of the helix and the bottom
of the groove affect both autoreactivity and α-GalCer recogni-
tion, these processes are not likely to be similar to superantigen-
type interactions. We also provide evidence that mCD1.1 auto-
reactive cells are heterogeneous, and by demonstrating that
some of the autoreactive cells are affected by mutations in the
bottom of the A’ pocket, the data suggest recognition of an
mCD1.1-bound autologous ligand by these T cells. Analysis of
mutations in the A’ pocket also indicates that α-GalCer binds to
the groove of mCD1.1, and that it is most likely to do so with the
sphingosine in the larger A’ pocket and the acyl chain in the
narrower F' pocket. If this were the case, then the entire 18-carbon sphingosine could be accommodated in the larger A' pocket. The 26-carbon acyl chain is too long to fit entirely into the F' pocket, although α-GalCer can be presented without intracellular Ag processing (10). The acyl chain therefore may extend out of the groove, with its terminal portion free, or it may interact with some other part of mCD1.1. It remains to be determined whether compounds with even longer hydrophobic chains will need to be processed to be bound in the CD1 groove or whether there is some other mechanism for accommodating the long hydrophobic chains of lipid Ags.

We thank Drs. Kyoko Hayakawa, Olga Naidenko, Jennifer Matsuda, and Yasuhiyo Koezuka for support and helpful discussions, and Dr. Chung-Ryu Wang for the gift of the 5C6 hybridoma. This work was supported by National Institutes of Health Grants RO1 CA52511 (M.K.) and RO1 CA58896 (I.A.W.) and by a grant from the Association pour la Recherche contre le Cancer (N.B.). This is manuscript no. 313 from the La Jolla Institute for Allergy and Immunology.