

Antigen-presenting Function of the Mouse CD1 Molecule^a

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INTRODUCTION

CD1 molecules were first identified as cell-surface molecules expressed by human thymocytes.¹ Like the thymus-leukemia (TL) antigen expressed by mouse thymocytes, CD1 molecules were found to be coexpressed with $\beta 2$ microglobulin ($\beta 2m$) and to be relatively nonpolymorphic. It was therefore speculated that CD1 might be the homologue of the mouse TL antigen. In 1986, the group of Cesar Milstein reported the first isolation of a gene encoding a CD1 molecule.² The translated nucleotide sequence revealed that unlike the TL antigen, CD1 is only distantly related to classical class I molecules with no statistically significant sequence similarity in the $\alpha 1$ and $\alpha 2$ domains that encode the peptide antigen-binding site. Therefore, although the TL antigen and human CD1 might be functional homologues, they clearly are not structural homologues.

CD1 genes and molecules have been discovered subsequently in a number of species including mice, rats, and rabbits.³ In humans there are five closely linked CD1 genes encoded on chromosome 1, unlinked to the major histocompatibility complex (MHC). Four of these genes, CD1a, CD1b, CD1c, and CD1d, are known to encode cell-surface

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proteins.⁴ Although all of the human CD1 genes share distinct sequence features that distinguish them from other class I genes, they are not particularly closely related to one another. For example, the human CD1d protein, which is the most divergent human CD1 molecule, shares no more than 37-40% sequence similarity in the $\alpha 1$ and $\alpha 2$ domains to the other human CD1 molecules. In addition, each human CD1 molecule has a distinct pattern of expression. As a group, CD1 molecules are expressed on a limited number of cell types that seem to have an antigen-presenting function. CD1a is expressed by Langerhans' cells in the skin,⁵ and CD1d is expressed by intestinal epithelial cells.⁶

The mouse has two closely related CD1 genes, CD1.1 and CD1.2. These two are most similar in sequence to human CD1d.^{2,3} As in the human, the mouse CD1 (mCD1) genes are encoded outside of the MHC located on mouse chromosome 3. The two CD1 genes in mice are closely linked and are presumed to have derived from a relatively recent gene duplication. mCD1 molecules are expressed by both small and large intestine epithelial cells.⁶ Using immunohistochemistry, some mCD1 expression also is detectable in lymph node and thymus. As in the mouse, the only known rat CD1 gene is CD1d-like,⁷ and the rat CD1 mRNA also is expressed in the intestine.⁷ Similarly, one of the two known rabbit CD1 genes, CtrbCD1, is a CD1d homologue.⁸ CD1d, therefore, is the only nonclassical class I molecule known to have been conserved throughout much of mammalian evolution.

It is thought that CD1 molecules are likely to have a specialized antigen-presenting function. Despite this, prior to the work reported in reference 9 and in this manuscript, there was no evidence for antigen presentation by mCD1. By contrast, T cells reactive to each of the four human CD1 molecules have been reported.¹⁰⁻¹³ Evidence from several laboratories indicates that antigen presentation by CD1 is distinct from MHC-encoded molecules. First, most of the T cells reactive with human CD1 are CD4, CD8 double negative,¹⁰ although there are some exceptions.¹⁴ The CD1-reactive T cells express either $\alpha\beta$ or $\gamma\delta$ T-cell antigen receptors (TCRs). Second, CD1b transfectants of T2 cells can present a mycobacterial antigen to reactive T cells.¹⁰ Because T2 cells are defective for both TAP (transporter associated with antigen processing) subunits and the LMP2 and LMP7 proteins, which are MHC-encoded subunits of the proteasome, this demonstrates that the pathway for antigen processing and presentation by human CD1b is distinct from that of MHC class I. Consistent with this, CD1b-mediated presentation of the mycobacterial antigen was inhibitable by chloroquine,¹⁰ suggesting that endosomal acidification is necessary for presentation. The processing and presentation pathway must also be different from the MHC class II pathway, as T2 cells lack the DM molecule that is required for efficient peptide loading of class II molecules.¹⁵ Third, Beckman and co-workers¹⁶ have recently demonstrated that the mycobacterial antigen presented to a CD1b-restricted T-cell clone is mycolic acid, a bacterial lipid.

There are several reasons why the antigen-presenting function of CD1 molecules might be relevant for investigators interested in oral tolerance. First, CD1 molecules are expressed in a TAP-independent fashion,¹⁷ and they may traffic to an endosomal compartment. If this were true, they might be capable of "sampling" antigens from the lumen of the gut. Third, because they are not polymorphic, they could prove to be useful targets for any specific immunotherapy directed to an antigen-presenting molecule. In this chapter, we present our recent data demonstrating that mCD1 also has a distinct non-class I, non-class II antigen-presenting function. We define one type of antigen that can bind to mCD1, and we characterize the first peptide-specific and mCD1-restricted T cells.

DEFINITION OF PEPTIDES THAT BIND TO mCD1

We initially made stable mCD1 transfectants in TAP-deficient RMA-S cells, in the hope that these transfectants would prove to be useful in the definition of mCD1-binding

peptides. Classical class I molecules, which are not loaded with peptide in RMA-S cells, can reach the cell surface when these TAP-deficient cells are cultured at 23°C. When the culture temperature is raised to 37°C, however, the empty molecules become unstable and they denature.¹⁸ If peptide capable of binding the class I molecule is added prior to the shift to 37°C, the class I molecules are stabilized. A heat stabilization assay, therefore, can be used to screen for peptides capable of class I binding. When mCD1 transfectants of RMA-S were tested in this way, however, we were surprised to find that equally high levels of surface expression of mCD1 were obtained in 23°C and 37°C cultures.¹⁹ We also tested *Drosophila melanogaster* cells, which are presumed to have a broader defect in peptide loading than the TAP-2-deficient RMA-S cells. Transfected *D. melanogaster* cells synthesize empty classical class I molecules that are thermally unstable.¹⁹ Stable mCD1 transfectants of *D. melanogaster* embryo cells showed equal levels of mCD1 surface expression when cultured at the normal temperature of 23°C or following a two-hour shift to either 33°C or 37°C. We, therefore, conclude that the mCD1 surface expression is TAP independent.

In an attempt to identify the possible peptide-binding ability of mCD1, recombinant soluble CD1-β2m complexes produced in *D. melanogaster* cells were used to screen a random peptide phage-display library (RPPDL). The phage-display library was generated by cloning oligonucleotides encoding random 22 amino acid sequences into the mature NH2 terminus of the gene VIII coat protein. When this library was screened with soluble and presumably empty mCD1 molecules derived from insect cells, 47 different clones were selected by mCD1 binding. Alignment of the amino-terminal sequences encoded by these phages shows a well-defined core motif consisting of an aromatic Phe or Trp amino acid at position one (100% of the clones), an amino acid with a long, aliphatic side chain at position four (38/47), and a Trp at position seven (35/47). Therefore, mCD1 seems to select phages with a hydrophobic binding motif, preferring aromatic residues at positions one and seven and aliphatic residues at position four. The sequence motifs obtained by screening RPPDL with classical class I molecules are characterized by an anchor amino acid in fixed positions relative to the N terminus,²⁰ consistent with the binding of the N terminus buried in a conserved pocket.²¹ This is not, however, a characteristic shared by the mCD1 motif; the N terminus of the mCD1-selected peptides is at a variable distance from the anchor binding motif, suggesting that mCD1, like class II molecules, is capable of binding peptides with extended N and C termini. The K_d determined for peptide binding to mCD1 is in the range of an intermediate to good binder for class I molecules and in the range of naturally processed peptides copurified with class II molecules.²¹

It was possible that the synthetic peptides bind to some portion of the mCD1 molecule other than the putative peptide binding groove formed by the α1 and α2 domains. To begin to assess the immunologic relevance of the biochemical data on peptide binding, we raised several peptide-specific and mCD1-restricted T-cell lines. Most of these mCD1-reactive T-cell lines were generated from lymph nodes of mice immunized with mCD1 transfectants of the RMA-S cell line that had been preloaded with peptide p99a-2.12 (EHFHIREWGNHWK); the putative anchor amino acids for mCD1 binding are underlined. The reactive T cells require both mCD1 and peptide for stimulation, as measured by γ-interferon synthesis, as shown in TABLE 1. Three different mCD1 transfectants, RMA-S T cells, L-cell fibroblasts, and J774 macrophages stimulated the T cells in the presence of peptide, indicating independence of any other MHC molecule for T-cell stimulation.

Using a variety of synthetic peptides, we found that there was a reasonably good correlation between the ability of a synthetic peptide to bind to soluble mCD1 and its ability to stimulate mCD1-restricted T cells *in vitro*, confirming the importance of the putative anchor amino acids and their relevance for antigen presentation. For example, single substitutions of each of the anchor residues with alanine reduced the peptide binding

TABLE 1. Peptide plus CD1 Are Recognized by T Cells as Assayed by IFN γ Production^a

Interferon- γ Production (U/mL)	
(a) RMAS CD1 ⁺ and peptide	25.2
(b) J774 CD1 ⁺ and peptide	17.4
(c) J774 (no peptide)	1.1

^a Table shows IFN γ produced by T cells when stimulated with either RMAS CD1⁺ cells pulsed with peptide (a), J774 CD1⁺ cells pulsed with peptide (b), or with peptide alone (c).

to mCD1 drastically, and these peptides could not effectively stimulate the peptide-specific, mCD1-restricted T cells. To rule out the possibility that stabilization of the mCD1 molecule by peptide is responsible for T-cell stimulation, we tested two peptides that had the same anchor amino acids for binding to mCD1 but differed in the probable T-cell binding sites. Even though these two peptides had the same binding constant to mCD1 as the original peptide, both of them were unable to stimulate the T cells. These data argue against the possibility that any mCD1-binding peptide causes a conformational change in mCD1 that can be sensed by the reactive TCR. Instead, they are most consistent with a more conventional model in which some amino acid side chains contribute to mCD1 binding, and others side chains point towards the TCR for recognition by specific clones.

Because we have shown that peptides up to 22 amino acids long, or longer, might bind to mCD1 *in vitro*, it was necessary to show that no further trimming of these peptides to the more typical 8-9 amino acid size was occurring. In order to do this, we used purified mCD1 molecules to eliminate the possibility of any intracellular peptide-antigen processing or trimming. Plates were coated with soluble mCD1 on 96-well plates. Some of the wells were incubated with appropriate antigenic peptide, mCD1-reactive cells were added to the cultures, and the production of γ -interferon was measured by ELISA. We demonstrated that the cell-free mCD1-stimulation system was reasonably effective when the correct peptide was present; plates coated with mCD1 molecules in the absence of peptide did not stimulate γ -interferon release. Studies using serum-free medium showed that proteases in serum are not required for extracellular processing of long peptides that are presented by mCD1.

We have carried out a preliminary phenotypic characterization of the T cells reactive to mCD1. Flow-cytometric analysis of the T cells in a line raised to the peptide p99a.-2.12 demonstrated that nearly all the cells express an $\alpha\beta$ TCR and the CD8 molecule (FIGURES 1A and 1B). These data demonstrate that the mCD1-reactive T cells can have a conventional phenotype for class I-reactive cells from the lymph node, in contrast to the CD1-reactive cells from the human peripheral blood that tend to be either TCR $\alpha\beta^+$ and double negative, or TCR $\gamma\delta^+$.

A MODEL FOR INTRACELLULAR TRAFFICKING OF mCD1

We propose a model for intracellular trafficking of mCD1 based on the following. (1) Expression of mouse and human CD1 molecules is TAP independent.^{11,19} (2) mCD1 molecules, as well as some CD1 molecules in other species, have an endosomal localization signal in their intracytoplasmic tail.²² For example, mCD1 has the sequence YQDI in its

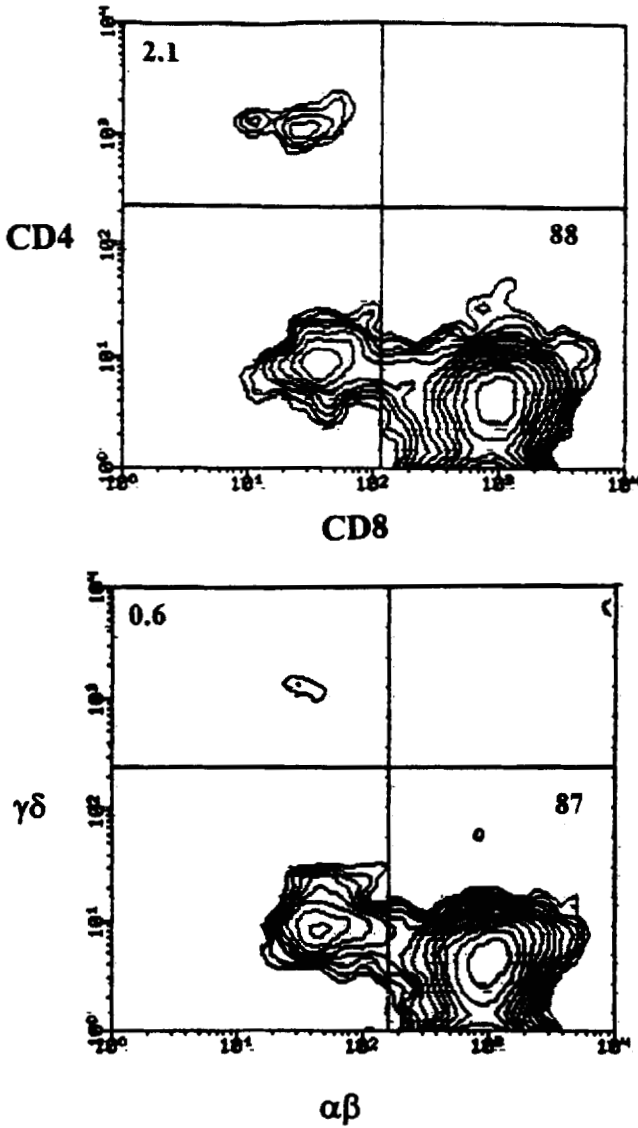


FIGURE 1. Flow cytometric analysis of a CD1-reactive T-cell line. Top: Staining of the cells with anti-CD4 (phycoerythrin, PE) and anti-CD8 (fluorescein isothiocyanate, FITC) antibodies. Bottom: Staining of the T-cell line with anti- $\gamma\delta$ TCR (PE) and anti- $\alpha\beta$ TCR (FITC).

intracytoplasmic domain. (3) Preliminary data indicate that the mCD1 molecules produced by *D. melanogaster* cells are free of peptide. (4) Mycobacterial antigen presentation by CD1b requires acidified endosomes.¹¹ Based on these facts, we hypothesize that assembly of CD1-class heavy chain and β_2m is sufficient to release the complex from binding to calnexin or any similar chaperons, and that the heavy-chain/ β_2m complex makes its way to an acidified vesicular compartment where it will contact antigens present in this compartment.

DISCUSSION

The results described above clearly establish that mCD1 can present a distinct set of peptides to CD8⁺, cytotoxic T cells. A number of important questions remain unanswered. First, what are the natural ligands presented by mCD1? A search of protein-sequence databases indicates that the mCD1-binding motif is present in a great variety of proteins of both microbial and nonmicrobial origin. From this search, there is nothing to suggest why an antigen-presenting molecule that presents peptides with a WXXLXXW sequence motif, or similar sequences consistent with the motif, might be conserved through evolution. Because all CD1 molecules are related to one another, the finding of peptide binding by mCD1 and lipid binding by human CD1b is puzzling. There are several possibilities. First, all CD1 molecules might naturally be lipid binding, and peptides were obtained for mCD1 only because a peptide display library was screened. The attractive feature of this model is that it can help to explain the relative lack of CD1 polymorphism.

Because lipids are the end product of a complex biosynthetic pathway, they may be presumed to change more slowly in evolution than the amino acids of antigenic peptides, many of which are likely to be nonessential from the viewpoint of function of that protein. In addition, bacterial lipids are distinct from those in eukaryotic cells, thereby allowing CD1 molecules to carry out a basic form of self/nonself description. Despite this conceptual appeal, the model requires that the hydrophobic mCD1 peptide-binding motif obtained from the bacteria phage display library is essentially a mimic for the natural lipid ligand. It is not known how the anchor amino acid side chains and the mycobacterial fatty acids would be capable of binding to the same peptide groove. A second model holds that CD1 molecules are selected primarily for their ability to traffic to a highly acidic late endosomal or lysosomal compartment where they might be capable of binding to hydrophobic ligands that are generated by antigen-processing enzymes there. These ligands could be either hydrophobic peptides, lipids, lipoproteins, or glycolipids. A third possibility is that there are at least two different categories of CD1 molecules. According to this view, CD1d-like molecules, which are distantly related to the others, present hydrophobic peptides, whereas molecules such as CD1b, which are not present in rodents, present lipids. It is interesting to note that the $\alpha 1$ and $\alpha 2$ domains of CD1b apparently are the most divergent in the CD1 family.⁸

A second major issue concerns the diversity and distribution of CD1-reactive T cells. It is unlikely that the synthetic peptide sequence p99.a-2.12 is uniquely capable of stimulating the generation of mCD1-restricted cells, although this remains to be formally proven by generating T lymphocytes specific for other mCD1-binding peptides. How might a diverse repertoire of mCD1-restricted T cells be generated? Are these cells thymus dependent? Do they require positive selection by mCD1, or by cross-reaction? Are they positively selected with another class I molecule? Are they all CD8 positive? Most important, from the point of view of this volume, what is the frequency and specificity of mCD1-restricted T cells in the intestine? In future experiments, we intend to determine if mCD1-restricted and peptide-reactive T lymphocytes can be detected in lamina propria and among intraepithelial lymphocytes following *in vivo* priming. If such cells exist in lamina propria, and

if they are capable of recirculating from that site, then mCD1-restricted cells sensitized in the gut could, in part, be responsible for the induction of systemic oral tolerance. Finally, it is possible that mCD1 can acquire long peptides in the lumen of the gut for further processing to 9 amino acids and presentation by classical class I molecules. This would require escape from the endosome into a classical class I peptide-loading pathway, a process that is known to occur for some bacterial proteins.²³

SUMMARY

CD1 molecules are distantly related to major histocompatibility complex (MHC)-encoded class I molecules, and they are coexpressed with $\beta 2$ microglobulin ($\beta 2m$). In the mouse, CD1 is expressed by intestinal epithelial cells and also by some cells in spleen and lymph node. We have shown that surface expression of mouse CD1 (mCD1) is not dependent upon a functional transporter associated with antigen processing (TAP). This, and other data, suggest that mCD1 may acquire peptides in an intracellular compartment other than the endoplasmic reticulum, where classical class I molecules bind peptide. mCD1 molecules also are distinct from classical class I molecules with regard to the types of peptides that they bind. We have demonstrated that mCD1 molecules preferentially bind peptides much longer than the 8-9 amino acids typical of the peptides that bind to classical class I molecules. The sequence motif for mCD1 peptide binding is characterized by the presence of bulky and hydrophobic amino acid side chains. We have generated mCD1-restricted and peptide-specific T-cell lines, thereby demonstrating the immunologic relevance of peptide binding to mCD1. The reactive T cells are TCR $\alpha\beta^+$ and CD8⁺, a phenotype typical of many lymphocytes in both lymph node and intestinal mucosae. We speculate that mCD1 molecules may be capable of sampling peptides from the gut lumen and presenting them to mucosal T lymphocytes. In this way, they may function in the maintenance of normal mucosal immune homeostasis, and perhaps also in the induction of systemic tolerance to antigens delivered by the oral route.

In summary, CD1 molecules are a novel category of antigen-presenting molecules that have features in common with class I molecules, features in common with class II, and properties distinct from either subset of antigen-presenting molecules. Further studies of the antigen-presenting function of these molecules are certain to yield new insight into immune regulation and perhaps also into the mechanism of oral tolerance.

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