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Murine CD1d-Restricted T Cell Recognition of Cellular Lipids

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Summary

NKT cells are associated with immunological control of autoimmune disease and cancer and can recognize cell surface mCD1d without addition of exogenous antigens. Cellular antigens presented by mCD1d have not been identified, although NKT cells can recognize a synthetic glycolipid, α-GalCer. Here we show that after addition of a lipid extract from a tumor cell line, plate-bound mCD1d molecules stimulated an NKT cell hybridoma. This hybridoma also responded strongly to three purified phospholipids, but failed to recognize α-GalCer. Seven of sixteen other mCD1d restricted hybridomas also showed a response to certain purified phospholipids. These findings suggest NKT cells can recognize cellular antigens distinct from α-GalCer and identify phospholipids as potential self-antigens presented by mCD1d.

Introduction

CD1 molecules are evolutionarily conserved β_2-microglobulin (β_2m)-associated proteins, with a similar domain organization to class I antigen-presenting molecules of the major histocompatibility complex (Porcelli, 1995). However, CD1 molecules have a deeper and more hydrophobic antigen-binding groove than class I molecules (Zeng et al., 1997). Correspondingly, while class I molecules present peptide antigens, CD1 molecules can present lipids and glycolipids. Studies of human CD1a, -b, and -c molecules first demonstrated that they can present microbial glycolipid antigens to T cells (Beckman et al., 1994, 1996; Sieling et al., 1995). Subsequently, both human and murine CD1d molecules have been shown to present α-glactosylceramide (α-GalCer), a synthetic acylphytosphingolipid originally isolated from a marine sponge (Kawano et al., 1997; Spada et al., 1998).

The T cells that recognize murine CD1d molecules are either $CD4^+$ or negative for both CD4 and CD8β (double negative, or DN) (Bendelac et al., 1994, 1995). At least two distinct populations of CD1d-restricted αβ T cells have been identified in the mouse, based on their T cell receptor (TCR) structures. One population has a characteristic invariant TCRα chain (Vα14/Jα281) paired preferentially with TCRβ chains utilizing Vβ8. These cells comprise a part of the NKT cell subset, T cells that express receptors of the NK complex (Lantz and Bendelac, 1994; Taniguchi et al., 1996). More recently, T cells expressing diverse TCRα and -β chains have also been found that recognize mCD1d molecules (Cardell et al., 1995; Behar et al., 1999; Chiu et al., 1999). Similar to those of the "NKT" subset, CD1d-restricted cells belonging to this "diverse TCR" population can secrete significant amounts of IL-4 and IL-10 in addition to IFNγ and may thus contribute to determining the TH_1/TH_2 cytokine balance in immune responses (Yoshimoto et al., 1995; Behar et al., 1999). CD1d-restricted T cells have also been associated with various immunologically mediated functions, such as preventing development of autoimmune diabetes, tumor rejection, and modulating IgG responses during protozoal infections (Cui et al., 1997; Wilson et al., 1998; Schofield et al., 1999).

The origin and the identity of the natural antigens recognized by CD1d-restricted T cells remain unknown. It has been postulated that mCD1d-restricted NKT cells may recognize a single or a conserved set of antigens, since their cannonical α chains and limited β chain diversity result in TCRs of comparatively little structural variability, whereas the diverse TCR population of mCD1d-restricted T cells may have heterogeneous antigenic specificities (Cardell et al., 1995; Behar et al., 1999; Chiu et al., 1999). Both T cell populations can recognize CD1d molecules on antigen-presenting cells (APCs) in vitro, without requiring addition of exogenous antigens (Bendelac et al., 1995; Behar et al., 1999). Whether this phenomenon is due to recognition of the CD1d heavy chain itself or represents recognition of CD1d complexed with cellular antigens or exogenous antigens derived from the culture medium is unclear. NKT cells have also been shown to respond to synthetic α-GalCer in a CD1d dependent manner, but this antigen has thus far not been found in mammalian tissues (Kawano et al., 1997). Hence, neither the nature of the cellular antigens bound by CD1d molecules nor whether these antigens are required for T cell recognition of CD1d molecules is well understood.

Here, we investigated the requirement for presentation of cellular antigens in T cell recognition of mCD1d molecules and examined the antigen specificities of mCD1d-restricted T cells of the NKT cell and diverse TCR populations. We developed a system to study recognition of mammalian lipids using an immobilized murine CD1d fusion protein and purified antigen preparations. Recognition of the recombinant mCD1d fusion

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protein in this system was dependent on the addition of particular lipids, permitting analysis of the lipid antigen specificities of mCD1d-restricted T cells. Our results provide evidence that mCD1d-restricted T cells require presentation of specific antigens for recognition of mCD1d molecules. Surprisingly, our findings suggest that the mCD1d-restricted NKT cell subset surveys multiple cellular antigens distinct from α-GalCer and implicate common phospholipids as potential autoantigens recognized by certain NKT cells.

Results

An mCD1d-Restricted NKT Cell Hybridoma Responds to a Lipid Extract from RMA-S Cells

Certain mCD1d-restricted T cells do not require exogenous antigens for mCD1d recognition, suggesting that they may recognize mCD1d molecules directly or may recognize cellular antigens complexed with mCD1d (Bendelac et al., 1995; Behar et al., 1999). To investigate whether cellular lipids are involved in such recognition, we studied an NKT cell clone called 24.8, which recognizes mCD1d expressed on murine splenocytes and dendritic cells as well as on mCD1d-transfected RMA-S tumor cells (Behar et al., 1999; S. M. B., unpublished data). Because hybridomas can produce IL-2 in response to antigenic stimulation in the absence of additional costimulatory signals, a T cell hybridoma, designated 24.8.A, was derived from this clone.

To investigate mCD1d recognition by the 24.8.A hybridoma, we tested a soluble mCD1d-IgGFc$_{2a}$ fusion protein that had been purified and immobilized on protein A–coated plates for its ability to stimulate IL-2 release. The 24.8.A hybridoma usually secreted a modest amount of IL-2 when incubated with the mCD1d fusion protein (50–300 pg/ml in 60% of the experiments), but it occasionally produced high levels of IL-2 ($>$600 pg/ml in 20% of the experiments) or did not generate quantifiable IL-2 (20% of the experiments; data not shown). In contrast, incubation with an immobilized anti-CD3 mAb consistently resulted in very high levels of IL-2 secretion (usually $>$2000 pg/ml IL-2; data not shown). No detectable IL-2 was secreted when the 24.8.A hybridoma was incubated with a negative control protein (IgG$_{2a}$ mAb RPC5.4 or UPC10) immobilized on the protein A plate (data not shown).

The poor stimulation of the 24.8.A hybridoma by the mCD1d fusion protein suggested that a specific cellular antigen might be required for efficient recognition of the recombinant mCD1d molecule. We reasoned that an appropriate antigen should be contained within a lipid extract made from RMA-S cells, since these cells can be efficiently recognized when they are transfected with mCD1d (Behar et al., 1999). A modified Folch extraction protocol was used to purify biochemical fractions from RMA-S and S49 T lymphoma cells (Folch et al., 1956; Hamilton et al., 1992). The resulting aqueous, organic, and interface fractions were tested for the ability to stimulate the 24.8.A hybridoma. Plate-bound mCD1d fusion protein or the negative control protein was preincubated with the cellular fractions, then repeatedly washed to remove unbound material prior to addition of the 24.8.A hybridoma. Pretreatment of the mCD1d fusion protein

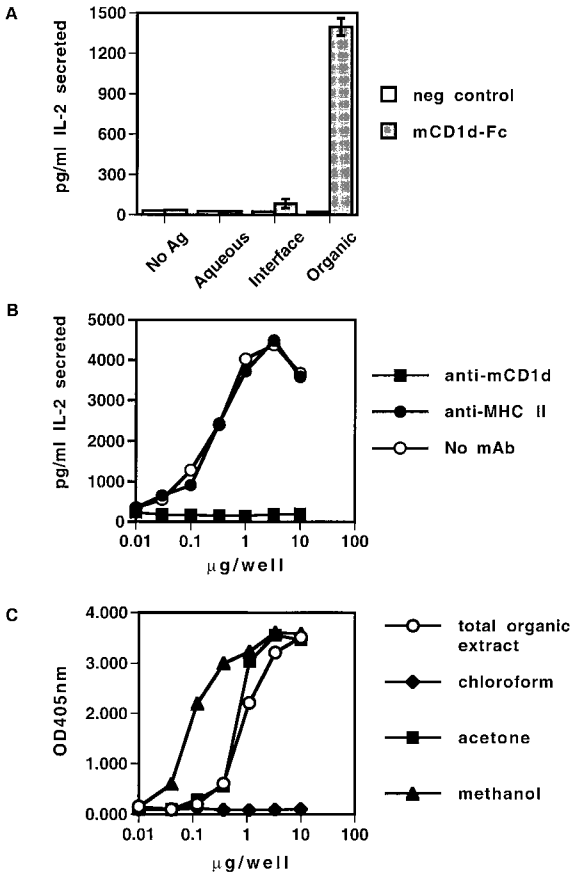

Figure 1. IL-2 Secretion by the 24.8.A Hybridoma in Response to Plate-Bound mCD1d Molecules and Antigens Extracted from Tumor Cell Lines

(A) The mCD1d fusion protein (mCD1d-Fc) or a negative control protein (neg control), pretreated with buffer (no Ag), or 3.3 μg/well of the aqueous, interface, or organic phase fractions from a Folch extraction of RMA-S tumor cells.

(B) The mCD1d fusion protein preincubated with serially diluted amounts of the organic phase of a Folch extraction of RMA-S cells. Open circles, assays performed without added antibody; filled circles, assays performed in the presence of a negative control anti-class II MHC mAb; filled squares, assays performed in the presence of the 19G11 anti-mCD1d mAb.

(C) The mCD1d fusion protein preincubated with serially diluted fractions eluted from a silica column after passage of an organic extract of S49 tumor cells. Open circles, unfractionated organic extract; filled diamonds, chloroform eluate; filled squares, acetone eluate; filled triangles, methanol eluate. The data represent the mean OD_{405} nm or the pg/ml IL-2 detected in an IL-2 ELISA of hybridoma culture supernatants from three replicate wells. Error bars show the standard deviations of the means.

with the organic phase of the RMA-S extract resulted in markedly augmented IL-2 release by the 24.8.A hybridoma compared to the mCD1d fusion protein treated with buffer (Figure 1A). In contrast, the mCD1d fusion protein preincubated with the interface induced only a small increase in IL-2 production, and treatment with the aqueous phase did not enhance IL-2 secretion compared to the buffer-treated control (Figure 1A). The negative control protein failed to induce significant IL-2 secretion when preincubated with any of the Folch fractions (Figure 1A). Thus, stimulation was dependent on

the presence of the mCD1d fusion protein and specific for the organic phase of the cellular extract, which contains mainly the cellular lipids (Folch et al., 1956; Hamilton et al., 1992).

To examine further the antigen dependence of the hybridoma, the amount of organic extract added to the plate-bound mCD1d fusion protein was titrated. Titration of the lipid extract from 0.03 μg/well to 10 μg/well produced a dose-dependent response that appeared saturated at 1 μg/well (Figure 1B). In the presence of a negative control anti-MHC class II mAb, the titration curve was nearly identical, but an anti-mCD1d blocking mAb completely abrogated the response (Figure 1B). Organic extracts from S49 cells gave similar results (data not shown). Hence, the lipid fraction of mammalian cellular extracts contained antigenic material that stimulated the 24.8.A hybridoma in an mCD1d- and dose-dependent manner.

To characterize the nature of the antigen contained in the cellular lipid extract, the organic phase preparations from the Folch extractions were further fractionated using a silica column. Lipids of increasing polarity were eluted sequentially from the column with chloroform, acetone, and methanol, resulting in separation of fractions that predominantly contained neutral lipids, glycolipids, and phospholipids, respectively. These fractions were tested for stimulation of the 24.8.A hybridoma, compared to the unfractionated organic phase of the extract, by titrating the amount of each fraction preincubated with the plate-bound mCD1d fusion protein. Addition of the chloroform fraction did not induce detectable IL-2 production (Figure 1C). In contrast, pretreatment of the mCD1d fusion protein with the acetone and methanol fractions resulted in dose-dependent stimulation of the 24.8.A hybridoma (Figure 1C). Hence, the 24.8.A hybridoma recognized fractions of the organic extract containing polar lipids but did not respond to a fraction enriched in neutral lipids.

Recognition of Synthetic Antigens by NKT Cell Hybridomas

Studies using human cells have shown that CDI molecules present lipid antigens, including mycolic acids and the glycosylated phosphatidylinositols (GPIs) lipoarabinomannan and phosphatidyl mannoside (Beckman et al., 1994; Sieling et al., 1995). Subsequently, GPI and the acylphytosphingolipid α-GalCer have been shown to bind and be presented by murine CD1d (Kawano et al., 1997; Joyce et al., 1998; Schofield et al., 1999). Our finding that addition of cellular organic extracts containing polar lipids permitted efficient recognition of the mCD1d fusion protein suggested that the 24.8.A hybridoma recognizes an abundant mammalian lipid. To investigate recognition of potential cellular lipid antigens, we tested a purified preparation of the phospholipid phosphatidylinositol (PI) and a series of purified and synthetic sphingolipids for recognition by the 24.8.A hybridoma and by another NKT cell hybridoma called 24.9.E. Plate-bound mCD1d fusion protein or a negative control protein was pretreated with α-GalCer, β-GalCer, unglycosylated ceramide, the naturally occurring gangliotriosyl-ceramide (asialo-GM_2), and PI prior to addition of the hybridomas. The 24.8.A hybridoma showed only a slightly enhanced response to the mCD1d fusion protein, which had been preincubated with the α-GalCer antigen or the other sphingolipids, compared to untreated fusion protein (Figure 2A). However, pretreatment of the mCD1d fusion protein with PI resulted in a marked increase of IL-2 production (Figure 2A). In contrast, the 24.9.E hybridoma responded strongly to the mCD1d fusion protein, which had been preincubated with α-GalCer, but showed only modestly increased IL-2 secretion in response to the PI-treated mCD1d fusion protein (Figure 2A). Consistent with the results of Kawano et al. (1997), stimulation of the 24.9.E NKT cell hybridoma required the α-linked galactose to be present on the galactosylceramide antigen, since neither the unglycosylated ceramide nor the closely related β-linked form, β-GalCer, was recognized (Figure 2A). The asialo-GM_2 sphingolipid also was not recognized (Figure 2A). Pretreatment of the negative control protein with any of the lipids failed to induce detectable IL-2 secretion by either hybridoma (data not shown). Thus, while the 24.8.A. and 24.9.E hybridomas both required addition of a lipid antigen to the mCD1d fusion protein for efficient activation, they appeared to have distinct antigen specificities.

Titration of the molar ratio of antigen to fusion protein from 10:1 to 80:1 confirmed the antigen-specific, dose-dependent responses of the 24.8.A and 24.9.E hybridomas (Figure 2B). IL-2 production by the 24.8.A hybridoma appeared saturated at a 40:1 molar ratio of PI to mCD1d fusion protein, while little IL-2 was secreted even at an 80:1 molar excess of α-GalCer (Figure 2B). In contrast, the 24.9.E hybridoma secreted IL-2 efficiently in response to α-GalCer-treated mCD1d fusion protein but generated significantly less IL-2 even at high ratios of PI to mCD1d (Figure 2B). To confirm that this antigen-dependent stimulation of the NKT cell hybridomas was mCD1d specific, the 19G11 anti-mCD1d blocking antibody was used. In a representative experiment, the 24.8.A hybridoma secreted a mean of 4746 pg/ml IL-2 in response to mCD1d fusion protein pretreated with PI, but in the presence of the 19G11 mAb, no detectable IL-2 was produced. For the 24.9.E hybridoma, pretreatment with α-GalCer resulted in production of a mean of 2089 pg/ml IL-2, which was reduced to 103 pg/ml when the 19G11 anti-mCD1d mAb was included. Hence, antigen-specific activation of the hybridomas by the mCD1d fusion protein could be blocked by addition of an anti-mCD1d antibody.

Specificity of Phospholipid Antigen Recognition

To examine the specificity of PI recognition by the 24.8.A hybridoma, analogs of PI were tested with the mCD1d fusion protein. Three synthetic PIs with one, two, or three additional phosphate groups attached to carbons of the inositol ring (PI3-P, PI3,4-P2, and PI3,4,5-P3, respectively) were compared to PI and to successively smaller constituent components of PI: phosphatidic acid (PA), which lacks the inositol ring of PI; diacyl glycerol (DAG), which lacks the phosphate of PA; palmitic acid, which corresponds to one free acyl chain of the DAG molecule; and free inositol. As previously observed, pretreatment of the mCD1d fusion protein with PI resulted in significantly enhanced IL-2 release (Figure 3A). Treatment of the fusion protein with components of PI lacking

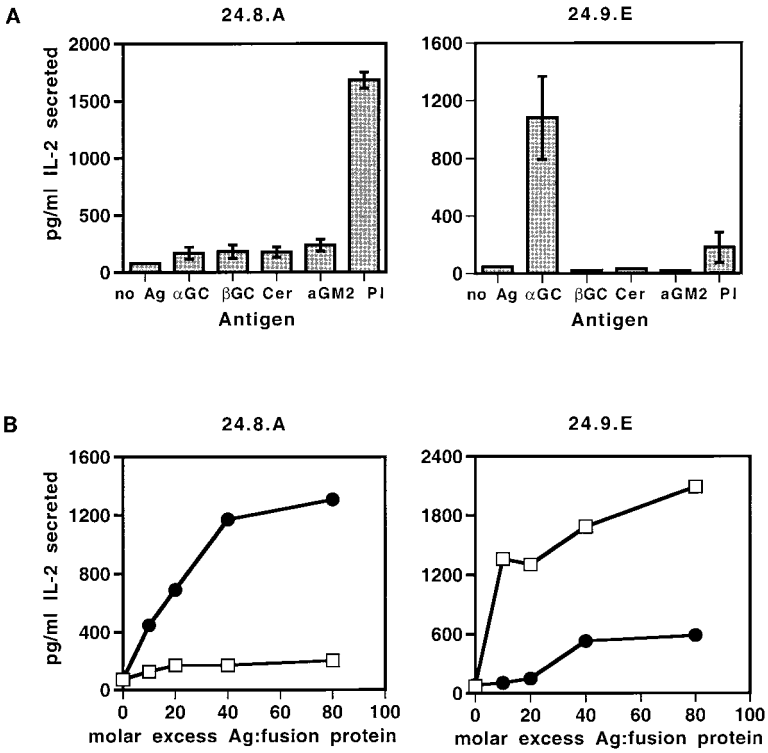

Figure 2. IL-2 Secretion by the 24.8.A and 24.9.E Hybridomas in Response to Plate-Bound mCD1d Molecules and Purified or Synthetic Lipid Antigens

(A) The mCD1d fusion protein incubated with buffer (no Ag), α-GalCer (αGC), β-GalCer (βGC), unglycosylated ceramide (Cer), asialo-GM_2 ganglioside (aGM_2), or phosphatidylinositol (PI).

(B) The mCD1d fusion protein incubated with serially diluted amounts of α-GalCer (open squares) or PI (filled circles). The data represent the mean pg/ml IL-2 detected in hybridoma culture supernatants from three replicate wells. Error bars show the standard deviations of the means.

the inositol ring attached to the acyl chains (PA, DAG, palmitate, and inositol) provided little or no stimulation (Figure 3A). IL-2 secretion induced by pretreatment with the synthetic phosphorylated PI antigens was also significantly greater than that for the mCD1d fusion protein incubated with buffer alone (Figure 3A). These results suggested that the inositol ring was an important antigenic determinant of PI for the 24.8.A hybridoma.

To confirm the importance of the inositol ring in recognition of PI, the PI was phospholipase treated prior to incubation with the fusion protein. Two different phospholipases were tested. Phospholipase D (PLD) removes the inositol ring from the phosphate, which links it to the diacyl glycerol backbone, to yield free inositol and phosphatidic acid (PA). PI-specific phospholipase C (PI-PLC) cleaves the bond between the phosphate and the glycerol to produce inositol phosphate and diacyl glycerol (DAG). Treatment of PI with PLC or PLD prior to preincubation of the antigen with the mCD1d fusion protein reduced IL-2 secretion by approximately 70%, approaching the IL-2 levels seen when synthetic preparations of DAG or PA were incubated with the fusion protein (Figure 3B). Thus, the inositol ring appears to be important for PI recognition by the 24.8.A hybridoma in this system, and forms of PI that are phosphorylated on the inositol ring can also be recognized.

We next examined the specificity of the 24.8.A hybridoma for PI compared to other common phospholipid antigens. Four additional phospholipids related to PI were tested: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylserine (PS). Titrations of the molar ratio of antigen to fusion protein from 10:1 to 80:1 were carried out for these antigens (Figure 3B). The 24.8.A hybridoma demonstrated dose-dependent responses to the PE and PG antigens, which appeared saturated at a molar ratio of 40:1 antigen to fusion protein (Figure 3C). Preincubation with PC or PS did not reproducibly significantly enhance reactivity to the mCD1d fusion protein (Figure 3C). Hence, recognition of the mCD1d fusion protein by the 24.8.A hybridoma was clearly augmented by pretreatment with PI, PE, and PG but not with PS or PC. Taken together these results are consistent with a model in which the acyl chains of the lipid tails are required for binding to CD1 molecules, but antigen specificity is determined by TCR recognition of features of the polar head group (Porcelli and Brenner, 1997).

The Effect of pH on Antigen Recognition

Previous studies have suggested that CD1d molecules may encounter antigens in intracellular vesicles that undergo substantial acidification during the process of antigen loading (Kawano et al., 1997; Brossay et al., 1998; Spada et al., 1998; Chiu et al., 1999). The 24.9.E hybridoma was used to examine the effect of acidic pH on α-GalCer presentation by the mCD1d fusion protein. The mCD1d fusion protein was incubated with α-GalCer antigen diluted into citrate/phosphate buffer solutions ranging from pH 7.5 to pH 3.0 at a 3:1 molar ratio of antigen to protein; the solutions were then neutralized to allow binding to the protein A–coated plate and assayed for recognition by the 24.9.E hybridoma (Figure 4). Recognition of the α-GalCer antigen was enhanced approximately 4-fold after antigen preincubation at pH 4.0 compared to pH 7.5. Maximal IL-2 release was reproducibly observed for the samples preincubated at pH 4.0, while IL-2 production dropped significantly for samples preincubated below this pH (Figure 1F). Negative

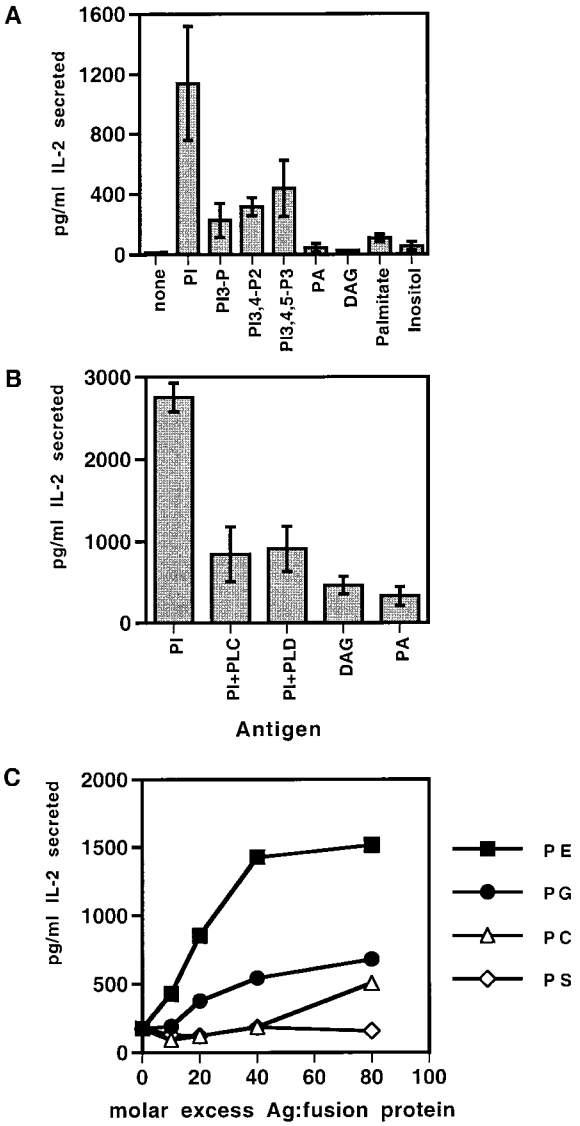

Figure 3. IL-2 Secretion by the 24.8.A Hybridoma in Response to Plate-Bound mCD1d Molecules and Purified or Synthetic Phospholipids and Analogs

(A) The mCD1d fusion protein preincubated with buffer (none), phosphatidylinositol (PI), phosphorylated phosphatidylinositols (PI3-P; PI3,4-P2; PI3,4,5-P3), phosphatidic acid (PA), diacyl glycerol (DAG), palmitic acid (palmitate), or free inositol (inositol).

(B) The mCD1d fusion protein preincubated with untreated phosphatidylinositol (PI), PI-specific phospholipase C treated PI (PI-PLC), phospholipase D treated PI (PLD), diacyl glycerol (DAG), or phosphatidic acid (PA).

(C) The mCD1d fusion protein preincubated with serially diluted amounts of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), or phosphatidylcholine (PC). The data represent the mean pg/ml IL-2 detected in hybridoma culture supernatants from three replicate wells. Error bars show the standard deviations of the means.

control wells containing the mCD1d fusion protein diluted into the pH-titrated citrate/phosphate buffer solutions with no antigen added or a negative control protein treated with α-GalCer at pH 7.2 did not induce detectable IL-2 production (data not shown). To ensure that

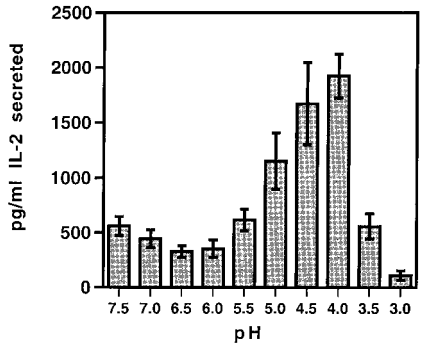

Figure 4. Analysis of the Effect of the Buffer pH during α-GalCer Preincubation with the mCD1d Fusion Protein on Stimulation of the 24.9.E Hybridoma

Plots represent the mean pg/ml IL-2 detected in hybridoma culture supernatants from six replicate wells. Error bars show the standard deviation of the mean.

preincubation at low pH did not affect binding of the fusion protein to the protein A plate, the assay plate was tested (after removal of the culture supernatants) for the presence of mCD1d using a biotinylated rat anti-mCD1d mAb (19G11), which does not bind to protein A, followed by detection with a streptavidin-enzyme conjugate and a chromogenic substrate. This analysis revealed that the amount of mCD1d fusion protein bound to the plate was not affected by the preincubation pH (data not shown). Therefore, although antigens incubated at physiological pH could be recognized, treatment of the mCD1d fusion protein with α-GalCer at pH 4.0 provided optimal antigen recognition in this system.

Comparison of Antigen Recognition by Diverse and NKT Cell mCD1d-Restricted Hybridomas

Our observation that two NKT cell hybridomas, 24.8.A and 24.9.E, differed in their antigen reactivity raised the possibility that NKT cells may have heterogeneous antigen specificities. To extend our analysis of NKT cells and to compare antigen recognition by mCD1d-restricted T cells of the diverse TCR population, we tested 9 NKT and 8 diverse TCR mCD1d-restricted hybridomas for recognition of 14 purified and synthetic lipid antigens (see Tables 1 and 2). None of the hybridomas produced detectable IL-2 in response to a negative control protein, and only the 24.8.A hybridoma secreted detectable IL-2 in response to untreated mCD1d fusion protein (Table 2). Eight out of nine NKT cell hybridomas were potently stimulated by α-GalCer-treated fusion protein, whereas none of the diverse TCR hybridomas reproducibly recognized this antigen (Table 2). Purified PI strongly stimulated the 24.8.A hybridoma and also stimulated some of the α-GalCer reactive NKT cell hybridomas, although with only about 10%–20% of the activity of the synthetic α-GalCer. Several diverse TCR hybridomas also secreted detectable IL-2 upon incubation with PI-, PE-, or PG-treated mCD1d fusion protein (Table 2). None of the hybridomas reproducibly recognized any of the other antigens tested. Thus, in this antigen screen, most (8/9) of the NKT cell hybridomas recognized α-GalCer, whereas all but one of the diverse TCR hybridomas failed to respond to this antigen. In contrast, approximately

Table 1. TCR Gene Usage of TT Hybridoma Cells Used for Analysis

Hybridoma	Lineage	Vα/Jα Genes	Vβ/Jβ Genes
24.8.A	NKT	Vα14/Ja281	Vβ8.2/Jβ2.5
24.7.C	NKT	Vα14/Ja281	Vβ6.1/Jβ2.6
24.9.E	NKT	Vα14/Ja281	Vβ8.3/Jβ2.4
DN32D3	NKT	Vα14/Ja281	Vβ8.2/Jβ2.4
KT/7	NKT	Vα14/Ja281	Vβ8.2/ND
KT/12	NKT	Vα14/Ja281	Vβ8.2/ND
KT/22	NKT	Vα14/Ja281	Vβ8.2/ND
KT/23	NKT	Vα14/Ja281	Vβ8.2/ND
Vβ/9	NKT	Vα14/Ja281	Vβ8.2/ND
14S.6.A	diverse	Vα17.1/JαTT11	Vβ14.1/Jβ2.1
14S.7.N	diverse	Vα15.1/JαNEW.02	Vβ8.2/Jβ2.5
14S.10.C	diverse	Vα11.3/JαNEW.15	Vβ8.1/Jβ2.6
14S.15.A	diverse	Va10.2/9/JaTA65	Vβ5.1/Jβ2.4
VII68	diverse	Vα4/Jα25	Vβ11/Jβ2.5
VIII24	diverse	Vα3.2/Jα20	Vβ9/Jβ1.4
XV19	diverse	ND	ND
XV104	diverse	Vα4/5/ND	Vβ8.3/Jβ2.6

TCR α and β gene usage for the 24.7.C, 24.8.A, 24.9.E, DN32D3, 14S.6.A, 14S.7.N, 14S.10.C, 14S.15.A, VII68, VIII24, and XV104 hybridomas was determined by DNA sequencing. For the KT/7, KT/12, KT/22, KT/23, and Vβ/9 hybridomas, the presence of the Vα14/Jα281 rearranged TCR α chain was determined by PCR analysis, and the Vβ chain usage was assessed by flow cytometry.

half of both the NKT and diverse TCR hybridomas tested showed some reactivity to certain phospholipid antigens.

Recognition of mCD1d-Transfected Tumor Cell Lines

The results of our analyses using the mCD1d fusion protein suggested mCD1d-restricted T cells may require presentation of specific antigens for recognition of mCD1d molecules. Previous studies have demonstrated differences in the abilities of CD1d-restricted T cells to recognize different APCs, indicating that different APCs may present distinct antigens, and CD1d-restricted T cell clones may have heterogeneous antigen specificities (Brossay et al., 1998; Couedel et al., 1998; Park et al., 1998; Chiu et al., 1999). Therefore, to investigate whether the antigen specificities of the hybridomas in the mCD1d fusion protein plate stimulation assay correlate with their ability to recognize mCD1d expressed by cells, we tested the panel of hybridomas for recognition of four different mCD1d-transfected tumor cell lines: RMA-S and EL-4 are derived from T lymphomas, A20 from a B lymphoma, and P815 from a mastocytoma. The hybridomas were incubated with the mCD1d-transfected tumor cell lines or the untransfected parental lines without addition of exogenous antigens. The untransfected tumor cells stimulated little or no detectable IL-2 release by any of the hybridomas (data not shown), whereas the mCD1d-transfected cells could induce high levels of IL-2 secretion by certain NKT and diverse TCR hybridomas (Table 3).

Surprisingly, despite their common specificity for α-GalCer-treated mCD1d fusion protein, there were three distinct patterns of recognition of the mCD1d-transfected cell lines among the eight α-GalCer-reactive NKT lineage hybridomas (Table 3). The α-GalCer-reactive 24.7.C hybridoma recognized all of the mCD1d-expressing cells well (>500 pg/ml IL-2 release for each transfectant), while the 24.9.E, DN32D3, and KT/23 hybridomas only responded to the mCD1d-transfected EL-4 cell line (Table 3). The remaining four α-GalCer-reactive hybridomas, KT/7, KT/12, KT/22, and Vβ/9, showed little or no recognition of any of the mCD1d-transfected cells (Table 3). The 24.8.A hybridoma, which had specificity for phospholipids rather than α-GalCer, responded well to all of the transfected cell lines (Table

Table 2. mCD1d-Restricted Hybridoma Responses to Plate-Bound mCD1d Fusion Protein Preincubated with Lipid Antigens

	Invariant TCRα NKT Hybridomas									Diverse TCR Hybridomas							
	24.8.A	24.7.C	24.9.E	DN32D3	KT/7	KT/12	KT/22	KT/23	Vβ/9	14S.6.A	14S.7.N	14S.10.C	14S.15.A	VII68	VIII24	XV19	XV104
No mCD1d	0	0	0	0						0	0	0	0				
No Ag	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-GalCer	+	+++	+++	+++	+++	+++	+++	+++	++	0	0	0	+	0	0	0	0
β-GalCer	+	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0
Cer	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sph	+	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0
aGM_2	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GD1a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PA	+	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0
PI	+++	0	+	0	0	+	+	+	0	+	+	0	0	0	0	0	0
PS	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PG	++	0	+	0	0	0	0	0	0	+	+		+	0	0	0	0
PE		0	0	0						+	0		0				
PC	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MGDG	+	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0
DAG		0	0	0	0	0	0	0	0	0	0		0	0	0	0	0

IL-2 secretion by mCD1d-restricted hybridomas in response to plate-bound mCD1d-$IgGFc_{2a}$ fusion protein and lipid antigens. A "0" indicates a mean of less than 50 pg/ml IL-2 was secreted, "+" indicates 50–250 pg/ml, "++" indicates 250–1000 pg/ml, "+++" indicates greater than 1000 pg/ml IL-2 secretion, and spaces left blank were not done in the experiment shown. Negative control wells contained neither fusion protein nor antigen (No mCD1d). The mCD1d fusion protein was preincubated with buffer alone (No Ag), α-galactosylceramide (α-GalCer), β-galactosylceramide (β-GalCer), unglycosylated ceramide (Cer), sphingomyelin (Sph), gangliotriosyl ceramide (aGM_2), disialoganglioside (GD1a), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), monogalactosyl diglyceride (MGDG), or diacyl glyceride (DAG). The results are compiled from six independent representative experiments.

Table 3. mCD1d-Restricted Hybridoma Responses to mCD1d-Transfected Tumor Cells

	Invariant TCRα NKT Hybridomas									Diverse TCR Hybridomas							
	24.8.A	24.7.C	24.9.E	DN32D3	KT/7	KT/12	KT/22	KT/23	Vβ/9	14S.6.A	14S.7.N	14S.10.C	14S.15.A	VII68	VIII24	XV19	XV104
CD1/P815	+++	+++	0	0	0	0	0	0	0	+++	+	+	+++	++	++	+	+
CD1/EL4	+++	+++	+++	+	0	0	0	+	0	+++	+	0	+++	++	+	0	+
CD1/RMA-S	+++	++	0	0	0	0	0	0	0	++	+	++	++	+	+	+	0
CD1/A20	++	+++	0	0	0	0	0	0	0	++	0	0	+++	+	+	0	0

IL-2 secretion by mCD1d-restricted hybridomas in response to mCD1d-transfected tumor cell lines. The untransfected parental cell lines induced little or no detectable IL-2 production by any of the hybridomas. A "0" indicates a mean of less than 50 pg/ml IL-2 was secreted, "+" indicates 50–250 pg/ml, "++" indicates 250–1000 pg/ml, and "+++" indicates greater than 1000 pg/ml IL-2 secretion. The results are compiled from three independent representative experiments.

3). All of the diverse TCR hybridomas recognized at least two of the mCD1d transfectants (Table 3). Thus, although the diverse TCR hybridomas did not respond strongly to any of the antigens screened in the mCD1d fusion protein stimulation assay, they could recognize mCD1d molecules expressed by different cell types. Additionally, hybridomas that shared specificity for α-GalCer differed in their recognition of mCD1d expressed by distinct APCs.

Discussion

Because cell surface mCD1d molecules may be complexed with cellular lipids, it has been difficult to evaluate the role of potential endogenous antigens in T cell recognition of mCD1d. The observation that a recombinant β_2m-linked mCD1d-IgGFc$_{2a}$ fusion protein did not stimulate high levels of IL-2 production from mCD1d-restricted T cell hybridomas allowed us to develop a system to analyze the contribution of lipid antigens to recognition of mCD1d molecules by T cells. Activation of the hybridomas using plate-bound mCD1d fusion protein was dramatically enhanced after preincubation with certain lipids or lipid-containing cellular extracts. The response could be blocked by an anti-mCD1d mAb, showing that the mCD1d molecule was required for stimulation. Preincubation of a negative control protein with the same lipids did not induce detectable IL-2 production, indicating that the lipids did not have a nonspecific stimulatory effect. Hence, although other mechanisms cannot be ruled out, together these results suggest that binding of certain lipid antigens to the plate-bound mCD1d molecules permitted efficient recognition of the mCD1d fusion protein by hybridomas expressing cognate TCRs.

Several investigations have now demonstrated that many NKT cells can respond to CD1d-mediated presentation of the unusual acylphytosphingolipid α-GalCer (Kawano et al., 1997; Brossay et al., 1998; Burdin et al., 1998; Spada et al., 1998). Additionally, glycosylated forms of PI have been implicated as determinants recognized by murine CD1d-restricted NKT cells during protozoal and mycobacterial infections, and PI-containing compounds have been shown biochemically to be associated with mCD1d molecules purified from transfected human T2 cells (Joyce et al., 1998; Apostolou et al., 1999; Schofield et al., 1999). Thus, sphingolipid and phospholipid compounds can apparently bind CD1d and function as antigens for CD1d-restricted NKT cells, but whether these molecules represent self- or foreign antigens, and whether the NKT cells that respond to α-GalCer are the same as those that see phospholipids, has been unclear.

Our finding that a lipid extract of RMA-S cells could reconstitute the recognition of plate-bound mCD1d molecules by an NKT cell hybridoma shows that self-lipids can serve as antigens for NKT cells. Further separation of the lipids within the organic phase extract revealed specificity for fractions containing mainly polar glycolipids and phospholipids, suggesting the 24.8.A hybridoma could recognize phospholipid antigens. This possibility was supported by experiments showing that the 24.8.A hybridoma responded to certain purified and synthetic phospholipids, including PI, PE, and PG, while PA, PS, and PC did not reproducibly induce IL-2 production. Whether the failure of PA, PS, and PC to stimulate IL-2 release resulted from lack of recognition by the 24.8.A hybridoma or was due to inefficient binding of these lipids to the fusion protein under the conditions of the plate stimulation assay is unclear. However, the 24.8.A hybridoma also did not respond to α-GalCer, which stimulated other hybridomas when added to the mCD1d fusion protein, indicating that it can bind. Thus, the 24.8.A hybridoma had specificity for three of the purified phospholipid antigens tested but not for α-GalCer.

Unlike other hybridomas tested, the 24.8.A hybridoma had a variable amount of reactivity to the fusion protein that had not been pretreated with a lipid antigen. This response could be due to recognition of the mCD1d molecule itself, independent of a specific antigen. Alternatively, the reactivity could result from recognition of an antigen that remained bound to the fusion protein after purification (e.g., an antigen that derived from the CHO cells used to produce the fusion protein, or from the culture supernatant from which the fusion protein was purified). Hence, given their abundance in cells and in culture supernatants, one of the phospholipids shown here to stimulate the 24.8.A hybridoma could also be responsible for its variable reactivity to the untreated fusion protein.

Eight of the NKT cell hybridomas tested responded strongly to α-GalCer preincubated with the mCD1d fusion protein. Surprisingly, four of these α-GalCer reactive hybridomas also had detectable reactivity to purified phospholipid antigens, suggesting that the cellular antigens they recognize may be related lipids. The eight diverse TCR hybridomas tested did not respond reproducibly to α-GalCer, but three also showed some response to purified phospholipids. In all, responses to

PI, PE, or PG were detected for 8 of the 17 hybridomas tested. Thus, phospholipids may represent a major class of self-antigens recognized by CD1d-restricted T cells, and some of the T cells that recognize these antigens may also respond to α-GalCer, while others do not.

The ability of the 24.8.A hybridoma to respond to phospholipids but not α-GalCer is particularly interesting with regard to its TCR gene usage. This hybridoma possesses a cannonically rearranged Vα14/Jα281 TCRα chain that is identical to those of the α-GalCer-reactive NKT cell hybridomas, implying that it is the TCRβ chain which is responsible for its distinct antigen specificity. Surprisingly, the 24.8.A hybridoma expresses TCR Vβ 8.2, a Vβ gene that is also used by most of the α-GalCer-reactive NKT cell hybridomas we tested (Table 1). Thus, it is unlikely that the Vβ of 24.8.A prevents recognition of α-GalCer and seems instead that residues of the CDR3 loop encoded by the D segment, Jβ, or by N-region addition may be critical in conferring its antigenic specificity. Hence, despite their invariant TCRα chains and limited TCR Vβ gene usage, the diverse TCRβ VDJ junctional regions of CD1d-restricted NKT cells may result in multiple different antigenic specificities within this T cell subset.

The potential for heterogeneous antigen specificities may explain our surprising finding that NKT cell hybridomas which responded similarly to α-GalCer presentation by the plate-bound mCD1d fusion protein varied in their patterns of recognition of a panel of four mCD1d-transfected tumor cells. One α-GalCer-reactive hybridoma recognized all of the transfectants well, while three of the hybridomas only responded to one of the transfectants, and the remaining four α-GalCer-specific hybridomas did not recognize any of the transfectants. This result suggests that the endogenous cellular antigen recognized by these hybridomas is not α-GalCer or a single analog, since in that case recognition of the mCD1d-transfected cells should correlate with the α-GalCer reactivity observed in the plate stimulation assay. Instead, based on the three patterns of reactivity with the mCD1d transfectants, there must be at least three different antigenic specificities among the eight α-GalCer-reactive NKT cell hybridomas tested. The α-GalCer antigen might stimulate many NKT cells because it possesses a common determinant of some diverse set of antigens, or it may function similarly to a superantigen and activate a large fraction of CD1d-restricted NKT cells, regardless of their other antigenic specificities. A recent analysis by Kawano et al. (1999) identifies an amino acid motif in the CDR3 region of TCRβ chains of human CD1d-restricted NKT cells that responded to selection by α-GalCer, indicating that this antigen preferentially stimulates a subset of the CD1d-restricted T cells.

Based on their diverse TCR structures, non-NKT lineage mCD1d-restricted hybridomas are thought to see a heterogeneous group of antigens (Cardell et al., 1995; Behar et al., 1999). The diverse TCR mCD1d-restricted hybridomas tested in this analysis could recognize multiple mCD1d-transfected cell lines, suggesting that they recognize broadly distributed cellular antigens. In contrast to most of the NKT hybridomas, the diverse TCR hybridomas did not respond strongly to α-GalCer. While this result suggests that the diverse TCR population sees a set of antigens that is distinct from those recognized by mCD1d-restricted NKT cells, some of the diverse TCR hybridomas reacted to the same purified phospholipids recognized by members of the NKT cell subset. Therefore, some of the diverse TCR mCD1d-restricted T cell population may recognize similar self-antigens to those recognized by mCD1d-restricted NKT cells.

The observation that mCD1d-restricted T cells varied in their recognition of different mCD1d-transfected tumor cells suggests that antigens presented by mCD1d molecules differ according to the cell type. Given the broad expression of murine CD1d on cells of hematopoietic origin, variation in antigen presentation among cells that express mCD1d could be a critical mechanism of regulating mCD1d-restricted T cells (Brossay et al., 1997; Mandal et al., 1998). Little is known about the factors that affect endogenous lipid antigen presentation by mCD1d molecules, although variations in antigen presentation could arise from differences among APCs in expression, trafficking, processing, or mCD1d loading of antigens.

Antigen recognition in our mCD1d fusion protein presentation assay could occur after preincubation at pH 7.2 but was significantly enhanced by preincubation at pH 4.0. Therefore, while acidic pH is not required, it may facilitate lipid binding to the fusion protein. This observation might help to explain apparently conflicting results regarding α-GalCer presentation by APCs. Burdin et al. (1998) found that α-GalCer could be presented in the absence of endosomal trafficking and acidification, while in the experiments of Kawano et al. (1997) and Spada et al. (1998) these elements of cellular antigen processing appeared necessary for α-GalCer presentation to NKT cells. Our results suggest that α-GalCer binding to mCD1d at the cell surface at neutral pH is possible, but that binding may be favored in endocytic vesicles that have an acidic pH. In contrast, recognition of cell surface mCD1d by diverse TCR hybridomas did not appear to require endosomal localization (Chiu et al., 1999). Thus, intracellular trafficking of CD1d molecules may play a critical role in determining the antigens presented by cells that express CD1d.

The in vitro mCD1d-specific antigen recognition system described here should prove useful in the isolation and identification of endogenous cellular antigens recognized by CD1-restricted T cells. Analysis of biochemically fractionated cellular lipids for their ability to stimulate mCD1d-restricted hybridomas after addition to the mCD1d fusion protein could provide a means of identifying physiological antigens presented by normal or neoplastic cells. Identification of the natural antigens recognized by mCD1d-restricted T cells will be critical to our future understanding of the role of these cells in disease processes such as autoimmunity and cancer.

Experimental Procedures

Hybridomas

The CD1d-restricted T cell clones 24.7, 24.8, 24.9 (NKT cell) and 14S.6, 14S.7, 14S.10, and 14S.15 (diverse TCRs) were all derived from spleen of wild-type C57BL/6 mice, as described previously (Behar et al., 1999). To generate T cell hybridomas, the activated T cells were fused to the aminopterin-sensitive BW5147 αβTCR$^-$

thymoma cell line using PEG1500, and hybrids were selected in HAT medium (Life Technologies). Resulting TT hybridomas were tested for recognition of RMA-S cells transfected with mCD1D1 compared to untransfected RMA-S cells, as described below. Hybridomas that demonstrated specific recognition of mCD1d were further subcloned by limiting dilution. The hybridomas are distinguished from the original T cell clones by the addition of a letter to their names. The KT/7, KT/12, KT/22, KT/23, and Vβ/9 NKT cell hybridomas were derived from NK1.1$^+$ T cells enriched from spleen of C57BL/6 mice by depletion of CD8$^+$ T cells, naive T cells, and B cells by mAbs (anti-B220, CD8, and CD62L, or anti-CD8α, CD8β, and Mel14) bound to magnetic microbeads or to plastic. The purified cells were stimulated either by the anti-CD3 KT3 mAb (KT/7, KT/12, KT/22, KT/23) or by an anti-Vβ8.2 mAb (Vβ/9) and addition of IL-2 or IL-2 and IL-7. After 4–5 days of culture, the cells were fused with BW5147 thymoma cells. The VII68, VIII24, XV19, and XV104 diverse TCR hybridomas were generated from CD4$^+$ T cells from class II^o mice, as described previously (Cardell et al., 1995). The DN32D3 hybridoma was derived as described (Lantz and Bendelac, 1994).

Generation of mCD1d Fusion Protein

A soluble murine CD1d fusion protein covalently linked to human β_2m at the N terminus by a glycine-serine (Gly-Ser) spacer peptide and at the C terminus to the Fc portion of murine IgG_{2a} by another Gly-Ser spacer peptide, was constructed as follows. All synthetic oligonucleotides were commercially obtained, (Operon Technologies). A cDNA of the full-length coding sequence of mCD1D1 was used as template DNA for PCR amplification. PCR primers were designed to create a truncated mCD1D1 gene, which eliminates the cytoplasmic, transmembrane, and leader peptide sequences. The 5′ primer oligonucleotide sequence, containing a SpeI restriction site, was 5′-GCGCGGACTAGTTCTGAAGCCCAGCAAAAGAATTA CACC-3′, and the 3′ primer sequence, containing a NotI restriction site, was 5′-TGCTTGGCGGCCGCTCCAGTAGAGGATGATATCCTG TCC-3′. A cDNA fragment encoding human β_2m fused to the Gly-Ser linker was generated by PCR, using as a template a cDNA construct encoding a human β_2m-linked single chain CD1a molecule. The 5′ primer sequence containing an XhoI site was 5′-GCGCGGC TCGAGCATGTCTCGCTCCGTGGCCTTAGC-3′, and the 3′ primer sequence containing an XbaI restriction site was 5′-CGGCTCTA GATCCACCTCCAGAACCGGATCCACCTG-3′. The PCR products were digested with the appropriate restriction enzymes, ligated and subcloned, and the fragment containing β_2m linked to mCD1d was excised by digestion with XhoI and NotI. This fragment was linked to a cDNA fragment encoding the hinge, CH2, and CH3 regions of murine IgG_{2a} using a synthesized DNA fragment encoding a 14–amino acid Gly-Ser spacer peptide sequence (SGPGGSGGSGGSGG), made from the following complementary oligonucleotides: 5′-GGCCCG GGAGGTTCTGGAGGTTCAGGAGGTTCTGGAGGG-3′ and 5′-GATC CCCTCCAGAACCTCCTGAACCTCCAGAACCTCCCG-3′. The 3 cDNA fragments were ligated and subcloned into the pBluescript SK vector (Stratagene). The resulting construct was fully sequenced with M13 reverse and T7 outside primers to ensure that no coding mutations were present, then excised by restriction digestion and subcloned into the pBJ1-neo expression vector for transfection (Lin et al., 1990).

Production and Purification of mCD1d Fusion Protein

Chinese hamster ovary (CHO) cells were transfected with the PBJ1-neo vector containing the β_2m-mCD1d-Fc_{2a} cDNA construct by electroporation, then selected for G418 drug resistance and subcloned by limiting dilution to isolate stably transfected cells with high protein expression levels. Culture supernatants were tested for the presence of the mCD1d fusion protein by a standard double antibody sandwich ELISA using the 1B1 anti-mCD1d monoclonal antibody (PharMingen) as a capture reagent and a biotinylated polyclonal rabbit anti-human β_2m anti-serum (DAKO, Glostrup, Denmark), followed by a streptavidin-alkaline phosphatase conjugate (Zymed) or an anti-murine IgG_{2a} antibody conjugated directly to alkaline phosphatase (Zymed), as the detection reagent. The fusion protein was detectable by both methods, indicating that the mCD1d was complexed with both human β_2m and murine IgG_{2a} Fc. The CD1d fusion protein was purified by passage over a protein A Sepharose column (Amersham-Pharmacia Biotech) and eluted with 50 mM sodium acetate buffer at pH 4.3, followed by immediate neutralization by addition of 1/10 vol of a 1 M Tris buffer at pH 8.8. Subsequent analysis of the protein A eluate by size exclusion chromatography using a Superose 6 column (Amersham-Pharmacia) revealed a single peak eluting slightly earlier than a polyclonal IgG standard, as expected for a homodimeric fusion protein complex. Analysis by reducing and nonreducing SDS-PAGE demonstrated single bands at the expected molecular weights of approximately 100 kDa and 200 kDa, respectively.

Cellular Extracts and Fractionation

Cellular lipid was extracted from RMA-S and S49 murine T lymphoma cells using the method of Folch et al. (1956), with modifications as described by Hamilton and Hamilton (Hamilton et al., 1992). Briefly, 1 g of pelleted cells was mixed with 20 ml of a 2:1 v/v chloroform:methanol solution (C:M), then homogenized and incubated at room temperature for 1 hr. The mixture was centrifuged to remove insoluble material, and the supernatant saved. A 1/5 vol of sterile dH_2O was added to the C:M supernatant and the mixture was shaken until an emulsion formed, then incubated 24 hr at room temperature to allow phase separation into an organic fraction, an aqueous fraction, and the interface. For analysis using the mCD1d fusion protein assay, the aqueous and interface fractions were lyophilized, and the organic fraction was dried under a stream of nitrogen. The samples were then quantified by weight and resuspended in dimethyl sulfoxide (DMSO). The organic phase was further fractionated by dissolving 35 mg of dried sample in chloroform and applying it to a silica column (400 mesh silicic acid; Selecto Scientific). Lipids of increasing polarity were eluted from the column using a stepwise gradient of chloroform, acetone, and methanol. The resulting fractions were dried, quantitated, and solubilized in C:M, then dried down and resuspended in DMSO prior to use.

Glycolipid Antigens

The following antigens were commercially obtained (Matreya Corporation): purified bovine brain sphingomyelin (Sph), purified bovine brain disialoganglioside (GD1a), purified bovine brain gangliotriosyl ceramide (aGM_2), purified plant monogalactosyl diglyceride (MGDG), purified bovine phosphatidylserine (PS), purified soybean phosphatidylinositol (PI), synthetic dipalmitoyl phosphatidylinositol 3-phosphate (PI3-P), synthetic dipalmitoyl phosphatidylinositol bis-3,4-phosphate (PI3,4-P2), synthetic dipalmitoyl phosphatidylinositol tris-3,4,5-phosphate (PI3,4,5-P3), synthetic distearoyl phosphatidylcholine (PC), purified distearoyl phosphatidylethanolamine (PE), synthetic dipalmitoyl phosphatidylglycerol (PG), and synthetic dipalmitoyl phosphatidic acid (PA). Palmitic acid (palmitate), free inositol, and dipalmitin diacylglycerol (DAG) were acquired from Sigma. The synthetic α- and β-galactosylceramide (α-GalCer, β-GalCer) and unglycosylated ceramide (Cer) were produced synthetically as previously described (Kawano et al., 1997). The antigens were dissolved at a stock concentration of 100 or 200 μg/ml in DMSO and were sonicated in a 37°C water bath for 10 min prior to use.

Plate-Bound mCD1d Fusion Protein Hybridoma Stimulation Assays

To test for recognition of the mCD1d fusion protein and purified or synthetic antigens, 96-well protein A–coated plates (Pierce Chemical Company) were incubated with 400–600 ng/well of the fusion protein or a negative control IgG_{2a} antibody, RPC5.4 or UPC10, in PBS at pH 7.2. Lipid antigens were diluted into PBS and added where specified at the indicated molar ratio of antigen to fusion protein (when not specified the ratio was 40:1). Protein A plates containing the fusion protein and antigen were incubated 24–48 hr at 37°C, then washed three times with 200 μl/well sterile PBS (pH 7.2) and two times with 200 μl/well sterile culture medium (containing RPMI supplemented with L-glutamine and penicillin/streptomycin [Life Technologies] and 10% bovine calf serum [Hyclone Laboratories]). For assays in which the PI was phospholipase treated, it was first diluted into 0.01 M Tris, 0.15 M NaCl (pH 7.5) containing 0.25 U PI-specific phospholipase C or 0.5 U phospholipase D (Sigma) and incubated 30 min at room temperature, then added to the protein A plates as described above. For assays in which the pH was varied

during antigen incubation with the fusion protein, the fusion protein and α-GalCer were diluted into a 20 mM citrate/phosphate buffer of the specified pH, which contained 0.15 M NaCl, and after incubation the samples were neutralized by addition of 1 M Tris (pH 7.5). Hybridoma cells were added to fusion protein/antigen-treated plates at a concentration of 1×10^5 cells/well, in a total volume of 150 μl/well. Assays were performed using 2–6 replicate wells. In some assays, an anti-mCD1d blocking antibody (19G11) was included at a final concentration of 20 μg/ml. The plates were incubated at 37°C for 16–20hr, and culture supernatants were withdrawn for analysis. Each experiment was performed at least three times.

Generation of mCD1d APC Transfectants and mCD1d Recognition Assay

CD1D1-transfected RMA-S cells were derived as described previously (Behar et al., 1999). A similar procedure was used to transfect the EL4, A20, and P815 cell lines. Briefly, the cells were transfected by electroporation with the pSRa-neo expression vector containing mCD1D1 cDNA and subjected to G418 drug selection to obtain stably transfected lines. Drug-resistant cells were stained using the 19G11 or 1B1 rat anti-mCD1d mAbs (graciously provided by Dr. Albert Bendelac, Princeton University, and Dr. Laurent Brossay, UCLA, respectively) and analyzed by flow cytometry. In some cases the cultures were sorted using a FACsort (Becton Dickinson) to obtain cells expressing high levels of mCD1d, then cloned by limiting dilution. Hybridomas were tested for IL-2 production in the presence of the mCD1d-transfected compared to the untransfected parental cell lines. Hybridomas and APCs were added at a concentration of 1×10^5 cells/well each, in a total volume of 150 μl/well, and incubated as described above.

Detection of IL-2 Secretion

IL-2 secreted in the hybridoma stimulation assays was quantitated in a double antibody sandwich ELISA, by comparison to a standard curve of purified murine IL-2 (PharMingen). Hybridoma plate stimulation supernatants (used either neat or diluted) and serially diluted IL-2 standards were added to 96-well ELISA plates coated with a rat anti-mouse IL-2 capture antibody (PharMingen). IL-2 was detected by addition of a biotinylated rat anti-mouse IL-2 antibody, followed by addition of a streptavidin-alkaline phophatase conjugate and a chromogenic substrate. The picograms per milliliter of IL-2 present in the hybridoma supernatants was quantitated by linear regression of the IL-2 standard curve.

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