The Journal of Immunology

The Paradox of Immune Molecular Recognition of α-Galactosylceramide: Low Affinity, Low Specificity for CD1d, High Affinity for αβ TCRs

Carlos Cantu III, Kamel Benlagha, Paul B. Savage, Albert Bendelac and Luc Teyton

J Immunol 2003; 170:4673-4682; ;
doi: 10.4049/jimmunol.170.9.4673
http://www.jimmunol.org/content/170/9/4673

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

The Paradox of Immune Molecular Recognition of α-Galactosylceramide: Low Affinity, Low Specificity for CD1d, High Affinity for αβ TCRs[1]

Carlos Cantu III,* Kamel Benlagha,† Paul B. Savage,‡ Albert Bendelac,† and Luc Teyton[2]*

CD1 resembles both class I and class II MHC but differs by the important aspect of presenting lipid/glycolipids, instead of peptides, to T cells. Biophysical studies of lipid/CD1 interactions have been limited, and kinetics of binding are in contradiction with functional studies. We have revisited this issue by designing new assays to examine the loading of CD1 with lipids. As expected for hydrophobic interactions, binding affinity was not high and had limited specificity. Lipid critical micelle concentration set the limitation to these studies. Once loaded onto CD1d, the recognition of glycolipids by αβ T cell receptor was studied by surface plasmon resonance using soluble Vα14-Vβ8.2 T cell receptors. The Vα14 Jα18 chain could be paired with NK1.1 cell-derived Vβ chain, or any Vβ8 chain, to achieve high affinity recognition of α-galactosylceramide. Biophysical analysis indicated little effect of temperature or ionic strength on the binding interaction, in contrast to what has been seen in peptide/MHC-TCR studies. This suggests that there is less accommodation made by this TCR in recognizing α-galactosylceramide, and it can be assumed that the most rigid part of the Ag, the sugar moiety, is critical in the interaction. ***The Journal of Immunology*****, 2003, 170: 4673–4682.**

Conventional T cell adaptive immune responses rely on the recognition of peptide Ags bound to MHC molecules by αβ TCRs. In more recent years, a family of MHC-like, β_2-microglobulin-associated molecules called CD1 has been shown to present lipid and glycolipid Ags rather than peptides to T cells (1, 2). Although there is little amino acid sequence homology between CD1 and classical MHC class I molecules, the crystal structure of the mouse CD1d protein showed overall superimposable structures between the two protein families (3). However, in line with the differences in Ag presentation, the groove of the CD1 molecule differs from that of the MHC by being deeper and more hydrophobic over its surface to accommodate a lipid/glycolipid Ag. Subsequent studies have shown that the molecular recognition of CD1 by TCR follows the same conventional manner as recognition of MHC molecules, as opposed to a superantigen-like manner, for example, although the specific rules of recognition may differ (4–6).

The recognition of mouse CD1d and the corresponding human CD1d has been linked to a subpopulation of mature T cells called NKT cells (7–9). NKT cells express activating and inhibitory NK receptors and a limited αβ TCR repertoire consisting mostly of an invariant Vα14-Jα18 chain preferentially paired to Vβ8.2 in the mouse and Vα24-Jα18 paired with Vβ11 in human. Although the physiological ligand(s) remain unknown for CD1d, a synthetic glycolipid, α-galactosylceramide (αGC)[3] has been shown to selectively activate NKT cells expressing the Vα14/Vβ8 TCR (10). Tetrameric CD1d molecules loaded with αGC have proved successful for the identification of CD1-restricted T cell populations (11–14). Although the functional studies have been extensive, biophysical characterization of glycolipid-CD1d interactions and CD1d/TCR interactions have been very limited. The binding of αGC to CD1d has previously been characterized only by surface plasmon resonance (SPR) (15). The half-life of interaction was reported to be <1 min, in contradiction to functional assays that reported a half-life of ~1 day (12). Similarly, an SPR study of CD1b established a submicromolar affinity (K_D 0.66 μM) for a phosphatidylinositol mannoside and a short half-life of ~1 h (16). We have revisited CD1d-lipid interactions by using biochemical and biophysical methods. It is clear from this study that lipid binding to CD1 cannot be studied like peptide binding to MHC class I and II molecules. The nature and physical properties of lipids, e.g., critical micelle concentration (CMC) and solubility, impose technical constraints on the assays that are used. Not surprisingly, binding of αGC is of fairly low affinity but rather stable. Because CD1d binds monomers of glycolipids, the CMC of these ligands influences binding critically and suggests eventually the need for lipid transfer proteins in the loading of integral membrane lipids to CD1.

We examined the biophysics of the recognition of glycolipid-CD1d complexes by using recombinant soluble Vα14-Vβ8.2 TCR molecules. This interaction is the strongest ever measured for an αβ TCR, stronger than the γδ TCR binding to T22 (17). We also show that the invariant α-chain can accommodate pairing with random, non-NK1.1 β-chains to keep high binding and specificity. In contrast to TCR binding of peptide-MHC complexes, the recognition of the glycolipid-CD1 complex does not appear to require or be associated with important structural remodeling of the interface as shown by thermodynamic measurements. This mode of binding is

*Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037; †Department of Pathology, University of Chicago, Chicago, IL 60637; and ‡Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84601

Received for publication November 14, 2002. Accepted for publication February 19, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants AI 42267 (to L.T.), AI 62267 and CA 87060 (to A.B.), and A153725P2 (to A.B., P.B.S. and L.T.); from the Leukemia and Lymphoma Society of America (to K.B.); and from the National Science Foundation (to P.B.S.) (CAREER). This is manuscript 15224-IMM from The Scripps Research Institute.

[2] Address correspondence and reprint requests to Dr. Luc Teyton, Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail address: lteyton@scripps.edu

[3] Abbreviations used in this paper: αGC, α-galactosylceramide; SPR, surface plasmon resonance; CMC, critical micelle concentration; PE-FITC, FITC-labeled phosphatidylethanolamine; PS, phosphatidylserine; IEF, isoelectric focusing; ITC, isothermal titration calorimetry; GM, asialoganglioside; GD, disialoganglioside; GT, trisialoganglioside; NEPHGE, nonequilibrium pH gel electrophoresis.

0022-1767/03/$02.00

suggestive of a rigid mode of recognition compatible with the binding of the invariant α-chain with the sugar moiety of the glycolipid. The high affinity of TCR binding could constitute a compensatory mechanism to sense low affinity, low abundance ligands.

Materials and Methods

Reagents and cell lines

The αGC was synthesized (18), purified, lyophilized, and stored neat or resuspended in chloroform; aliquoted; and stored at −80°C after solvent evaporation under a stream of nitrogen gas. Aliquots were resuspended at 200 μg/ml in 0.05% Tween-PBS and sonicated for several minutes. FITC-labeled phosphatidylethanolamine (PE-FITC) and phosphatidylserine (PS) were ordered from Avanti Polar Lipids (Alabaster, AL). Ganglioside GM1 was from Calbiochem (La Jolla, CA). Trisialoganglioside GT1b was purchased from Sigma-Aldrich (St. Louis, MO). DN32DN3 NKT hybridoma cells have been previously reported (19).

Protein expression and purification

Recombinant soluble murine CD1d molecules were produced in a fly expression system as previously described (12). The same system was used to produce soluble Vα14-Vβ8 TCRs, as done for other TCRs (20). The cDNA coding the Vα14 and Vβ8.2 chains from DN32 were cloned from the cell line by RT-PCR using flanking primers. cDNAs for the β-chain of the 2C (21) and D10 TCR (22) were available in the laboratory. Each chain was modified by PCR to remove its transmembrane and cytoplasmic domains and replace it with a leucine zipper (acid sequence with the α-chain, basic sequence with the β-chain, respectively) followed by a six-histidine tag. Additional constructs of the α-chain were made to add a C-terminal biotinylation sequence to the modified construct to allow biotinylation and tetramerization. SC2 *Drosophila melanogaster* cells were transfected with 15 μg of each α- and β-chain-modified cDNA and 0.5 μg of a constitutive neomycin resistance gene by calcium phosphate precipitation. After 3 wk of G-418 selection (0.5 mg/ml), resistant cell lines were checked for protein expression after a 2-day induction with 0.7 mM copper sulfate. Single cell cloning of each line was performed to optimize production. Large scale expression was done in X-press serum-free medium (Cambrex Bioscience, Walkersville, MD) and expanded to 12 L in roller bottles. After a 3-day induction, supernatants were harvested by centrifugation and concentrated by tangential flow to a volume of ~400 ml. Proteins were affinity purified using nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA) chromatography. This step was followed by anion exchange chromatography on a MonoQ 10/10 column (Amersham Biosciences, Piscataway, NJ). Purification was monitored by SDS-PAGE. Biotinylation (CD1d or TCR) was done according to the manufacturer's instructions for 20–24 h at room temperature (Avidity, Denver, CO). The level of biotinylation was checked by immunoprecipitation with streptavidin-agarose beads (Pierce, Rockford, IL). Protein concentration was checked with the bicinchoninic acid reagent kit (Pierce).

Loading of CD1d with αGC and generation of tetramers

For generation of αGC-loaded CD1d complexes, αGC from a 200 μM stock made in 0.05% Tween 20 in PBS was mixed at a 20:1 molar ratio with soluble empty CD1d for 48 h. Free αGC was removed by centrifugation dialysis in a Microcon YM-30 tube (Millipore, Billerica, MA) for small amount of CD1 or by gel filtration on a Superdex 200 column (Amersham Biosciences) for large scale preparation. Vα14-2C tetramers were generated by mixing the biotinylated monomers with streptavidin (Jackson Immuno Research Laboratories, West Grove, PA) at a 5:1 molar ratio.

Isoelectric focusing (IEF) electrophoresis

The PhastGel system from Amersham Biosciences was used to perform IEF gel analysis. A constant 6 μM CD1d was used in measuring lipid interactions. When PE-FITC, GT1b, or GM1 binds to CD1d, a shift in the position of the CD1d bands can be observed. Competition assays were set up with 20 μM PE-FITC or GT1b as tracers. For quantification, the gels were scanned and digitized on an Agfa (Ridgefield Park, NJ) scanner and quantified using the UN-SCAN-IT software program (Silk Scientific, Orem, UT). To account for variations in loading per lane, the pixel count for the CD1d-lipid band was divided by the total pixel count for the lane. This number represents the bound CD1d-lipid complex, relative to a control lane for which no lipid was present. Each binding assay was repeated a minimum of three times, independently. For testing association and dissociation of lipid with CD1d, excess GT1b was mixed with CD1d for 24 h and then separated by MonoQ to specifically isolate and concentrate the CD1d-GT species. Association of αGC is measured by incubating 2.5 μM αGC with 2 μM CD1-GT1b for various times. Dissociation is measured by incubating 2 μM CD1d/GT1b at 37°C for various times.

Isothermal titration calorimetry (ITC)

ITC experiments to measure binding of lipids to CD1d were done at 25°C using a MicroCal MCS calorimeter (MicroCal, Northhampton, MA) following standard instrumental procedures with a 250-μl injection syringe and 400 rpm stirring. PBS-0.05% Tween was the buffer used for αGC experiments, whereas PBS only was used for phosphatidylethanolamine experiments. Protein was dialyzed against PBS and degassed briefly before loading into the calorimeter cell. A typical binding experiment involved 25 × 10-μl injections of ligand solution into the ITC cell (~1.3 ml active volume) containing protein. Control experiments were performed under identical conditions by injection of buffer into protein. Integrated heat effects, after correction for heat of dilution, were analyzed by nonlinear regression using the simple one-site binding model of the ORIGIN software package (OriginLab, Northhampton, MA).

SPR experiments

All SPR experiments were done using a BIAcore 2000 machine (BIAcore, Uppsala, Sweden). For analysis of CD1d-αGC interaction, αGC was modified by addition of a biotin molecule to position 6 of the sugar moiety (18). The biological activity of this biotin-αGC compound was verified by stimulation of the DN32D3 T cell hybridoma and found to be identical with that of unmodified αGC.

For analysis of TCR-CD1d interaction, standard amine coupling of CD1 or TCR or directed cysteine coupling of TCR to a CM5 Research Grade sensor chip (BIAcore) was used. Filtered and degassed PBS was used as running buffer. In experiments where TCR was immobilized, sensorgrams for empty CD1d were subtracted from sensorgrams for CD1d-αGC of the same flow cell to control for nonspecific binding. In experiments in which TCR was flowing, sensorgrams for TCR over one flow cell with immobilized CD1-empty were subtracted from sensorgrams for TCR over another flow cell with immobilized CD1d-αGC. For thermodynamic studies, a range of temperatures from 10°C to 34°C (in 3°C increments) was used, controlled by the temperature unit of the machine as well as an external cooling/heating water unit. A concentration of 2 μM CD1d-empty or CD1d-αGC was used in the flow. For the analysis of the effect of ionic strength, a range of NaCl concentrations from 50 mM to 1 M was used.

T cell stimulation assays

The CD1d-restricted DN32D3 NKT cell hybridoma (18) expressing the canonical Vα14-Jα281/Vβ8 TCR was used in all T cell stimulation assays. Empty or αGC-loaded CD1d molecules were coated for 16–24 h at 1 or 2.5 μg/well in PBS on 96-well plates. For testing of preloaded CD1d-αGC, wells were washed three times with PBS; then 2×10^4 hybridoma cells were added in RPMI (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 2 mM L-glutamine, and 10 mM HEPES buffer. Supernatants were harvested after 24 h to measure IL-2 release using a [^{3}H]thymidine uptake assay. For testing the loading of αGC onto CD1d, after the PBS washings, αGC was added at various concentrations from a 200 μM stock solution in 0.05% Tween 20-PBS and incubated for 48 h. The wells were washed another three times with PBS before adding the hybridoma cells. For testing the association and dissociation requirements of αGC with CD1d, CD1d was plate coated at 1 μg/well and loaded with 25 μM αGC. For dissociation, the plate was loaded for 48 h and then resuspended in PBS and washed at indicated times before addition of cells. For association, the plate was washed at indicated times before addition of cells. To monitor TCR-CD3 down-regulation, 8×10^4 cells were incubated with CD1d-coated wells and various concentrations of αGC in a 96-well U-bottom plate at 37°C. After 30 h, cells were stained either with CD3ε-PE (2C11; BD PharMingen, San Diego, CA) or F23.1-biotin followed by streptavidin-PE (BioSource International, Camarillo, CA) to detect surface TCR.

Results

Measuring the functional kinetics of αGC-CD1d binding

Using a functional assay of DN32D3 T cell activation, it was possible to estimate minimum functional association and dissociation times of αGC to CD1d (Fig. 1*A*). To appreciate binding, empty CD1 molecules were coated onto plastic and loaded with constant amounts of αGC. At indicated times, wells were washed and cells were added. As seen in Fig. 1*A*, αGC was loaded very slowly and bioactivity did not reach a plateau until ~7 h. Once loaded, dissociation also proceeded very slowly taking >24 h to lose ~25%

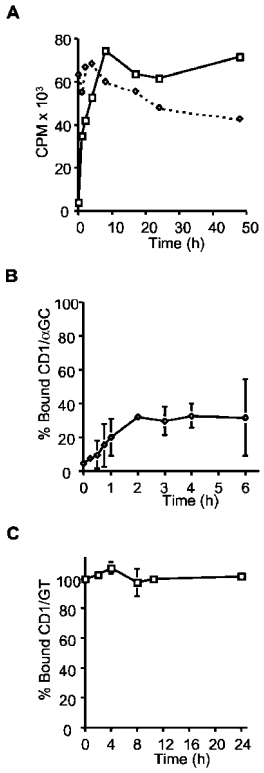

FIGURE 1. Slow association and dissociation of lipid with CD1d. *A*, DN32D3 NK T cell stimulation assay to establish association (□) and dissociation (◇) of 25 μM αGC loading onto 1 μg of CD1d. *B*, IEF analysis of αGC loading/displacement of CD1d-GT1b at approximately a 1:1 molar ratio. *C*, GT1b dissociation from the CD1d-GT1b complex, in the absence of other lipids, after incubation at 37°C.

bound. These values were in agreement with the previously estimated half-life of 1 day also measured by functional assay (12). Although these are indirect measurements for association and dissociation, they indicated much slower on and off rates than direct measurements of the lipid interaction with CD1 by SPR (15, 16). For CD1d, these estimated to 6.3×10^4 M^- s^{-1} and 0.023 s^{-1} on and off rates, respectively, translating into a half-life of 30 s. However, we were able to directly measure association and dissociation of lipid with CD1d using a newly established IEF binding assay, described in more detail in *Alternative lipid binding assays*. In line with results from the functional assay (Fig. 1*A*), αGC loading, as measured by GT1b displacement, did not plateau until ~2 h (Fig. 1*B*). Although complete GT1b displacement did not occur unless αGC was at least in 10-fold molar excess, the kinetics of loading remained the same when analyzed at CD1d/GT1b-αGC molar ratios of 1:1, 1:2, 1:5, and 1:10. Dissociation of GT1b from the CD1d-GT1b complex without additional lipid present was virtually nonexistent in a 24-h time span (Fig. 1*C*). In fact, the CD1d-GT1b complex remains stable for up to 2 wk at 4°C before showing any loss of GT1b binding. The slightly faster association of αGC seen in the IEF direct binding assay than in the functional assay may be a reflection of having to displace only a single bound lipid of the CD1d-GT1b purified complex as opposed to a heterogeneous mix of lipids bound to the empty CD1d.

To understand the discrepancy between our results and the published SPR data, we first re-examined the SPR experiments using a biotinylated αGC. In contrast to the biotin-αGC compound used in the previous study, which was modified by addition of a biotin molecule to the acyl chain (15), the present compound was modified by addition of a biotin molecule to position 6 of the sugar moiety. This new site is preferred given that it is the lipid acyl chains that are expected to bind in the groove of CD1 (3–6). The biological activity of the present biotin-αGC compound was verified by stimulation of the DN32D3 T cell hybridoma and found to be identical with that of unmodified αGC (C. Cantu III and L. Teyton, unpublished observations). The αGC-biotin was coupled to a streptavidin sensor chip on a BIAcore 2000, and empty CD1d was injected over it at increasing concentrations, using empty H-2D^d molecules as a negative control (Fig. 2*A*). Subtracted sen-

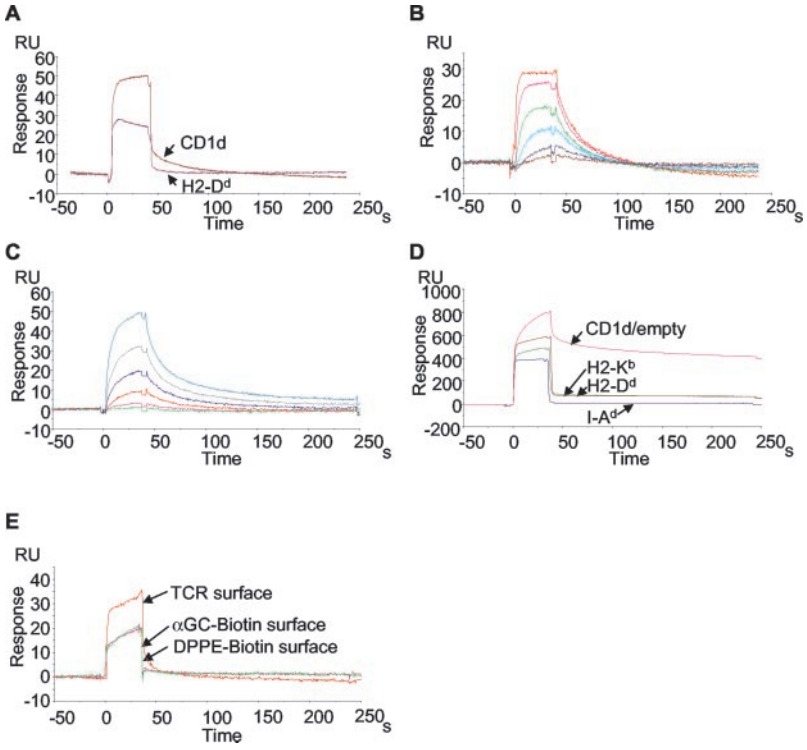

FIGURE 2. Fast association and dissociation of αGC with CD1d is an artifact of the SPR system. *A* and *B*, Binding of empty or αGC-loaded CD1 to the biotin-αGC surface. Controls included using αGC-loaded CD1d over biotin-αGC surface, (*C*) a hydrophobic surface to test class I and II MHC binding as well as CD1/αGC (*D*), and 0.05% Tween in the running buffer plus CD1d-empty over additional control TCR and DPPE-biotin surfaces (*E*). RU, Resonance units.

sorgrams (Fig. 2*B*) could be used to derive fast kinetic parameters, (k_{on} 1.2 × 10^5 M^{-1} s^{-1}; k_{off} 0.0227 s^{-1}; K_D 1.89 × 10^{-7} M) comparable with the published data (15), which was unexpected given the new preferred location of the biotin modification. To further investigate this interaction, preloaded CD1d molecules were injected onto the same surface. Blocking of the groove with a ligand would be expected to prevent interaction with the immobilized αGC as we observed for MHC-peptide interactions. For these experiments, CD1 molecules were loaded with αGC during a 48-h period at room temperature, separated from free lipids by gel filtration, and tested functionally (C. Cantu III and L. Teyton, unpublished observations). Surprisingly, binding of CD1d-αGC preloaded complexes was readily observed over the αGC-biotin surface and had kinetic parameters very similar to those of the empty CD1d molecules (Fig. 2*C*) (k_{on} 8 × 10^4 M^{-1} s^{-1} and k_{off} 0.025 s^{-1}; K_D 3.1 × 10^{-7} M). Similar pseudo-binding curves were obtained for both empty CD1d and αGC-loaded CD1d on a biotin-dipalmitoylphosphatidylethanolamine (DPPE-biotin) surface (C. Cantu III and L. Teyton, unpublished observations). Suspicious of nonspecific hydrophobic interactions between CD1d and biotinylated lipid surfaces, empty CD1d molecules were injected directly over the hydrophobic surface on an HPA BIAcore sensor chip. Compared with MHC class I and class II molecules, CD1 molecules exhibit high nonspecific interaction with this surface (Fig. 2*D*). Finally, CD1d molecules were injected onto the αGC-biotin surface with 0.05% Tween 20 in PBS as running and injection buffer to reproduce the loading conditions currently used by most investigators. The result was the loss of all specific binding (Fig. 2*E*). The presence of DMSO, another condition used in αGC binding studies, was also tested and produced the same effect as the Tween 20 addition (C. Cantu and L. Teyton, unpublished observations). We concluded from that series of experiments that CD1 would interact nonspecifically with hydrophobic surfaces regardless of the nature of the hydrophobic group used to coat the surface. Even though we could detect slight qualitative differences in binding between empty and αGC-loaded CD1d, SPR appears ill suited to measure lipid-protein interactions in the present system. The presence of a number of bound lipids in the so-called empty CD1d molecules was shown by lipid extraction followed by mass spectrometry analysis (C. Cantu III and L. Teyton, manuscript in preparation). This feature probably explained some of the high nonspecific hydrophobic binding of CD1d.

Because a high number of tryptophans are in the binding groove of CD1d, tryptophan fluorescence was used to compare empty and loaded CD1d molecules. Free or unperturbed tryptophans would give fluorescence at a wavelength of ~350 nm, whereas the presence of a ligand in the binding site would cause a blue shift in emission maximum (23, 24). Spectra of empty as well as PE-FITC- or αGC-loaded CD1d were all comparable and shifted to a maximum of 338 nm indicating the presence of ligand in the binding groove (Fig. 3*A*). Treatment with 6 M guanidinium chloride shifted the emission maximum for each to 350 nm (Fig. 3*B*). The presence of lipids in the groove of empty CD1 molecules is in all points comparable with the presence of a mixture of peptides in empty MHC class I and class II molecules (25, 26). This is an important parameter to consider for interpreting binding studies, because the association phase will truly be an exchange-competition reaction. In contrast to the case of peptide binding to MHC, for which the increase in peptide concentration will limit the impact of this exchange reaction, an increase in lipid concentration will only limit the availability of monomers in the reaction as the CMC is exceeded and the micelle population becomes dominant.

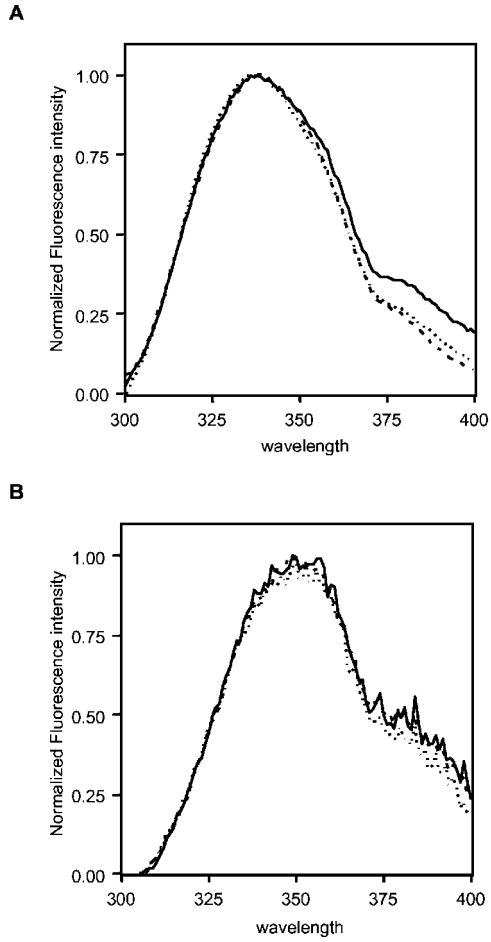

FIGURE 3. Tryptophan fluorescence analysis of CD1d, excitation at 295 nm. *A*, Emission spectrum of mCD1d. (——, empty; · ·· ··, αGC; – – – –, PE-FITC. *B*, Emission spectrum of same mCD1d-lipid complexes after denaturation with 6-M guanidine-HCl.

Alternative lipid binding assay

Empty CD1 molecules resolve on native IEF gels as two discrete bands of approximately neutral pI. This migration pattern can be altered by the binding of charged phospholipids, such as PE-FITC (two charges), or charged glycolipids, such as asialoganglioside (GM) (one charge), disialoganglioside (GD) (two charges), or trisialoganglioside (GT) (three charges) (Fig. 4). Shifts were seen with PE-FITC, GM1, and GT1b (mix of GM, GD, and GT) as shown in Fig. 4*B*, whereas neutral αGC did not induce changes in the migration of CD1. Because of the charge heterogeneity of the GT1b mix, separation of the GT1b-bound CD1d complexes into the individual GM-, GD-, and GT-CD1d complexes was then possible using anion exchange chromatography (Fig. 4*C*). The anionic PS did not load and therefore caused no shift. PE-FITC, GT1b, and GM1 binding to CD1d were each titrated to saturation and quantified from the IEF gels (Fig. 5*A*) to determine equilibrium binding constants (Table I). The binding of αGC could be titrated in a competition format using a constant amount of PE-FITC or GT1b to establish its equilibrium binding constant (Fig. 5*A*). One concern with these assays is using lipid concentrations that exceed their respective CMC, the concentration at which a given lipid begins transition from a monomeric species to complex structures and micelles. Whereas the CMC of GM1 is 20 nM and that of the phospholipid, phosphatidylcholine, is on the order of 1 nM (from www.avantilipids.com), the CMCs for the other lipids used in these experiments were not available. Because lipid concentrations

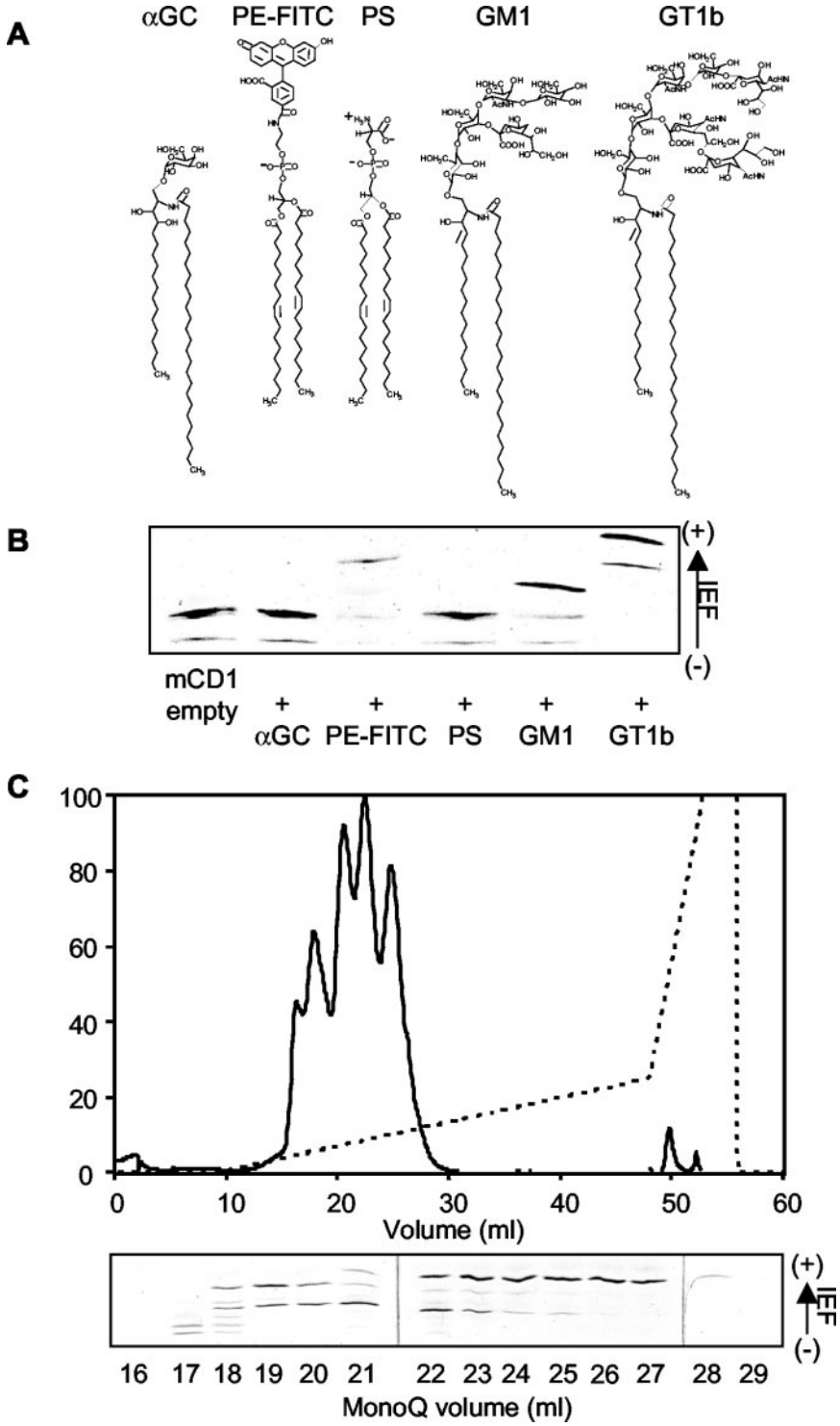

FIGURE 4. IEF analysis of CD1d lipid binding. *A*, Schematic diagrams of lipids used in this study. *B*, IEF gel of empty and loaded CD1d. *C*, Separation of CD1d/GT1b mix into specific GM-, GD-, GT-bound CD1d complexes by anion exchange chromatography with analysis of specific fractions by IEF. The scale of the *y*-axis of the chromatogram represents both the normalized OD_{280} values (——) of MonoQ fractions and the salt gradient (– – – –) used for protein elution.

used were in the micromolar range, it is worth mentioning that for both PE-FITC and GM1, saturation of binding was achieved with 20–40% CD1d still unbound, limitations possibly due to CMC effects. However, saturation with GT1b completely shifted all of CD1d to the bound state (Fig. 4*B*), suggesting that micelle formation is not a concern for the competition assay.

ITC as a second method to measure CD1d-lipid binding

ITC experiments done at 25°C suggest that the binding of PE-FITC or αGC to CD1d is exothermic (Fig. 5*B*). However, in using the one-site model to obtain equilibrium binding constants, the parameter of the number of binding sites had to be held constant at 1.0 to obtain reasonable fits. This may be due to the fact that the actual interaction involves a lipid exchange, or displacement, that is not inferred in the binding model. Also, at high concentrations of αGC or PE-FITC, the CMC problem that we mentioned above appeared (Table II) with an apparent drop in affinity (10-fold) for both lipids when added at high micromolar concentrations. Also, at low concentrations, the sensitivity of the calorimeter was greatly tested, and the curve fitting was not ideal. Even so, the equilibrium binding constants obtained by this method were in general agreement with those obtained by IEF analysis (Table I).

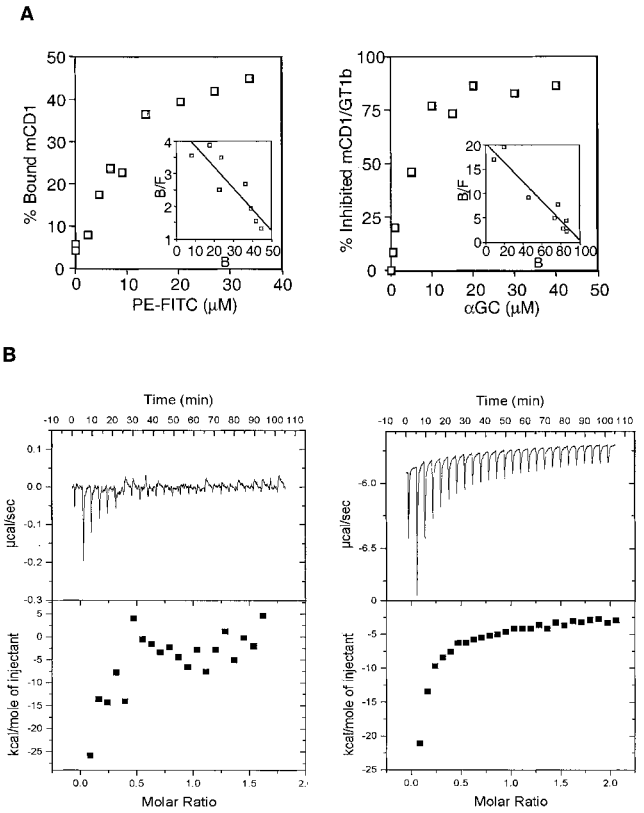

FIGURE 5. Alternative lipid binding assays. *A*, Representative binding curves obtained through IEF analysis and their respective Scatchard plots. *B*, ITC results for 30 μM αGC interacting with 3 μM CD1d (*left*) and 200 μM αGC interacting with 20 μM CD1d (*right*). Buffer only injection used as control. B, Bound; F, free.

In conclusion, CD1d appeared to bind lipids with moderate affinity (μM range) and to be competing for binding endogenous lipids with slow kinetics, explaining long half-life loading times for lipids such as αGC. However, the dissociation of the lipid-CD1 complexes is remarkably slow with half-lives in excess of 1 day. These two kinetic parameters would explain most of the functional results presented in *Measuring the functional kinetics of αGC-CD1d binding*. It is also apparent that SPR is not a satisfactory method for examination of lipid binding to CD1 molecules and that more traditional methods also have limitations due to the chemical/physical nature of lipids.

Expression and characterization of recombinant Vα14-Vβ heterodimers

Once formed, the CD1-glycolipid complex is recognized by the TCR. In the case of CD1-αGC, this recognition event is limited to NKT cells. Thus, we expressed recombinant forms of the semi-invariant TCR used by these cells. TCR expressed by NKT cells have two distinct characteristics: 1) they express a monomorphic Vα chain with a unique junctional region; 2) they pair it with a limited set of Vβ chains with highly polymorphic D and J regions. To address partly the role of the β-chain in ligand recognition, we expressed the Vα14 chain in combination with the β-chain from an NKT cell, as well as with two random β-chains derived from classical $\alpha\beta$ TCRs directed against MHC class I and class II molecules plus peptide (Table III). The invariant α-chain of the NKT cell line DN32D3 and its Vβ8.2 counterpart were cloned from RNA by RT-PCR and sequenced. The β-chain from the K^b/L^d-restricted 2C TCR and I-A^k-restricted D10 TCR were already present in the laboratory. cDNAs to produce soluble recombinant TCRs were constructed by PCR by deleting the transmembrane and cytoplasmic segments of both chains and adding leucine zippers and a six-histidine tag following the last cysteine of the constant domains (19). The modified cDNAs were transfected pairwise in the *D. melanogaster*-derived SC2 cell line. After selection in G-418-containing medium and single-cell cloning, expression was tested by nickel-nitrilotriacetic acid-agarose immunoprecipitation and Coomassie blue gel electrophoresis. The three V$\alpha14^+$ TCR molecules were expressed at similar low levels. Large scale preparations were purified to >90% purity by a succession of nickel affinity chromatography, anion exchange chromatography, and size exclusion gel chromatography. The presence of the interchain disulfide bond within the TCR was verified by comparing reduced and nonreduced SDS-PAGE (Fig. 6*A*). The 1:1 α-β ratio and the presence of an acidic leucine zipper on the Vα14 chain and a basic leucine zipper on the Vβ8 chain were verified by two-dimensional gel electrophoresis, the first dimension by nonequilbrium pH gel electrophoresis (NEPHGE) and the second by SDS-PAGE (Fig. 6*B*). The functionality of these TCRs has been shown by inhibition of DN32D3 T cell hybridoma activation (C. Cantu III and L. Teyton, unpublished observations).

Table I. *IEF equilibrium mCD1 binding constants*

Lipid	K_D (μM)
PE-FITC	16.4 ± 7.0
GT1b	8.0 ± 5.6
GM1	47.1 ± 7.7
αGC (mCD1-PE-FITC)	2.3 ± 0.5
αGC (mCD1-GT1b)	5.9 ± 1.3

Table II. *Calorimetry equilibrium binding constants*

Binding Interaction	K_D (μM)
mCD1 (5 μM)-PE-FITC (50 μM)	1.9 ± 2.4
mCD1 (20 μM)-PE-FITC (200 μM)	11.1 ± 6.4
mCD1 (3 μM)-αGC (30 μM)	1.0 ± 0.3
mCD1 (20 μM)-αGC (200 μM)	9.7 ± 7.7

Recognition of αGC-loaded CD1d molecules by rVα14-bearing TCRs

Because of the invariant nature of the CD1d-restricted NK1.1 TCR and the unique nature of the ligand, the rules of recognition for the TCR-CD1d interaction are likely to vary somewhat from those established for conventional TCR-pMHC interactions (i.e., low affinity interaction; plasticity of Ag-binding surface). SPR was used to determine the affinities of CD1d-V$\alpha14^+$ TCR interactions. Each of the V$\alpha14^+$ TCRs was immobilized by standard amine coupling or cysteine coupling on a BIAcore CM5 sensor chip. Then, either size-purified CD1d/empty (not loaded with αGC) or CD1d-αGC molecules were flowed over the TCR surface to measure binding. Comparison of the sensorgrams for empty and loaded CD1d revealed specific binding only for CD1d-αGC (Fig. 6*C*). Using CD1d-empty to establish control sensorgrams for subtraction, at least six concentrations of CD1d-αGC were flowed over each TCR surface (Fig. 6*D*). The subtracted curves were fit to a 1:1 Langmuir binding model in the global fitting algorithm of the BIAevaluation 3.0.2 software (BIAcore) to obtain kinetic parameters and affinity constants (Table IV). The absence of binding of the 2C β-containing TCR and D10 β-containing TCR to their cognate ligands, L^d-p2CA and I-A^k Con A, respectively, was verified accordingly (C. Cantu III and L. Teyton, unpublished observations).

Table III. *Vα14⁺ TCR constructs*

Name of Recombinant TCR	Vα	Vβ	Origins of Vβ Selection	Expression Levels[a]
Vα14-DN32	14	8.2	mCD1	+/−
Vα14-2C	14	8.2	H2-L^d-p2CA (Vα3.1)[b]	+
Vα14-D10	14	8.2	I-A^k-Con A (Vα2)[c]	+/−

[a] +/−, 50–100 μg/L expression; +, 100–200 μg/L expression.
[b] Kranz et al. (21).
[c] Kaye et al. (22).

The binding affinity, K_D, of the NKT cell receptor DN32D3 was measured at 98 nM with a dissociation half-life of 185 s. This high affinity, when compared with classical αβ TCRs, was mostly due to slower off rates (higher half-lives, $t_{1/2}$) whereas on rates were fairly comparable (27). Most interestingly, the substitution of the β-chain of DN32D3 by a noncognate β-chain did not result in the loss of binding, but, in contrast, sustained similar binding affinities with K_Ds of 57 and 31 nM for Vα14-D10 and Vα14-2C, respectively. The reverse orientation of binding with immobilization of CD1/αGC on the chip was tested with the Vα14-2C using CD1d-empty immobilized on the adjacent flow cell as a negative control. Kinetics parameters and resulting affinities were very similar in that reverse setting (Table IV). Tetrameric Vα14-2C TCR was also flowed over the CD1d surfaces and showed a 35-fold increased half-life compared with monomers. However, due to the nature of the tetramers and their four independent binding sites, no mathematical model can be built to derive accurate kinetics parameters for those molecules, and the comparison of monomers/tetramers can be only approximate.

In conclusion, it appeared that CD1-αGC was recognized by Vα14-Vβ8.2 TCRs with high affinities regardless of the origin of the β-chain. These affinities 1- to 2-fold higher than classical TCR-MHC peptide interactions prompted us to examine further the biophysical nature of these interactions.

Thermodynamics of the CD1-αGC-Vα14-2C interaction

Thermodynamic parameters reflect accommodation and stability of interacting molecules. SPR has been used to analyze the temperature dependence of the binding of TCR-MHC interactions and gauge the plasticity and reorganization of TCR-MHC interfaces (28–30). CD1αGC-Vα14-DN32 TCR interactions were examined using a similar strategy. Over the temperature range 10–34°C, the K_D of this interaction was measured by SPR. Numbers stayed fairly constant ranging from 28 to 87 nM, indicating essentially no

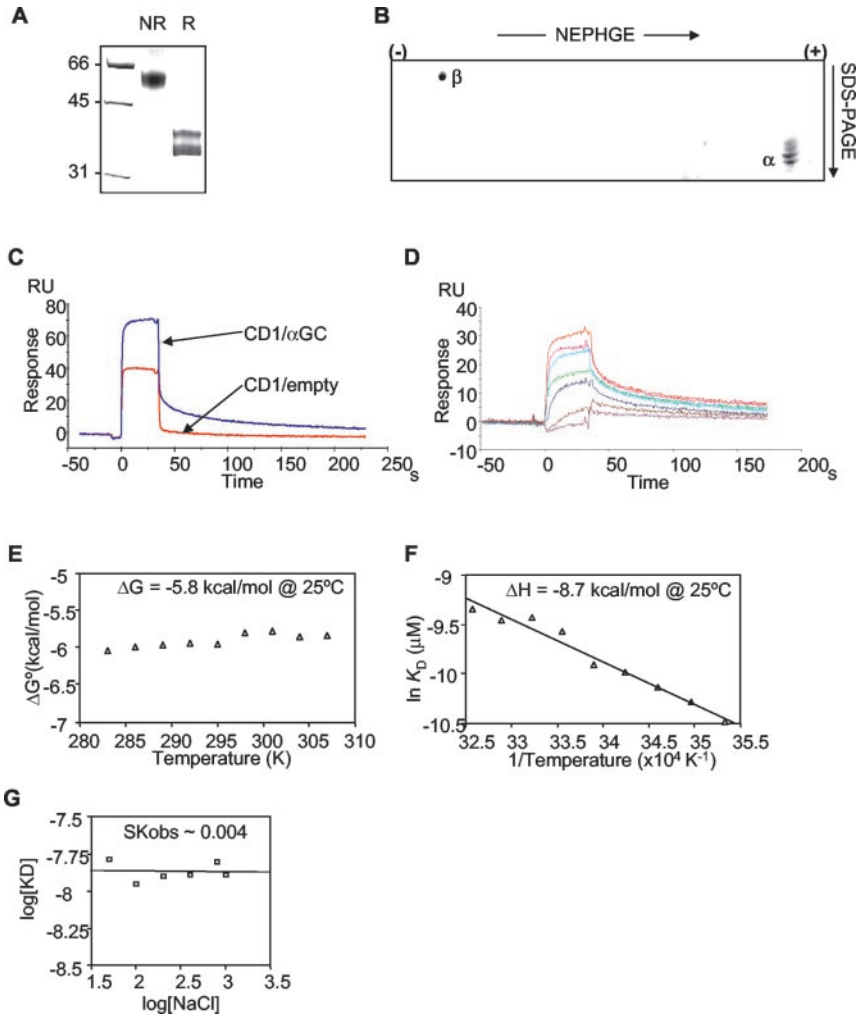

FIGURE 6. Characterization of Vα14 TCR constructs and SPR analysis of CD1/αGC binding to a Vα14-DN32 surface. *A*, SDS-PAGE of Vα14-2C construct under reducing (R) and nonreducing (NR) conditions. *B*, Two-dimensional gel electrophoresis analysis of the Vα14-2C construct. The presence of the basic and acidic leucine zippers is evident by the distinct one-dimensional NEPHGE separation. *C*, Single sensorgrams of 5 μM CD1-empty and CD1-αGC over the Vα14-DN32 surface. RU, Resonance units. *D*, Curves represent CD1-αGC concentrations ranging from 0.78 to 5 μM over the Vα14-DN32 surface after subtraction of the respective concentrations of CD1-empty sensorgrams. *E*, Plot of free energy, $\Delta G°$, vs temperature (in Kelvin). ΔG is determined from the relationship $\Delta G = -RT\ln K_D$, where R is the gas constant and the K_D value is obtained from SPR analysis of sensorgrams of 2 μM CD1-αGC injected over the Vα14 TCR surface at the respective temperature T. *F*, van't Hoff analysis of affinity, K_D, as a function of temperature. Enthalpy, ΔH, is derived from the slope (slope = $\Delta H/R$) of a linear fit to the data points. *G*, The measure of contribution of electrostatic attraction to binding is obtained from the slope, SK_{obs}, of the plot of log of K_D vs the log of the sodium chloride concentration for which the K_D was obtained.

Table IV. *Kinetic parameters for TCR-CD1-αGC interaction*

Immobilized Protein	Flowing Protein	k_{on} ($1/M^{-1}s^{-1}$)	k_{off} ($1/s^{-1}$)	K_D (M)	Half-life (s)
Vα14-D10	CD1d-αGC	6.69E+04	3.79E−03	5.67E−08	183
Vα14-DN32	CD1d-αGC	8.44E+04	8.19E−03	9.81E−08	85
Vα14-2C	CD1d-αGC	2.48E+05	6.40E−03	3.04E−08	109
CD1d-αGC	Vα14-2C	2.75E+05	3.02E−03	1.10E−08	230
CD1d-αGC	Vα14-2C tetramer	4.30E+05	8.20E−05	9.10E−11	8453

dependence of the interaction on temperature. Calculating the free energy (5.8 kcal/mol at 25°C) from the relationship $\Delta G = -RT\ln K_D$ (Fig. 6*E*), the enthalpy (−8.7 kcal/mol) from the slope ($= \Delta H/R$) of the van't Hoff plot (Fig. 6*F*), and the entropy (−2.9 kcal/mol) from the relationship $\Delta G = \Delta H - T\Delta S$ confirms the temperature independence. Actually, the enthalpy value is essentially identical with the average (−8.6 kcal/mol) for protein-protein interactions (31). For comparison, the average of published thermodynamic parameters (at 25°C) for two class I MHC-TCR and two class II MHC-TCR were: $\Delta G = -6.7$ kcal/mol, $\Delta H = -19.7$ kcal/mol, and $T\Delta S = -13$ kcal/mol (29, 30). Large changes in parameters were attributed to conformational adjustments and/or loss of flexibility at binding surfaces. Conversely, the almost negligible changes in CD1d-αGC-Vα14 TCR thermodynamic parameters indicated the rigidity of the recognition of the CD1d-αGC complex by the Vα14-2C TCR.

Additionally, the electrostatic contribution to binding was tested by measuring the affinity of interaction over a range of sodium chloride concentrations (32). The slope (SK_{obs}) of the plot of the log of K_D vs the log of sodium concentration of the CD1d-αGC-Vα14 TCR interaction was almost flat, indicating a very negligible contribution of electrostatic forces to this interaction (Fig. 6*G*).

The thermodynamic characteristics of CD1d-αGC-Vα14 TCR interactions suggest that the sugar moiety, the most rigid part of the glycolipid, is the most important part of the ligand seen by the TCR during this interaction. These results also indicate a correlation between higher affinity and limited rearrangement of the interactive surfaces.

Biological consequences of the high affinity

In the present study, NKT cell activation, as measured by IL-2 release, occurred at high concentrations of αGC, but with no sustained TCR down regulation (C. Cantu III and L. Teyton, unpublished observations). In comparison, sustained down-regulation of TCR by activated T cells for a high affinity pMHC-TCR system has been previously observed (33). The down-regulation, however, was observed only for TCR transfectants that displayed reduced IL-2 levels at high peptide concentrations. For a TCR transfectant that had optimal IL-2 release at high peptide concentrations, no TCR down-regulation was observed (33).

Discussion

While there have been many studies done on lipid antigen presentation by the different CD1 family members (reviewed in Refs. 1 and 2), few biochemical studies have been done to elucidate the lipid-binding properties of CD1. The crystal structure of mouse CD1d showed a very hydrophobic binding groove with two deep, large pockets (A′ and F′) occupied by discontinuous electron density (3) which was thought to be an acyl chain-containing ligand (15). Recently, the structure of human CD1b with phosphatidylinositol or ganglioside GM2 bound has been solved (34). In comparison with the mCD1 structure, it contains an additional hydrophobic pocket (C′) as well as a connective tunnel that connects the A′ and F′ pockets. Cellular GPI has been identified as a major natural ligand of CD1d (35), although it does not appear to provide $V\alpha14^+$ NKT cell reactivity (36). An SPR study by Naidenko et al. (15) established a submicromolar affinity (K_D, 0.34 μM) between mCD1d and αGC, with a half-life of 30 s established for the complex. Similar results were seen for CD1b and phosphatidylinositol mannoside using SPR. This is in sharp contrast to a half-life estimated by an NKT cell hybridoma activation assay (on the order of 1 day) and suggested by the long-lasting stability of CD1d-αGC tetramers further used in that study (12). We have since revisited the SPR experiments and established that indeed the CD1d-αGC interaction observed, including the short half-life of the complex, was an artifact of the system.

In characterizing CD1d-αGC and CD1d-empty molecules by IEF analysis, we were able to establish an equilibrium binding assay to measure the affinities of several lipids to CD1d.

The IEF equilibrium binding constants have been verified by ITC experiments. Also, both the IEF direct binding assay and the NKT cell activation assay agree on a CD1-αGC long half-life on the order of at least 1 day. Overall, these results suggest that regardless of lipid antigenicity, CD1d binds lipids with low micromolar affinities in a similar manner. In fact, a comparison of affinities between other lipid-binding proteins and their respective ligand reveals affinities also in the low micromolar range (24, 37, 38) and suggests a low specificity of lipid-binding proteins in general. Thus, the various synthetic and cellular lipids that have been shown to be presented by CD1 molecules likely achieve specific presentation more as a result of CD1 transfer and processing (39–43) than from CD1 binding specificity. Indeed, given that the lipid acyl chains are what the CD1 proteins bind, the amount of variability is limited to the number of acyl chains and their respective lengths and saturated bonds. The recent structural analysis of human CD1b highlights the adaptability of the CD1 family to accommodate acyl chains of various lengths and number (34).

To date, several studies have used soluble αGC-loaded CD1d molecules to address the questions of T cell activation through the invariant Vα14-Vβ8 TCR (12, 13). Although these reagents have proved very useful in studying the various populations of $V\alpha14^+$ TCR-bearing T cells (9), the nature of the direct CD1d-αGC-TCR interaction remains unclear. For instance, TCR down-regulation in Ag-activated T cells has been shown to be a result of the prevention of TCR recycling as opposed to increased TCR internalization (44). Kranz and colleagues (33) suggest that the valency state of the TCR-pMHC interaction is the connection relating T cell activation and TCR recycling. At high peptide concentrations, monovalent TCR interactions could predominate, preventing activation through TCR dimerization. However, TCR ligation also leads to intracellular prevention of recycling (44). For NKT cells, the activation seen at very high concentrations of αGC may be occurring through monovalent TCR-CD1-αGC interactions. As such, absence of TCR ligation would explain the lack of sustained TCR down-regulation. Determination of whether or not TCR dimerization is necessary for activation of NKT cells and TCR down-regulation awaits further studies.

To begin to identify the rules of recognition for this interaction, recombinant soluble Vα14-Vβ8 TCRs have been constructed for direct binding analysis. SPR analysis has revealed a high affinity interaction, in line with the invariant nature of the Vα14-Vβ8 TCR. The high affinity is due to much slower off rates (longer half-life) than seen for conventional MHC-TCR interactions (27, 29, 30). Similar results have just been reported in a recent paper for one other V$\alpha14^+$ TCR (45). In that study, SPR analysis, using a bacterially expressed single chain V$\alpha14^+$ TCR construct flowing over a CD1d-αGC surface, and CD1-αGC tetramer staining of NKT cell hybridomas were used to assess the high affinity-avidity interaction. In addition, we have shown that this high affinity interaction is also independent of temperature and electrostatic forces, which again makes this interaction contrary to conventional peptide-MHC-TCR interactions (28–30). Overall, these findings suggest that TCR recognition of CD1d-αGC is based on a inflexible interface implicating the more rigid structure of the lipid sugar moiety and the invariant Vα14 chain of the NKT cell receptor. This lock-and-key fit is reminiscent of that seen with affinity-matured hapten Abs that acquire rigid binding interfaces and nanomolar affinity through somatic mutations (46, 47). Further studies into the selection or maturation of the NK TCR are limited by uncertainty regarding the natural ligand. TCR transfectant cell lines with the new Vα14-Vβ8 pairs described in this paper are being developed for future functional studies.

In conclusion, monomers of lipids and glycolipids bind to CD1d with low affinity using a monomorphic, hydrophobic interaction. CD1d-glycolipids are seen by V$\alpha14^+$ TCRs with high affinity using nonadaptable interacting surfaces, in contrast with conventional $\alpha\beta$ TCRs that bind MHC-peptide complexes.

Acknowledgments

We thank Jamie Williamson for the use of the Microcal MCS calorimeter and Michael Recht for assistance with isothermal titration calorimetry. Special thanks to Randy Stefanko, Michael Wallace, and Berta Lyles for technical assistance; Thomas Stratmann and Valerie Mallet-Designe for discussions; and Amanda Moore for administrative assistance.

References

1. Porcelli, S. A., and R. L. Modlin. 1999. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu. Rev. Immunol. 17:297.*
2. Jayawardena-Wolf, J., and A. Bendelac. 2001. CD1 and lipid antigens: intracellular pathways for antigen presentation. *Curr. Opin. Immunol. 13:109.*
3. Zeng, Z., A. R. Castano, B. W. Segelke, E. A. Stura, P. A. Peterson, and I. A. Wilson. 1997. Crystal structure of mouse CD1: an MHC-like fold with a large hydrophobic binding groove. *Science 277:339.*
4. Melian, A., G. F. M. Watts, A. Shamshiev, G. De Libero, A. Clatworthy, M. Vincent, M. B. Brenner, S. Behar, K. Niazi, R. L. Modlin, et al. 2000. Molecular recognition of human CD1b antigen complexes: evidence for a common pattern of interaction with $\alpha\beta$ TCRs. *J. Immunol. 165:4494.*
5. Burdin, N., L. Brossay, M., Degano, H. Iijima, M. Gui, I. A. Wilson, and M. Kronenberg. 2000. Structural requirements for antigen presentation by mouse CD1. *Proc. Natl. Acad. Sci. USA 97:10156.*
6. Grant, E. P., M. Degano, J.-P. Rosat, S. Stenger, R. L. Modlin, I. A. Wilson, S. A. Porcelli, and M. B. Brenner. 1999. Molecular recognition of lipid antigens by T cell receptors. *J. Exp. Med. 189:195.*
7. Bendelac, A., O. Lantz, M. E. Quimby, J. W. Yewdell, J. R. Bennink, and R. R. Brutkiewicz. 1995. CD1 recognition by mouse NK1.1$^+$ T lymphocytes. *Science 268:863.*
8. Brossay, L., M. Chioda, N., Burdin, Y. Koezuka, G. Casorati, P. Dellabona, and M. Kronenberg. 1998. CD1d-mediated recognition of an α-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J. Exp. Med. 188:152.*
9. MacDonald, H. R. 2002. Development and selection of NKT cells. *Curr. Opin. Immunol. 14:250.*
10. Kwano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science 278:1626.*
11. MacDonald, H. R. 2000. CD_{1d}-glycolipid tetramers: a new tool to monitor natural killer T cells in health and disease. *J. Exp. Med. 192:F15.*
12. Benlagha, K., A. Weiss, A. Beavis, L. Teyton, and A. Bendelac. 2000. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J. Exp. Med. 191:1895.*
13. Matsuda, J. L., O. V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C.-R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med. 192:741.*
14. Park, S.-H., A. Weiss, K. Benlagha, T. Kyin, L. Teyton, and A. Bendelac. 2001. The mouse CD1d-restricted repertoire is dominated by a few autoreactive T cell receptor families. *J. Exp. Med. 193:893.*
15. Naidenko, O. V., J. K. Maher, W. A. Ernst, T. Sakai, R. L. Modlin, and M. Kronenberg. 1999. Binding and antigen presentation of ceramide-containing glycolipids by soluble mouse and human CD1d molecules. *J. Exp. Med. 190:1069.*
16. Ernst, W. A., J. Maher, S. Cho, K. R. Niazi, D. Chatterjee, D. B. Moody, G. S. Besra, Y. Watanabe, P. E. Jensen, S. A. Porcelli, M. Kronenberg, and R. L. Modlin. 1998. Molecular interactions of CD1b with lipoglycan antigens. *Immunity. 8:331.*
17. Crowley, M. P., A. M. Fahrer, N. Baumgarth, J. Hampl, I. Gutgemann, L. Teyton, and Y. Chien. 2000. A population of murine $\gamma\delta$ T cells that recognize an inducible MHC class Ib molecule. *Science 287:314.*
18. Zhou, X. T., D. Forestier, R. D. Goff, C. Li, L. Teyton, A. Bendelac, and P. B. Savage. 2002. Synthesis and NKT cell stimulating properties of fluorophore- and biotin-appended 6″-amino-6″-deoxy-galactosylceramides. *Org. Lett. 4:1267.*
19. Lantz, O., and A. Bendelac. 1994. An invariant T cell receptor α chain is used by a unique subset of MHC class I-specific CD4$^+$ and CD4$^-$8$^-$ T cells in mice and humans. *J. Exp. Med. 180:1097.*
20. Scott, C. A., K. C. Garcia, F. R. Carbone, I. A. Wilson, and L. Teyton. 1996. Role of chain pairing for the production of functional soluble IA major histocompatibility complex class II molecules. *J. Exp. Med. 183:2087.*
21. Kranz, D. M., D. Sherman, M. V. Sitkovsky, M. S. Pasternack, and H. N. Eisen. 1984. Immunoprecipitation of cell surface structures of cloned cytotoxic T lymphocytes by clone-specific antisera. *Proc. Natl. Acad. Sci. USA 81:573.*
22. Kaye, J., S. Porcelli, J. Tite, B. Jones, and C. A. Janeway, Jr. 1983. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen presenting cells in the activation of T cells. *J. Exp. Med. 158:836.*
23. Eftink, M. R., and C. A. Ghiron. 1976. Exposure of tryptophanyl residues in proteins: quantitative determination by fluorescence quenching studies. *Biochemistry 15:672.*
24. Kennedy, M. W., A. P. Heikema, A. Cooper, P. J. Bjorkman, and L. M. Sanchez. 2001. Hydrophobic ligand binding by Zn-α_2-glycoprotein, a soluble fat-depleting factor related to major histocompatibility complex proteins. *J. Biol. Chem. 276:35008.*
25. Fremont, D. H., E. A. Stura, M. Matsumura, P. A. Peterson, and I. A. Wilson. 1995. Crystal structure of an H-2Kb-ovalbumin peptide complex reveals the interplay of primary and secondary anchor positions in the major histocompatibility complex binding groove. *Proc. Natl. Acad. Sci. USA 92:2479.*
26. Stratmann, T., V. Apostolopoulos, V. Mallet-Designe, A. L. Corper, C. A. Scott, I. A. Wilson, A. S. Kang, and L. Teyton. 2000. The I-A^{g7} MHC class II molecule linked to murine diabetes is a promiscuous peptide binder. *J. Immunol. 165:3214.*
27. Davis, M. M., J. J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien. 1998. Ligand recognition by $\alpha\beta$ T cell receptors. *Annu. Rev. Immunol. 16:523.*
28. Boniface, J. J., Z. Reich, D. S. Lyons, and M. M. Davis. 1999. Thermodynamics of T cell receptor binding to peptide-MHC: evidence for a general mechanism of molecular scanning. *Proc. Natl. Acad. Sci. USA 96:11446.*
29. Willcox, B. E., G. F. Gao, J. R. Wyer, J. E. Ladbury, J. I. Bell, B. K. Jakobsen, and P. A. van der Merwe. 1999. TCR binding to peptide-MHC stabilizes a flexible recognition interface. *Immunity 10:357.*
30. Garcia, K. G., C. G. Radu, J. Ho, R. J. Ober, and E. S. Ward. 2001. Kinetics and thermodynamics of T cell receptor-autoantigen interactions in murine experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. USA 98:6818.*
31. Stites, W. E. 1997. Protein-protein interactions: interface structure, binding thermodynamics and mutational analysis. *Chem. Rev. 97:1233.*
32. O'Callaghan, C. A., and J. I. Bell. 1998. Structure and function of the human MHC class Ib molecules HLA-E, HLA-F and HLA-G. *Immunol. Rev. 163:129.*
33. Holler, P. D., A. R. Lim, B. K. Cho, L. A. Rund, and D. M. Kranz. 2001. CD8$^-$T cell transfectants that express a high affinity T cell receptor exhibit enhanced peptide-dependent activation. *J. Exp. Med. 194:1043.*
34. Gadola, S. D., N. R. Zaccai, K. Harlos, D. Shepherd, J. C. Castro-Palomino, G. Ritter, R. R. Schmidt, E. Y. Jones, and V. Cerundolo. 2002. Structure of human CD1b with bound ligands at 2.3 Å, a maze for alkyl chains. *Nat. Immunol. 3:721.*
35. Joyce, S., A. S. Woods, J. W. Yewdell, J. R. Bennik, A. D. de Silva, A. Boesteanu, S. P. Balk, R. J. Cotter, and R. R. Brutkiewicz. 1998. Natural ligand of mouse CD1d1: cellular glycosylphosphatidylinositol. *Science 279:1541.*
36. Molano, A., S.-H. Park, Y.-H. Chiu, S. Nosseir, A. Bendelac, and M. Tsuji. 2000. The IgG response to circumsporozoite protein is MHC class II-dependent and CD1d-independent: exploring the role of GPIs in NK T cell activation and antimalarial responses. *J. Immunol. 164:5005.*
37. LaLonde, J. M., M. A. Levenson, J. J. Roe, D. A. Bernlohr, and L. J. Banaszak. 1994. Adipocyte lipid-binding protein complexed with arachidonic acid. *J. Biol. Chem. 269:25339.*
38. Douliez, J.-P., S. Jegou, C. Pato, D. Molle, V. Tran, and D. Marion. 2001. Binding of two mono-acylated lipid monomers by the barley lipid transfer protein,

LTP1, as viewed by fluorescence, isothermal titration calorimetry and molecular modeling. *Eur. J. Biochem. 268:384.*
39. Chiu, Y.-H., J. Jayawardena, A. Weiss, D. Lee, S.-H. Park, A. Dautry-Varsat, and A. Bendelac. 1999. Distinct subsets of CD1d-restricted T cells recognize self-antigens loaded in different cellular compartments. *J. Exp. Med. 189:103.*
40. Gumperz, J. E., C. Roy, A. Makowska, D. Lum, M. Sugita, T. Podrebarac, Y. Koezuka, S. A. Porcelli, S. Cardell, M. B. Brenner, and S. M. Behar. 2000. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity 12:211.*
41. Chiu, Y.-H., S.-H. Park, K. Benlagha, C. Forestier, J. Jayawardena-Wolf, P. B. Savage, L. Teyton, and A. Bendelac. 2002. Multiple defects in antigen presentation and T cell development by mice expressing cytoplasmic tail-truncated CD1d. *Nat. Immunol. 3:55.*
42. Dharshan De Silva, A., J.-J. Park, N. Matsuki, A. K. Stanic, R. R. Brutkiewicz, M. E. Medof, and S. Joyce. 2002. Lipid protein interactions: the assembly of CD1d1 with cellular phospholipids occurs in the endoplasmic reticulum. *J. Immunol. 168:723.*
43. Moody, D. B., Briken, V., T.-Y. Cheng, C. Roura-Mir, M. R. Guy, D. H. Geho, M. L. Tykocinski, G. S. Besra, and S. A. Porcelli. 2002. Lipid length controls antigen entry into endosomal and nonendosomal pathways for CD1b presentation. *Nat. Immunol. 3:435.*
44. Liu, H., M. Rhodes, D. L. Wiest, and D. A. A. Vignali. 2000. On the dynamics of TCR:CD3 complex cell surface expression and downmodulation. *Immunity 13:665.*
45. Sidobre, S., O. V. Naidenko, B.-C. Sim, N. R. J. Gascoigne, K. C. Garcia, and M. Kronenberg. 2002. The Vα14 NKT cell TCR exhibits high-affinity binding to a glycolipid/CD1d complex. *J. Immunol. 169:1340.*
46. Wedemayer, G. J., P. A. Patten, L. H. Wang, P. G. Schultz, and R. C. Stevens. 1997. Structural insights into the evolution of an antibody combining site. *Science 276:1665.*
47. Yin, J., E. C. Mundorff, P. L. Yang, K. U. Wendt, D. Hanway, R. C. Stevens, and P. G. Schultz. 2001. A comparative analysis of the immunological evolution of antibody 28B4. *Biochemistry 40:10764.*