Natural killer T (NKT) cells are a subset of T lymphocytes that recognize glycolipid antigens presented by the CD1d molecule (CD1d). They rapidly respond to antigen challenge and can activate both innate and adaptive immune cells. To study the role of antigen presentation in NKT cell activation, previous studies have developed several anti-CD1d antibodies that block CD1d binding to T-cell receptors (TCRs). Antibodies that are specific to both CD1d and the presented antigen can only be used to study the function of only a limited number of antigens. In contrast, antibodies that bind CD1d and block TCR binding regardless of the presented antigen can be widely used to assess the role of TCR-mediated NKT cell activation in various disease models. Here, we report the crystal structure of the widely used anti-mouse CD1d antibody 1B1 bound to CD1d at a resolution of 2.45 Å and characterized its binding to CD1d-presented glycolipids. We observed that 1B1 uses a long hydrophobic H3 loop that is inserted deep into the binding groove of CD1d where it makes intimate nonpolar contacts with the lipid backbone of an incorporated spacer lipid. Using an NKT cell agonist that has a modified sphingosine moiety, we further demonstrate that 1B1 in its monovalent form cannot block TCR-mediated NKT cell activation, because 1B1 fails to bind with high affinity to mCD1d. Our results suggest potential limitations of using 1B1 to assess antigen recognition by NKT cells, especially when investigating antigens that do not follow the canonical two alkyl-chain rule.

Besides a role in immunotherapies, antibodies that block receptor–ligand interactions are often used to assess the importance of a specific signaling axis in immune activation or inhibition. Receptor–ligand interactions can be complex and often involve more than two molecules. For example, T-cells use their antigen receptor (TCR) to recognize the ligand, which forms a composite epitope formed by the antigen-presenting molecule major histocompatibility complex (MHC) and the small antigen that it presents (1). Therefore, antibodies that block TCR engagement of MHC molecules ideally have an overlapping binding site with the TCR on the MHC molecule. Antibodies that block TCR-mediated T-cell activation exist for all MHC molecules including MHC class I (2–5), MHC class II (6), and CD1d (7–9) and are widely used to study or modulate T-cell function by specifically blocking antigen-mediated TCR activation. Although some antibodies are specific for the MHC molecule and the antigen, such as the natural killer T (NKT) cell antigen receptor-blocking antibody L363 that recognizes mouse (m)CD1d presenting α-galactosylceramide (αGalCer), the anti-mCD1d antibody 1B1 binds to mCD1d regardless of the presented antigen (9).

NKT cells are a population of T lymphocytes that recognize glycolipid antigens presented by the nonclassical MHC I homolog CD1d. NKT cells are activated within hours after antigen stimulation and rapidly produce both pro- and anti-inflammatory cytokines (10). Type I and Type II NKT cells are the major classes of NKT cells, and they differ in both their TCR usage and their antigen-specificity (11). Type I NKT cells express a semi-invariant αβ T-cell receptor (TCR) α chain (Vα14-Jα18 in mouse, Vα24-Jα18 in humans) that pairs with a limited number of TCR β chains (Vβ8.2, and to a lesser extend Vβ7 and Vβ2 in mouse, Vβ11 in human) (10, 12). The prototypical antigen α-galactosylceramide (αGalCer) is the common antigen for Type I NKT cells (13). Type II NKT cells do not have a conserved TCR rearrangement and do not recognize a common antigen, making them difficult to identify and characterize. Although Type II NKT cells can recognize a variety of different antigens, a well-characterized and major subset recognizes sulfatide self-antigens (14–17).

In addition to Type I and Type II NKT cells, minor subsets of unconventional NKT cells have been identified and structurally characterized. Although these NKT cells exhibit specificity toward αGalCer, or related glycolipids the TCR repertoire...
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is markedly different from the conventional Type I NKT cells (18, 19).

Glycolipids are bound by CD1d with the lipid backbone deeply inserted into a hydrophobic binding groove that is composed of two major pockets, A' and F'. Each pocket accommodates one alkyl-chain of a dual alkyl-chain lipid. For ceramide-based lipids, such as αGalCer or sulfatide, the acyl chain is accommodated in the larger A' pocket, whereas the sphingosine moiety is bound in the F' pocket (20–22). The binding orientation of diacylglycerolipids is less restricted and the sn-1 and sn-2 linked acyl chain can go in either of the two pockets, depending on number and positions of unsaturations located within the fatty acyl chains (23–28). The different headgroups of the various glycolipids are exposed at or above the CD1d-binding groove for TCR recognition.

NKT cells generally recognize the exposed headgroup and a few polar groups of the lipid backbone. Type I NKT cells maintain a conserved binding footprint centered over the F' pocket of CD1d, whereas the sulfatide-restricted Type II NKT cell clone XV19 binds over the A' pocket (29–33). Both TCRs contact their antigens from the side with only one chain, TCRα for Type I and TCRβ for Type II NKT cells. There is no overlap in the mCD1d-binding footprint between Type I NKT cells and the XV19 Type II NKT cell clone. The anti-mCD1d antibody 1B1 is able to block both TCR-mediated activation of Type I and Type II NKT cells, suggesting it has an overlapping binding site with the TCRs from both NKT cell subsets. However, because the glycolipid is presented in the center of the CD1d-binding groove between both TCR-binding sites this raises the questions as to how 1B1 is able to bind mCD1d and compete with both TCRs, whereas not being impacted by the presented glycolipid.

To understand how 1B1 binds to mCD1d and blocks both Type I and Type II NKT cell activation, we assessed the binding of 1B1 to CD1d presenting different glycolipids using surface plasmon resonance studies. We have further assessed the ability of 1B1 Fab and 1B1 IgG in blocking Type I and Type II NKT cell responses toward different lipid antigens and determined the crystal structure of 1B1 Fab bound to mCD1d at a resolution of 2.45 Å.

Results

Generation of 1B1 Fab and mCD1d for structural studies

We have generated 1B1 Fab by papain digestions and formed complexes with insect cell-expressed mCD1d that was either loaded with αGalCer or not incubated with a particular lipid. Whereas we were able to obtain crystals of the complex, we were not able to obtain a diffraction dataset beyond 3.9 Å. Therefore, we have generated 1B1 Fab recombinantly in SF9 insect cells, to limit heterogeneity introduced during the papain digestion. We have further generated mCD1d via refolding to avoid the presence of N-linked glycans. Because mCD1d contains an unpaired cysteine at the bottom of the A' pocket (Cys-12), we first replaced this residue against serine (mCD1d C12S). We formed complexes of all possible combinations of the 4 proteins (2 Fab and 2 mCD1d) and finally obtained high quality diffracting crystals for the complex consisting of refolded mCD1d and the insect cell expressed 1B1 Fab.

1B1 Fab-mCD1d crystal structure

To determine the structural basis of how 1B1 blocks NKT cell activation, we have determined the crystal structure of the 1B1/Fab-mCD1d complex to 2.45 Å resolution (Table 1, Fig. 1). CD1d forms the typical heterodimeric arrangement in which the CD1d heavy chain noncovalently associates with β2-microglobulin. However, because CD1d has been refolded, no N-linked glycans are present in the structure. In addition, during refolding, three spacer molecules have been incorporated into CD1d to stabilize the hydrophobic pocket. Two molecules are incorporated into the larger A' pocket and one into the F' pocket of CD1d (Fig. 1). Based on the extent of the observed electron density, we modeled three C16 alkyl-chains, which mimic the position of the alkyl-chains generally associated with glycolipid antigens (Fig. 2).

The 1B1 Fab binds perpendicular across the lipid-binding groove of mCD1d, centered above the F' pocket. The majority of contacts are formed between the H chain and CD1d. The H chain uses 575 Å² and the L chain 45 Å² of protein surface and together they contact 610 Å² of CD1d (Table 2). A single contact is formed between CDR L1 residue Tyr-31 and CD1d Glu-83, whereas the H chain binding is dominated by hydrophobic contacts (Table 3). Only H2 and H3 participate in CD1d binding and together they form three hydrogen bonds and two salt bridges. H2:Asp55 forms both a salt bridge and H bond with Lys-148 of CD1d, whereas H3:Arg-109 forms a salt bridge with Asp-80 of CD1d, H3:Gly-105 forms a H bond with Asp-153.
and Leu-106 forms a H bond with Asp-80. A characteristic feature of the interaction between 1B1 Fab and CD1d is an elongated H3 loop that inserts into the F’ pocket of CD1d like a hydrophobic finger and forms many hydrophobic contacts with surrounding CD1d residues (Figs. 1 and 2, Table 3). The H3 loop has two tyrosine residues (Tyr-103 and Tyr-104) that extend outward and slightly upwards from this finger and together appear to dictate how far the loop inserts into the binding groove of CD1d (Fig. 2B). Surprisingly, the H3 loop is also in close contact with one of the spacer molecules that mim-
ics the position of the sphingosine chain of glycolipids, such as sulfatide or αGalCer (Fig. 2). H3:Tyr-104, Gly-105, and Leu-106 each form one VDW contact with the spacer lipid, but the overall intimate binding position of H3 leads to a combined total buried surface interface of 212 Å².

### 1B1 Fab binding kinetics

When we superimpose the 1B1-mCD1d complex with that of mCD1d/sulfatide (PDB ID 2AKR [20]), we notice that the H3 loop of 1B1 is very close to sulfatide, especially to the C4’-OH of the galactose (Fig. 2B). Therefore, we wanted to test the impact of 1B1 binding to CD1d that presents glycolipids with different sizes of headgroups. We chose sulfatide and GT1b as model antigens, because they are negatively charged and their loading efficiency can be visualized. Because 1B1 binds to CD1d without presenting a given lipid, we needed to achieve a near to 100% loading efficiency to avoid having an overlapping binding response contributed by unloaded CD1d molecules. After glycolipid loading, both sulfatide and GT1b bind 100% to CD1d (Fig. 2C) and we used these complexes for surface plasmon resonance studies with the 1B1 Fab (Fig. 3D). However, the binding affinity of 1B1 to mCD1d is nearly identical in all three analyzed CD1d-lipid complexes, suggesting that variations in the glycolipid headgroups can be accommodated by 1B1 and do not affect its binding affinity toward CD1d. 1B1 Fab binds with a $K_D$ of $\sim12.5$ nM calculated from an association rate ($K_{on}$) of 5.4–$6.1 \times 10^{5}$ and a dissociation rate ($K_{off}$) of 6.2–7.3 $\times 10^{-3}$ (Fig. 2C).

### Comparison between 1B1 and the TCR of iNKT cells

When we compared the binding site of 1B1 with that of the Vα14Vβ8.2 TCR of iNKT cells, we noticed a similar footprint on CD1d (Figs. 1E, 3, and 4). Although 1B1 binds perpendicularly across the F′ pocket of CD1d, the TCR binds parallel along both α-helices (Fig. 1E). In addition, whereas TCR binding seems to be dominated by the CDR3α region, 1B1 uses its elongated H3 loop to form the majority of contacts. In both molecules, a hydrophobic finger, Leu-99α of the TCR and Leu-106h of the antibody, binds at the F′ pocket of CD1d. In striking contrast, however, 1B1 inserts its hydrophobic finger much deeper into the F′ pocket, compared with the TCR (Fig. 3A). That results in a different binding mode. Although the TCR sits above the F′ pocket and binds to the F’ roof, the antibody prevents F’ roof formation and instead sits as a wedge inside the F’ pocket (Fig. 2, B–D). In fact, CDR3α of the TCR flattens out at the tip and hovers above CD1d, whereas the H3 loop is straight and reaches into the CD1d binding groove (Fig. 3, C and D). The deep location of the H3 loop raises the question whether the nature of the lipid chain that is bound inside the F′ pocket can impact 1B1 binding. Although common dual alkyl-chain–based lipids, such as αGalCer are nestled underneath Leu-106h of 1B1, lipids containing modifications could potentially impact 1B1 binding. We chose a modified lipid αGSA [26, P5p], and superimposed its crystal structure (PDB ID 6C6F) with the CD1d/1B1 structure. Leu-106h is very close to the phenyl group contained in the modified sphingamide chain (2.4 Å).

### 1B1 blocking of Type I and Type II NKT cell activation

We then analyzed the ability of 1B1 (both Fab and IgG) to block NKT cell activation by various glycolipids. We loaded antigens for both Type I and Type II NKT cells into mCD1d and performed a NKT cell hybridoma activation assay using the NKT hybridomas 1.2 (Type I) and XV19 (Type II) in the presence or absence of the TCR blocking antibody 1B1 (Fig. 4). A 1B1 dose titration demonstrated that the activation of both Type I and Type II NKT cells by αGalCer and sulfatide can be fully blocked with both 1B1 Fab and 1B1 IgG. 1B1 Fab appears to block Type I NKT cell activation at a lower dose (15.6 μg/ml) compared with Type II NKT cells (100 μg/ml), whereas 1B1 IgG blocks both hybridoma activation at the lowest dose used in
this assay (1.5 μg/ml, equivalent to the equimolar ratio with CD1d). Using a structure-guided approach, we generated the 1B1 Fab Y103K/I56S double mutant, which does not bind mouse or human CD1d (Fig. 2). The 1B1 mutant is unable to block NKT cell activation as expected (Fig. 4). However, using the modified sphingamide antigen αGSA[26,P5p], we noticed that 1B1 Fab is unable to block NKT cell activation, suggesting that the 1B1 binding affinity to mCD1d is greatly reduced when αGSA[26,P5p] is bound, due to steric clashes with the elongated H3 loop and the phenyl moiety of the ligand. Because we could not load αGSA[26,P5p] to mCD1d at 100%, we were unable to precisely measure the 1B1-binding kinetics via SPR. Our data suggests the reduced binding affinity drops below a threshold necessary for blocking NKT cell responses. This is evident when using 1B1 IgG, which in its natural bivalent form has an increased binding avidity. 1B1 IgG is fully able to block NKT cell activation by αGSA[26,P5p]. This demonstrated that whereas common NKT cell antigens can be blocked by 1B1, one must be mindful when using 1B1 to block NKT activation of structurally diverse glycolipids, such as cholesterol antigens or hydrophobic peptides, which do not follow the dual alkyl-chain rule.

Discussion

In this study we have determined the crystal structure of the 1B1 Fab bound to mouse CD1d. 1B1 binds to mCD1d in a perpendicular binding orientation across the F’ pocket, directly overlapping with the binding site of the TCR of iNKT cells but with minimal overlap with the binding site of the Type II NK TCR clone XV19 that binds above the A’ pocket of CD1d (31, 34–37). Nevertheless, 1B1 blocks TCR-mediated activation of both Type I and Type II NKT cells, as 1B1 overlaps with XV19 TCR in the central region between the A’ and F’ pocket. 1B1 is more potent in inhibiting Type II NKT cell responses correlating with a greatly reduced binding affinity of the XV19 TCR compared with the αGSA[26,P5p] complex of Type I NKT cells. The XV19 TCR binds with micromolar affinity (K_D = 6–24 μM) to CD1d-sulfatide, whereas the αGSA[26,P5p] TCR binds with a K_D of 10–30 nM to CD1d-αGalCer complexes (28, 30, 32, 33). The characteristic feature of 1B1 is its long H3 loop that inserts into the F’ pocket of CD1d to prevent the closure of the F’ roof. The F’ roof is a hallmark and a major binding site for the CDR3α region of the αGSA[26,P5p] TCR of Type I NKT cells. Because the H3 loop of 1B1 is deeply inserted into the CD1d groove, one side of the loop runs alongside the alkyl-chain of a CD1d-bound glycolipid. As alkyl-chains generally do not differ greatly in structure for the various glycolipids, 1B1 binding is not affected by the nature of the presented lipid. However, when considering other ligands, such as the hydrophobic peptide p99 or a cholesterol antigen, 1B1 binding may be greatly impaired or even abrogated (38, 39). We showed the influence of lipid modifications in 1B1 binding using the α-galactosylsphingamidine antigen αGSA[26,P5p]. The 1B1 Fab is unable to prevent Type I NKT cell activation using this antigen, suggesting that the binding affinity of 1B1 to CD1d presenting αGSA[26,P5p] is reduced below a threshold necessary for competing with TCR binding. However, when the intact IgG is used, 1B1 is able to block activation, yet slightly less efficiently. Together these data suggest that whereas 1B1 is a potent blocker of TCR-mediated T-cell activation for both Type I and Type II NKT cells, its binding strength to CD1d can be affected
by the antigen that is presented by CD1d. This could result in a scenario where the activity of an antigen for NKT cells cannot be blocked by 1B1, leading to the incorrect assumption that the antigen activates NKT cells through a mechanism that does not involve TCR binding.

**Experimental procedures**

**Glycolipid Ags**

Bovine brain sulfatides and GT1b were purchased from Avanti Polar Lipids Inc. C16 αGalCer, α-GSA[26,P5p], and C24:1-sulfatide were synthesized as previously reported (40–42).

**Cell line and cell culture**

The hybridoma cell line expressing the rat anti-mouse IgG2b mAb 1B1 was grown in RPMI 1640 medium (Invitrogen) supplemented with 10 mM HEPES, pH 7.5, 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 55 μM 2-mercaptoethanol, 20 μg/ml of gentamicin (Gibco), and 10% heat-inactivated fetal calf serum. The hybridoma was maintained in an incubator with a humidified atmosphere containing 5% CO₂ at 37 °C.

**Antibody production and purification**

The 1B1 hybridoma cells were gradually adapted to culture in protein-free hybridoma medium (PFHM-II, Gibco), supple-
mented as indicated above. Cells from two T175 tissue culture flasks were transferred into one 2-liter roller bottle, filled up to 1.5 liters with supplemented PFHM-II. Roller bottles were equilibrated with CO₂ by placing in the 37 °C + 5% CO₂ incubator with the lid loosened for ½-1 h, then grown with a closed lid at 37 °C while rolling for ~2 weeks or until the media turned yellow. Cells were spun down (1250 rpm for 6 min) and supernatant was filtered (0.22 μm) and concentrated to 300 ml using a tangential flow-through filtration unit (Millipore, Pellicon 2) while exchanging buffer to PBS. IgG was collected from the supernatant using affinity chromatography using a 5-ml HiTrap Protein G column (GE Healthcare). IgG was eluted from the column with 0.1 mM glycine, pH 3.0, whereas 0.7-ml fractions were collected in 1.5-ml test tubes containing 0.3 ml of 1 M Tris, pH 8.5, for neutralization. IgG containing fractions were pooled and buffer exchanged against PBS using centrifugal filtration devices (Amicon Ultra, Millipore). Final yield of purified 1B1 IgG was 12 mg/liter of culture.

**Cloning and sequencing of 1B1 VH and VL genes**

RNA isolation and sequencing was performed as reported previously for the αGalCer-specific mAb L363 (43), with some modifications including a poly-G tailing method (44). Briefly, total RNA was isolated from 5 × 10⁶ hybridoma cells using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. First-strand cDNA synthesis of 5’-RACE was performed according to the protocol for Clontech’s SMART-RACE using the Clontech cDNA amplification kit and the Invitrogen SuperScript II Reverse Transcriptase at 42 °C for 50 min in a 20-μl reaction volume containing: 500 ng of total RNA, 0.6 μM 5’-RACE CDS Primer A, 0.6 μM SMART II A oligonucleotide, 1× RT buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 5 mM MgCl₂, 0.01 M DTT, and 4 units of RNase out recombinant RNase inhibitor. The first strand cDNA was then poly-G-tailed using terminal deoxynucleotidyl transferase (15 units, Promega). The terminal deoxynucleotidyl transferase was then heat-inactivated at 70 °C for 10 min. The tailed cDNA was then amplified by PCR using a sense primer containing the poly-C sequence and a rat IgG-specific antisense primer (second strand reaction). For the PCR, 48 μl of PCR mix containing 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 1 μM ANCTAIL sense primer 5’-ctgcttgagctcttagaatgcgttgacag-tgtcgtgtagttcccccctccc-3’, 1 μM ratκC643-rev antisense primer 5’-aggatgtgctttagacaa-3’, and 1.5 units of high-fidelity TaqDNA polymerase were added to 2.5 μl of cDNA (from the first-strand reaction) as suggested (45). The cycling profile used for the PCR was: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 3 min. At the end of the 30 cycles, a 10-min incubation step at 72 °C was done to complete elongation. The resulting PCR products were cloned into the pGEM-T easy vector (Promega) for sequencing. The VJ and VDJ gene rearrangement was determined as IGKV9S1*01 F/IGKJ2–3*01 F (light chain), and IGHV5–25*01 F, IGHJ1*01 F, and IGHD1–6*01 F (heavy chain) using the IMGT server (46).

**Fab digestion and purification**

Purified 1B1 mAb (rat IgG2b) at 1 mg/ml in PBS was incubated with 1% (w/w) activated papain (Sigma number P3125) for 3 h at 37 °C in digestion buffer. Papain was activated by incubating 20 μl of papain with 100 μl of 10X papain buffer (1 M NaOAc, pH 5.5, 12 mM EDTA) and 100 μl of cysteine (12.2 mg/ml) for 15 min at 37 °C. The papain digestion was stopped by adding 20 μl iodoacetamide. The digestion mix was concentrated and adjusted with PBS for subsequent protein A purification to remove undigested IgG and Fc. The protein A flow-through containing Fab was dialyzed against 20 mM NaOAc, pH 5.5, overnight and purified to homogeneity by cation-exchange chromatography using MonoS (GE Healthcare).

**Mouse CD1d/β2m expression in insect cells**

The expression and purification methods of fully glycosylated mouse CD1d/β2m heterodimer proteins using the baculovirus expression system were reported previously (31, 34, 47).

**Gene cloning and site-directed mutagenesis**

cDNA cloning of mouse CD1d (mCD1d) was subcloned into the Escherichia coli expression vector pET-22b for subsequent expression in inclusion bodies. Single amino acid mutation C12S (TGC>TCC) was introduced into mCD1d using the QuickChange II site-directed mutagenesis kit (Stratagene) with primers C12S-forward (5’-GAATTACACTTCCGCTCCCT-GCAGATGTCTTCC-3’) and C12S-reverse (5’-GGAAGAC-TCTCGAGGAGGGAGGTGAATTCC-3’). Successful mutation was verified by sequencing. Mouse β2-microglobulin (β2M) without leader peptide was cloned into a separate pET-22b vector. Synthetic DNA for WT 1B1 Fab and the 1B1 Fab mutant Y103K/I56S, which is unable to bind to mCD1d, was synthesized by GenScript and subcloned into the dual promoter vector pBACP10 with a C-terminal His₅ tag on the heavy chain and expressed in SF9 insect cells using the baculovirus expression system. SF9 cells were removed from the cell culture media by centrifugation, and the media was further concentrated to 0.3 liter and washed twice with PBS buffer. The protein was purified from the concentrated media by affinity chromatography on a nickel-nitrilotriacetic acid resin (Takara Bio.) in 50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0, followed by size-exclusion chromatography (Superdex S200 16/60 GL; GE Healthcare) in 50 mM HEPES, pH 7.5, and 150 mM NaCl.

**Mouse CD1d refolding and purification**

mCD1d and β2M were separately expressed in E. coli and purified from inclusion bodies as reported for Qa-1a (48). The mCD1d-C12S/β2M complex was generated by refolding. First, 1 mg of mCD1d and 2.4 mg of mβ2M denatured inclusion bodies were loaded into two separate 1-ml syringes. Next, mCD1d and mβ2M were injected into 100 ml of high-speed stirred cold refolding buffer (400 mM l-arginine, 100 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM reduced GSH, 0.5 mM oxidized GSH, and 0.2 mM PMSF) through 26-gauge needles as close to the stirring bar as possible and incubated at 4 °C with medium-speed stirring. After 12 and 24 h, an additional 1 mg of mCD1d was added to

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Structure of the mouse CD1d/1B1-Fab complex

the refolding reaction and continued to incubate until 72 h under constant stirring. Finally, the refolding mixture was concentrated (Millipore Amicon 30K centrifugal filter) and purified by size exclusion chromatography on a Superdex S200 10/300 in 50 mM HEPES, pH 7.5, 150 mM NaCl. Refolded mCD1d-C9S/β2M complex was analyzed by SDS-PAGE.

CD1d/1B1-Fab complex formation and crystallization

Refolded or insect cell-expressed mCD1d was incubated with equimolar amounts of recombinantly expressed 1B1 Fab or 1B1 Fab obtained by papain digestion for 4 h at room temperature. The ternary mCD1d/1B1-Fab complex was isolated by size exclusion chromatography using a Superdex S200 10/300 in 50 mM HEPES, pH 7.5, 150 mM NaCl and further concentrated to 3–4 mg/ml in 10 mM HEPES, pH 7.5, 30 mM NaCl for subsequent crystallization. Crystals were obtained in various combinations of CD1d and 1B1 Fab.

Crystallization and structure determination

The initial crystals of recombinant 1B1 Fab and refolded mCD1d complexes were grown as small plates by mixing 200 nl of precipitant (100 mM MES, pH 6.5, 12% (w/v) PEG 20K) with 200 nl of protein solution at 22 °C using the sitting drop vapor diffusion method and the liquid handling robot Phoenix (Art Robbins Instruments). Other combinations such as insect cell mCD1d and papain-digested 1B1 Fab also yielded crystals but of limited diffraction power. The diffraction quality plate-like crystals were manually optimized and obtained in 80 mM MES, pH 6.5, 8–10% (w/v) PEG 20K. Crystals were flash-cooled at 100 K in mother liquor containing 22% glycerol. Diffraction data were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) beamline 9-2 and processed with HKL2000 (49). The 1B1 Fab/mCD1d crystal belong to space group C222, with unit cell parameters \( a = 84.05 \text{Å} \); \( b = 160.96 \text{Å} \); \( c = 165.42 \text{Å} \). The asymmetric unit contains one 1B1/mCD1d-Fab complex. The crystal structure was determined by molecular replacement using PHASER as part of the CCP4 suite (50, 51). Protein coordinates from the mCD1d-iGb3 structure (PDB code 2Q7Y) (52) and the L363 Fab (PDB code 3UBX) (43) were used as the search models for mCD1d and 1B1 Fab, respectively. The phased model was rebuilt into \( \sigma_\text{A-weighted} 2F_\text{o} - F_\text{c} \) and \( F_\text{o} - F_\text{c} \) difference electron density maps using the program COOT (53) and refined in REFMAC5 (54). The complex structure was refined to 2.45 Å to an Rcryst and Rfree of 21.0 and 24.7%, respectively. The quality of the model was examined with the program Molprobity (55). Data collection and refinement statistics are presented in Table 1.

Glycolipid loading and native isoelectric focusing (IEF) gel electrophoresis

For SPR or native IEF, 10 μl (~5 μg) of insect cell-expressed mouse CD1d was loaded overnight with 6–10 times molar excess of C24:1 sulfatide, bovine brain sulfatides, or GT1b (dissolved at 5–10 mg/ml in DMSO) in 100 mM HEPES, pH 7.5, 150 mM NaCl without the addition of any detergents. Loading efficiency was analyzed via native IEF gel electrophoresis. For each protein-lipid complex, 4 μl of solution were loaded onto a 6-well pH 5–8 native IEF gel and analyzed via the PhastSystem (GE Healthcare).

Surface plasmon resonance (SPR) studies

The real-time binding kinetics between 1B1 Fab and the CD1d-glycolipid complexes was analyzed by SPR using a Biacore T200 (Biacore, GE Healthcare) instrument. Briefly, insect cell-expressed mCD1d protein containing a C-terminal birA-tag (LHHILDAQKVNWHR) was enzymatically biotinylated and purified according to established methods (31, 47). Biotinylated mCD1d was loaded with glycolipids overnight as reported above, and ~400 response units of the individual mCD1d-glycolipid complexes were immobilized on a CAP sensor chip (GE Healthcare). A series of increasing concentrations (3-fold dilutions from 1.1 to 90 nM) of 1B1 Fab were passed over the immobilized CD1d-glycolipid complexes at 25 °C with a flow rate of 30 μl/min in a single cycle kinetic experiment. For background subtraction, flow channel 1 was similarly prepared but no protein was immobilized and the binding response of 1B1 Fab to flow channel 1 was subtracted from the other flow channel. The experiments were performed at least twice and kinetic parameters were calculated using a simple Langmuir 1:1 model in the BIA evaluation software.

Cell-free antigen-presenting assay

APC-free antigen-presenting assay for stimulation of mouse iNKT cell hybridoma by soluble insect cell-expressed mCD1d was carried out following published protocols (56, 57). 96-Well-plates were coated with 1 μg of CD1d and incubated overnight at room temperature with 100 ng of glycolipids (1 μg/ml) in triplicates. Buffer was removed and the indicated concentrations of 1B1 Fab (0–100 μg) or 1B1 IgG (0–150 μg) in 50 μl or culture media was added for 2 h at 37 °C. Hybridoma cells (5 × 10⁴ of either Type I NKT 1.2 or Type II NKT XV19) in 50 μl of culture media were added to each well and co-incubated with CD1d-glycolipid complexes overnight at 37 °C in a CO₂ incubator. IL-2 release in the APC-free antigen-presenting assay was measured after 16 h of culture in a sandwich ELISA according to standard protocols to assess the extent to which 1B1 blocked both Type I and Type II NKT cell activation. As a negative control, the 1B1 Fab double mutant Y103K/I56S that lost mouse CD1d binding was generated.

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