Loading of the Antigen-Presenting Protein CD1d with Synthetic Glycolipids

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*CD1 proteins present mammalian and microbial lipid and glycolipid antigens to different subsets of T cells. Few such antigens have been identified and the binding of these to CD1 molecules has mainly been studied by using responding T cells in cellular assays or recombinant solid-phase CD1 proteins. In the present study we use four different glycolipids, some of which contain tumor-associated carbohydrate antigens, to develop a procedure to easily detect binding of glycolipids to CD1 proteins on viable cells. Two of these glycolipids are novel glycoconjugates containing α-D-N-acetylgalactosamine (α-GalNAc) that were prepared by a combined solution and solid-phase approach. The key step, a Fischer glycosylation of 9-fluorenylmethoxycarbonylaminoethanol with GalNAc, furnished the α-glycoside **4** in 34% yield. Cells were incubated with glycolipids and stained with monoclonal antibodies specific for the carbohydrate part. The level of glycolipid bound to cells was then determined by flow cytometry with a secondary antibody labeled with fluorescein isothiocyanate. All four glycolipids were found to bind to CD1d but with different selectivity. The loading was dose dependent and could be inhibited by an established CD1d ligand, α-galactosylceramide. Through use of this procedure, glycolipids were selectively loaded onto CD1d expressed on professional antigen-presenting cells for future use as cellular vaccines. Moreover, the glycolipids described in this study represent novel CD1d-binding ligands that will be useful derivatives in the study of CD1d-dependent immune responses, for example, against tumors.*

Introduction

Presentation of peptide antigens by major histocompatibility complex (MHC) molecules and subsequent T cell activation are fundamental and well-studied mechanisms of our immune system.[1] Recent findings have made it clear that T cells also recognize and respond to lipids and glycolipids in a specific manner.[2,3] These nonpeptide antigens are presented by monomorphic CD1 molecules, an enigmatic class of antigen-presenting proteins that provide a unique solution to the challenge of displaying lipid antigens.[2,3] In humans there are two groups of CD1 proteins, according to amino acid sequence homology. Group 1 consists of CD1a–c and group 2 of CD1d.[2,3] CD1e is intermediate between the group 1 and 2 CD1 proteins in terms of its homology and has not been studied extensively.[2] In mice, no homologues to group 1 CD1 molecules have been found but CD1d proteins are expressed.[2,3]

Although few lipid and glycolipid antigens have been identified, it is clear that CD1 proteins have the capacity to present both mammalian and microbial antigens, with resulting different types of T cell responses.[2,3] Group 1 CD1 proteins present mycobacterial lipids and glycolipids for the generation of diverse, CD1-restricted T cell responses. Among these antigens, structurally defined glycophospholipids[4,5] and mycolic acids and their glucose derivatives[6] have been identified. Some mammalian glycolipids like gangliosides[7] and sulfatide[8] have also been identified as group 1 CD1-presented antigens. For group 2 CD1 proteins, that is, CD1d, an α-galactosylated ceramide from a marine sponge is the best-studied antigen.[9] Injection of α-galactosylceramide (α-GalCer) into mice activates a special population of NKT cells that express an invariant set of T cell receptors.[9] This response generates a cascade of cytokines, including IFN-γ, which can completely eradicate some tumors.[10] However, it is not clear what antigens CD1d normally presents.

The crystal structures of mouse CD1d[11] and human CD1b[12] provide information on how CD1 proteins present lipid and glycolipid antigens. The mCD1d structure shows a narrow hydrophobic groove with two deep pockets lined almost entirely with nonpolar or hydrophobic amino acid side chains. This, together with the ligands found to bind CD1d and CD1b, indicates a binding mode where a double-chained lipid inserts its alkyl tails into the two pockets, while leaving the polar head exposed for T cell recognition. The crystal structures of the human CD1b protein in complex with glycolipids demonstrate more flexibility in the binding mode with the possibility for a third alkyl chain or accommodation of very long alkyl chains.

Although some studies have shed light on the CD1-restricted immune response, the limited number of known immunologically active lipids and glycolipids restricts the possibilities to fully explore the antigenic repertoire of CD1 and establish the range of CD1-restricted immune responses.[2,3] Furthermore, with the exception of α-galactosylceramide,[13] the po-

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 DOI: 10.1002/cbic.200300655

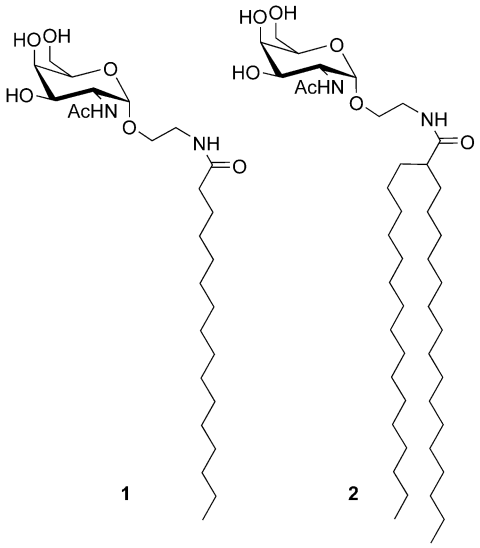
tential for immune intervention with CD1-binding lipids and glycolipids has not been explored. So far, most of the assays for measuring ligand binding to CD1 proteins have been T cell dependent, a method that makes it difficult to distinguish specific binding to the CD1 molecule from unspecific cellular binding based on hydrophobic interactions. Moreover, the failure of an antigen to activate T cells cannot be attributed to loss of critical interactions with the T cell receptor since reduced or abolished CD1 binding can be the underlying reason. Direct binding of lipids and glycolipids to soluble or immobilized CD1 molecules has been investigated in the absence of T cells by using surface plasmon resonance analysis. This method offers an alternative way of measuring CD1 binding in a cell-free environment.[14, 15]

In this paper we describe a combined solution and solid-phase synthesis of lipids carrying the immunologically relevant saccharide α-D-*N*-acetylgalactosamine (α-GalNAc), with a revised Fischer glycosylation as a key step in the synthesis. The resulting α-GalNAc glycolipids and two previously described lipids bearing tumor-associated carbohydrate antigens have been used in the detection of binding to CD1d on viable cells. The method relies on carbohydrate-specific monoclonal antibodies for the detection of cell-bound glycolipids by flow cytometry. In addition, new CD1d-binding structures were identified by using this strategy.

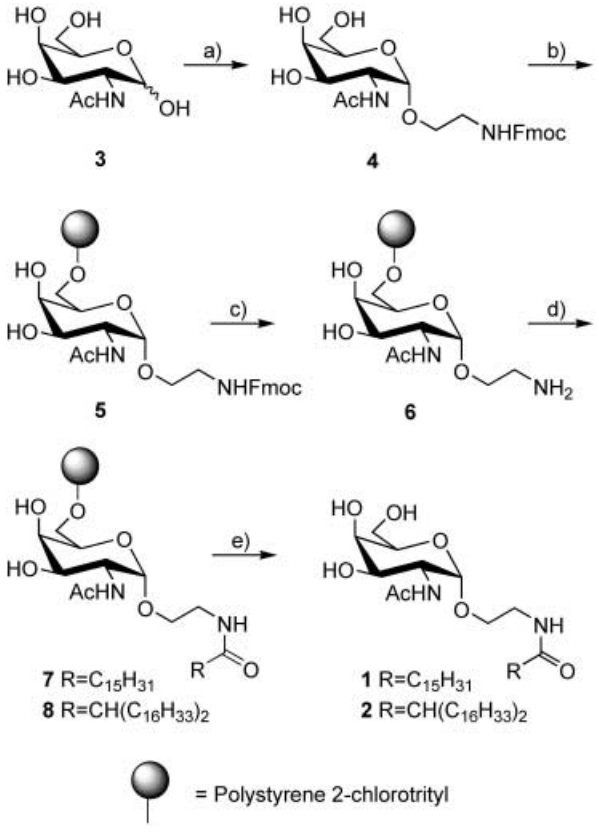
Results and Discussion

Synthesis of glycolipids

In order to study the binding of glycolipids to living cells that display a wide variety of carbohydrate epitopes, glycolipids with carbohydrate motifs not existing on normal cells were needed. Preferably, such carbohydrate motifs should be immunologically relevant and structural modifications through chemical synthesis should be straightforward. GalNAc is a common carbohydrate building block present in numerous glycoconjugates. However, with the exception of the determinant for blood group A, elevated levels of terminal α-GalNAc monosaccharides are associated with malignancies, predominantly cancer, where this epitope is present both in glycolipid and glycoprotein form.[16, 17] Single α-GalNAc units attached to Ser or Thr constitute the smallest tumor-associated carbohydrate antigens identified on mammalian cells.[17] Moreover, the monosaccharide is commercially available and the chemistry of this important carbohydrate is well studied,[18] thus making this epitope a suitable candidate for this project. In order to make sure that the saccharide protrudes from the CD1 protein, aminoethanol was used as a spacer between the sugar and the lipid. Two commercially available fatty acids, 2-(*n*-hexadecyl)-stearic acid and palmitic acid, were then attached to the spacer through an amide bond to result in the two α-GalNAc glycolipids **1** and **2**. The 2-(*n*-hexadecyl)-stearic acid is an analogue of the mycolic acids,[19] a class of microbial lipids known to bind CD1 proteins.[6] The combined solution and solid-phase synthesis of the two α-GalNAc-containing lipids **1** and **2** is summarized in Scheme 1.



α-Aminoethylglycosides of GalNAc have previously been prepared through Fischer glycosylation with azidoethanol or chloroethanol.[20, 21] The sugar hydroxy groups are then protect-



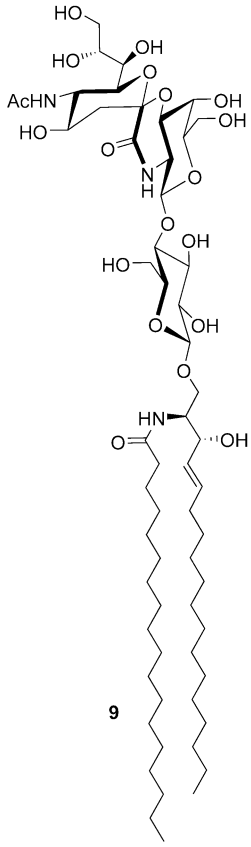
Scheme 1. *Combined solution and solid-phase synthesis of the single- and double-chain α-GalNAc glycolipids **1** and **2**. Reagents and conditions: a)* N-Fmoc-aminoethanol, boron trifluoride etherate, THF, reflux, 19 h; b) resin, DMAP, dichloromethane, reflux, 24 h; c) 20% piperidine in DMF; d) 2-(n-hexadecyl)-stearic acid or palmitic acid, DIC, HOAt; e) 5% TFA in CH_2Cl_2. Fmoc = 9-fluorenylmethoxycarbonyl, THF = tetrahydrofuran, DMAP = 4-dimethylaminopyridine, DIC = diisopropylcarbodiimide, HOAt = 1-hydroxy-7-azabenzotriazole, TFA = trifluoroacetic acid.

ed and in the case of chloroethanol the chlorine atom is substituted with an azide moiety. Finally the azide moiety is reduced to give the *O*-protected aminoethylglycoside. In a typical Fischer glycosylation the thermodynamically most stable glycoside is formed by acid-catalyzed acetal formation with the accepting alcohol as solvent.[22] This protocol is excellent for glycosylations of simple alcohols, while in the case of GalNAc more complex acceptors are generally glycosylated with a 2-deoxy-2-azido-galactose donor to give the corresponding α-glycoside.[18] Our aim was to use the Fischer glycosylation protocol with more complex and crystalline acceptors, for example, Fmoc-protected amino alcohols, in modest excess, by using a suitable aprotic solvent. We found that the reaction of five equivalents of the Fmoc-protected aminoethanol with GalNAc (**3**) in the presence of four equivalents of boron trifluoride etherate in refluxing THF for 19 h gave the desired α-glycoside **4** in 34% yield (Scheme 1). Thus, the spacer glycoside **4** can be assembled in one step from commercially available starting materials in a preparatively useful yield.

The first attempts to prepare glycolipids **1** and **2** were performed in solution. Removal of the Fmoc group from **4** was facile but the coupling of 2-(*n*-hexadecyl)-stearic acid to the primary amine turned out to be sluggish. The problems most likely originated from the large difference in polarity and hence solubility between the amine and the fatty acid. Our focus was then directed towards a combined solution and solid-phase approach (Scheme 1). The spacer glycoside **4** was attached to a polystyrene support functionalized with 2-chlorotrityl chloride by using DMAP in dichloromethane, to give resin **5** with a loading of 0.2 mmol g^{-1} according to Fmoc quantification. The linkage to the solid support most likely occurs through the primary 6-hydroxy group of the GalNAc moiety. Removal of the Fmoc group with piperidine in DMF gave the resin-bound amino glycoside **6**. Amine **6** was acylated with palmitic acid or 2-(*n*-hexadecyl)-stearic acid by using DIC and HOAt, to furnish the resin-bound glycolipids **7** and **8**. Using a large excess of the acids and monitoring the reaction with bromophenol blue ensured quantitative yields in the acylations. Cleavage of the glycoplipids with TFA in dichloromethane, followed by flash chromatography, gave glycolipid **1** in 75% and **2** in 66% yield, based on the resin-bound Fmoc-protected amine **5** (Scheme 1). In conclusion, we have developed a very short and straightforward combined solution and solid-phase method to prepare GalNAc-based glycolipids. The method can be used to prepare a wide variety of glycolipids with different saccharides, spacers, and lipids. In parallel to our study, a similar approach was employed in the preparation of glycolipid organo- and hydrogelators.[23]

Loading CD1d on living cells with glycolipids

The α-GalNAc-bearing lipids **1** and **2** were used in the development of a CD1-binding assay with living cells, together with two previously synthesized glycolipids. Glycolipid **9**[24] is a lactam derivative of the ganglioside GM3[17] and glycolipid



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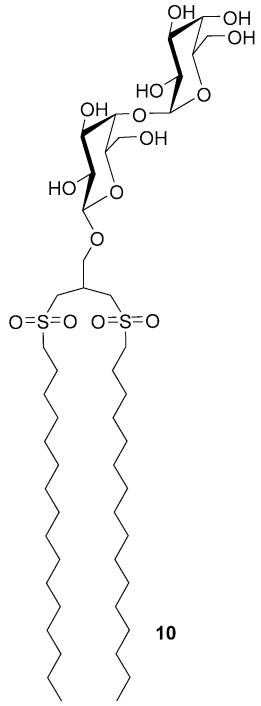

10

10[25] is constructed from the disaccharide galabiose, which is part of a tumor-associated antigen in Burkitt's lymphoma,[26] and a nonnatural bis-sulfone lipid. The CD1-restricted antigens that have been identified so far indicate that CD1 proteins can accept considerable variation in both the lipid and carbohydrate structure (see ref. [2]). However, only a small number of structurally defined antigens have been identified and comparative studies of the binding of different glycolipids to CD1 molecules on cells have not been carried out. Thus, our set of glycolipids addresses the impact of different lipid and carbohydrate structures on the binding to CD1 proteins as well as nonspecific binding or incorporation into the cell membrane. Cells were incubated with glycolipids in 5% dimethylsulfoxide (DMSO) buffer solution, washed, and incubated with monoclonal antibodies specific for the carbohydrate structures. Subsequently the presence of glycolipids on the cells was analyzed by flow cytometry by using a fluorescein isothiocyanate (FITC) labeled secondary antibody and fluorescence-activated cell sorting (FACS).

As a first step, a dose-response relationship in the binding of the galabiose glycolipid **10** to EL-4 cells was established (Figure 1). Loading of EL-4 cells was then examined for all four glycolipids, **1**, **2**, **9**, and **10** (Figure 2). To be able to estimate nonspecific binding and CD1-unrelated incorporation into the cell membrane, loading experiments were also carried out with EL-4 β_2-microglobulin-knockout cells. These cells lack all β_2-microglobulin-associated proteins including the CD1 molecules.

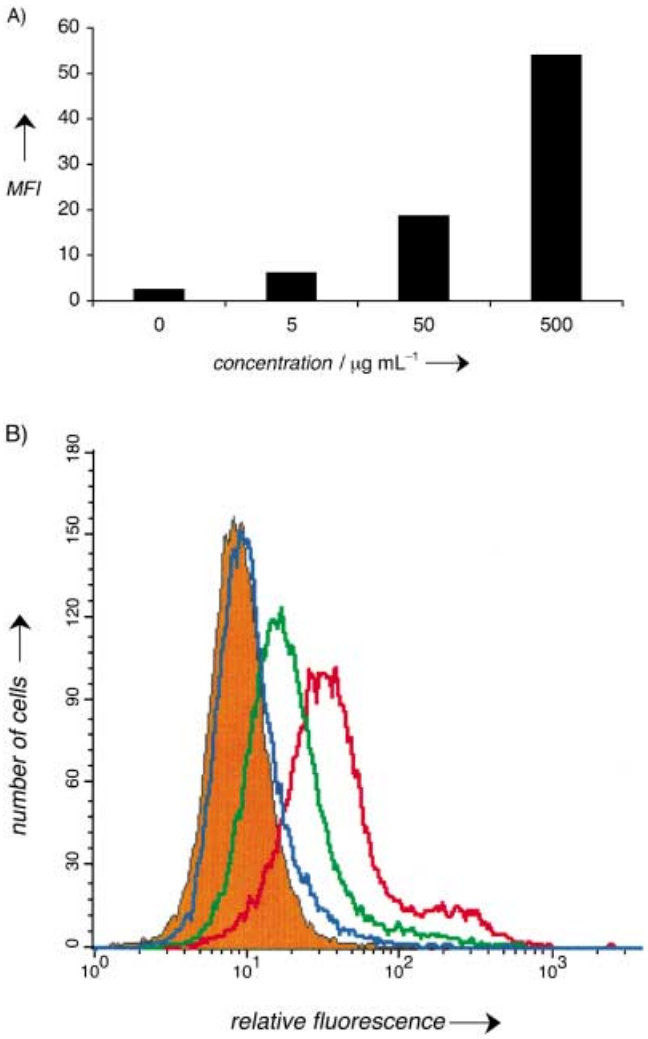

Figure 1. *Dose-dependent binding of glycolipid **10** to EL4 cells. EL4 cells were incubated with different doses of glycolipid **10** (5, 50, and 500 μg mL⁻¹) in a phosphate-buffered saline (PBS) buffer containing 5% DMSO at 26°C for 2 h, followed by washing. Cells were then stained with the mouse monoclonal antibody MC2102, which recognizes galabiose, and with a secondary FITC-labeled F(ab')₂ antimouse antibody, as described in the Experimental Section. A) The mean fluorescence intensity (MFI) was measured with flow cytometry by using FACS. B) FACS curves for different concentration of glycolipid **10** in PBS buffer with 5% DMSO. Filled orange: 0; blue: 5; green: 50; red: 500 μg mL⁻¹.*

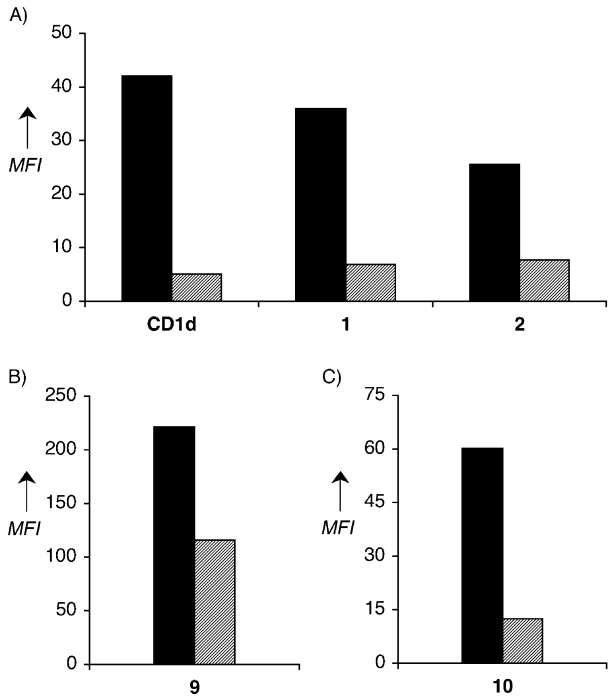

Figure 2. *Selective glycolipid loading of CD1d-positive EL4 cells. EL4 cells (black bars) and β₂-microglobulin −/− EL4 cells (gray bars) were incubated with A) glycolipid **1** and **2**, B) glycolipid **9**, and C) glycolipid **10** (50 μg mL⁻¹ of each) as described in the Experimental Section. The expression of CD1d was measured by using an anti-CD1d antibody (bars labeled CD1d in A). Cells were stained with the corresponding anticarbohydrate monoclonal antibodies after loading and the MFI was measured as described in the legend to Figure 1.*

Cells were also stained with anti-CD1d antibodies to verify the presence or absence of CD1d (Figure 2). All glycolipids resulted in significantly higher loading of wild-type EL-4 cells compared to EL-4 β_2-microglobulin-knockout cells (Figure 2), a result indicating that the loading is dependent on the CD1 proteins. In a control experiment, the α-(2-acetamidoethyl)glycoside of GalNAc, that is, an analogue to glycolipid **1** and **2** that carries an acetyl group instead of a fatty acid, failed to load EL-4 and EL-4 β_2-microglobulin-knockout cells (data not shown). Thus, the lipid parts of **1** and **2** are required for CD1d-dependent loading of the cells. Interestingly, the highest selectivity for CD1-dependent loading was obtained with the single-chain glycolipid **1.** Investigations of CD1d presentation of α-GalCer derivatives and subsequent activation of NKT cells have also shown that a single-chain glycolipid is capable of binding CD1d and that the complex could activate NKT cells.[27] CD1c-restricted T cell activation by single-chain isoprenoid glycolipids from *Mycobacterium tuberculosis* was recently described.[4] However, a glucose monomycolate analogue that possesses a single acyl chain did not bind to CD1b in an experiment that used surface plasmon resonance analysis.[14] The ceramide-based glycolipid **9** gave a relatively high unspecific binding, presumably due to the ability of this lipid, which naturally occurs on mammalian cells, to bind directly to the cell membrane. CD1b-restricted recognition of endogenous gangliosides, that is, glycosylceramides containing sialic acid, has been reported.[7] The carbohydrate-specific antibodies did not cross-react with any epitopes present on the EL-4 cells (data not shown). Importantly, these experiments also indicate that the carbohydrate epitopes are exposed and accessible to the monoclonal antibodies. The complex between the galabiose lipid **10** and CD1d is stable, since overnight incubation of the washed cells in buffer at 37°C did not significantly affect the FACS result (data not shown). To further establish the CD1 selectivity, blocking experiments with the known CD1d ligand α-GalCer[9] were performed. Cells preincubated with α-GalCer in a suitable vehicle showed a lower loading of the glycolipid **2** and the inhibitory effect proved to be dose dependent (Figure 3). The vehicle alone did not inhibit the loading of glycolipid **2** (data not shown). These results indicate that glycolipid **2** binds to CD1d in the same manner as α-GalCer, that is, by specific interactions with the CD1d protein.

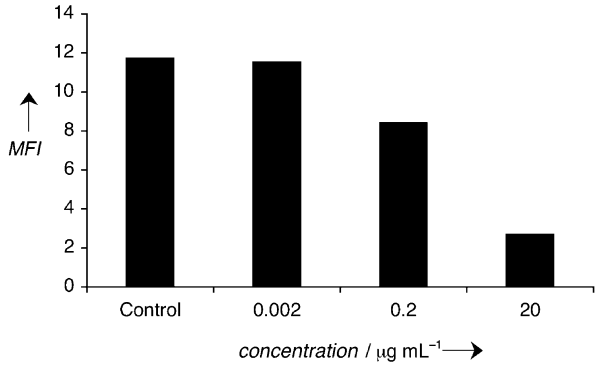

Figure 3. *α-GalCer competition for CD1d presentation of glycolipid **2**. EL4 cells were first incubated with different doses of α-GalCer, then washed and further incubated with glycolipid **2** (10 μg mL^{-1}). Treated cells were washed and stained for α-GalNAc expression as described in the legend to Figure 1.*

The synthetic glycolipids employed in this study all have the potential to be used for immune intervention since they carry immunologically relevant carbohydrate antigens. It has been observed that strong immune responses can be obtained if dendritic cells (DCs) preloaded with glycolipid antigen are injected into mice.[28] DCs are highly specialized antigen-presenting molecules that constitute a corner stone in our immune system.[29] DCs from B6 and B6-CD1d-knockout mice were loaded with glycolipid **2** as described for the EL-4 cells. Again, highly selective binding was observed in the wild-type cells in contrast to the results with the knockout cells (Figure 4). Importantly, this experiment rules out glycolipid binding to other β_2-microglobulin-associated proteins than CD1d, since these DCs originate from CD1d-knockout mice.

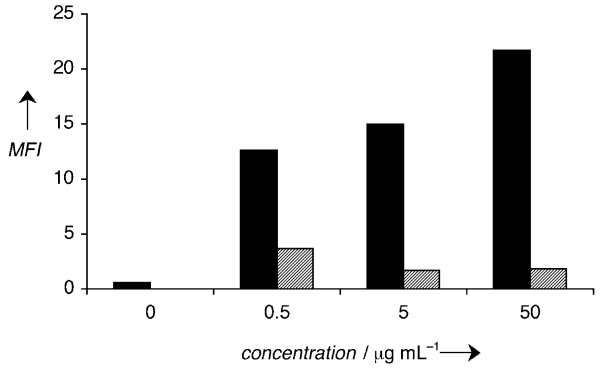

Figure 4. *Dose-response data for glycolipid **2** binding to dendritic cells. Immature dendritic cells from B6 (black bars) and CD1d −/− B6 (gray bars) mice were incubated with different doses of glycolipid **2**. This was followed by washing and staining for α-GalNAc expression as described in the legend to Figure 1.*

The CD1 proteins clearly have a role in innate and adaptive immune responses but the true nature of this role is presently not clear. A number of mammalian and microbial antigens have been found to bind to class 1 CD1 proteins and to activate classical CD1-restricted and diverse T cell responses, but for CD1d no natural lipid or glycolipid antigen has been identified. Even though α-GalCer has aided the study of the role of CD1d in the generation of an immediate, innate type of immune response, mediated by invariant NKT cells, it remains unclear which glycolipid antigens CD1d normally presents. As tumor-associated carbohydrate antigens can appear as a consequence of the truncation of carbohydrate chains in glycolipids, such structures could be potential endogenous ligands for CD1d and important for the immune surveillance against cancer. Furthermore, the possibility for cross-talk between the innate and adaptive immune systems and activation of one of the systems by MHC- or CD1-restricted T cell recognition is poorly understood. Interestingly, it was found that immunization of mice with a class I MHC-binding peptide carrying the galabiose antigen (see **10**) generated T cells, of which the γδ population could lyse cells loaded with glycolipid **10** in an MHC-unrestricted manner.[30] A strategy based on immunizations with tumor-associated carbohydrate antigens bound to different carrier molecules, such as MHC-binding peptides, CD1-binding lipids, or proteins activating helper T cells, might thus allow the selective activation of different effector mechanisms in the immune system with distinct antitumor effects.

Conclusion

A short and straightforward combined solution and solid-phase synthesis of α-GalNAc-bearing glycolipids was developed and single- and double-chain α-GalNAc lipids were prepared. The protocol allows incorporation of different saccharides, spacers, and alkyl chains and can easily be adopted for parallel synthesis of glycolipid libraries. The capacity of these and two other lipids carrying carbohydrate tumor-associated antigens to bind selectively to CD1d on viable cells was tested with a new rapid procedure that uses monoclonal antibodies against the carbohydrate epitopes to measure cell binding of the glycolipids by flow cytometry. All tested glycolipids showed CD1d-selective binding on the EL-4 cell line as well as on professional antigen-presenting cells, DCs. The fact that DCs can be selectively loaded with glycolipids in a controlled manner is promising, since injection of α-GalCer-treated DCs has been shown to be more effective in generating an immune response than direct injection of the glycolipid. In contrast to methods relying on T cell activation, the use of monoclonal antibodies allows detection of CD1-specific, as well as -unspecific, binding. However, the two different procedures are complementary, as the present method detects binding to CD1 proteins and the assay of T cell activation establishes the immunogenicity of the particular glycolipid. Moreover, the lipid parts of glycoconjugates **1**, **2**, and **10** represent novel lipid structures capable of binding CD1d. These lipids can be used to construct CD1-binding molecules, a step towards the ultimate goal of being able to modulate CD1-restricted immune responses in vivo. The immunogenicity of conjugates constructed with these lipids and which type of T cell response they might generate remains to be established.

Experimental Section

General chemistry: Dichloromethane was distilled from calcium hydride and THF from potassium. Solvent mixtures are reported as *v/v* ratios. TLC was run on silica gel 60 F_{254} (Merck) and the spots were detected in UV light and stained with 10% aq. H_2SO_4 and heat. Silica gel (Matrex, 60 Å, 35–70 μm, Grace Amicon) and solvents of analytical grade were used for flash column chromatography. The NMR spectra were recorded on a Bruker DRX-400 spectrometer with [D_6]DMSO or [D_5]pyridine as solvents; residual DMSO ($\delta_H = 2.50$ ppm; $\delta_C = 39.51$ ppm) and pyridine ($\delta_H = 8.74$ ppm; $\delta_C = 150.35$ ppm) were used as the internal standards. Chemical shifts and coupling constants were determined from one-dimensional spectra; assignments were made by using COSY and HETCOR experiments as well as the one-dimensional spectra. Positive fast-atom-bombardment mass spectra were recorded on a Jeol SX 102 A mass spectrometer. Ions were produced by a beam of Xenon atoms (6 keV).

***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-(*N*-acetyl-2-amino-2-deoxy-α-D-galactopyranosyl)-2-aminoethanol (4)**: Boron trifluoride diethyl ether complex (0.11 mL, 0.88 mmol) was added to a mixture of *N*-acetyl-2-amino-2-deoxygalactose **3** (50 mg, 0.22 mmol) and *N*-Fmoc-aminoethanol (339 mg, 1.19 mmol) in THF (2 mL). The resulting solution was heated under reflux conditions for 19 h, the mixture was allowed to cool to room temperature, the solvents were evaporated, and the residue was concentrated in vacuo. After chromatography (SiO_2, dichloromethane/MeOH (10:1→5:1) and toluene/MeOH (3:1)), **4** (36.8 mg, 34% yield) was obtained: ^{1}H NMR ([D_6]DMSO): $\delta = 7.89$ (d, 2H, $J = 7.5$ Hz; Fmoc), 7.68 (d, 2H, $J = 7.4$ Hz; Fmoc), 7.53 (d, 1H, $J = 8.9$ Hz; NHAc), 7.41 (t, 2H, $J = 7.4$ Hz; Fmoc), 7.37–7.28 (m, 3H; Fmoc, NHFmoc), 4.61 (d, 1H, $J = 3.5$ Hz; H-1), 4.60–4.47 (m, 2H; OH-6 OH-4), 4.41 (brs, 1H; OH-3), 4.37–4.26 (m, 2H; Fmoc), 4.26–4.17 (m, 1H; Fmoc), 4.12–4.02 (m, 1H; H-2), 3.72 (brs, 1H; H-4), 3.67–3.42 (m, 5H; H-3, H-5, H-6, H-6′, OC*H*HCH$_2$N), 3.28–3.04 (m, 3H; OCH*HC*H$_2$N), 1.83 (s, 3H; COCH$_3$) ppm; ^{13}C NMR ([D_6]DMSO): $\delta = 169.6$, 156.2, 143.9, 143.8, 140.7, 127.6, 127.0, 125.1, 120.1, 97.8, 71.4, 68.2, 67.8, 66.6, 65.4, 60.7, 49.5, 46.7, 22.7 ppm; HRMS (FAB): *m/z* calcd for $C_{25}H_{30}N_2NaO_8$: 509.1900 $[M+Na]^+$; found: 509.1913.

Resin 5: The spacer glycoside **4** (48 mg, 0.099 mmol), polystyrene functionalized with 2-chlorotrityl chloride (1% cross-linked poly(styrene-co-divinylbenzene), 75–150 μm, 100–200 mesh, 204 mg, 0.20 mmol), and 4-dimethylaminopyridine (14 mg, 0.12 mmol) were dissolved/swelled in dichloromethane (3 mL). The mixture was stirred slowly under reflux conditions for 24 h. MeOH (2 mL) was added and the resin was filtered and washed with dichloromethane/MeOH (1:1, 3×4 mL). After drying under a high vacuum, resin **5** (221 mg) was obtained with a loading of 0.22 mmol g^{-1} according to Fmoc determination.[31]

***N*-palmitoyl-*O*-(*N*-acetyl-2-amino-2-deoxy-α-D-galactopyranosyl)-2-aminoethanol (1)**: Piperidine (20% in DMF, 2 mL) was added to resin **5** (95 mg, 0.021 mmol) and the mixture was shaken at room temperature for 10 min. The resin was filtered, piperidine (20% in DMF, 2 mL) was added, and the mixture was shaken for 2 h 15 min. The resin was filtered and washed with DMF and dichloromethane (3×4 mL each). Palmitic acid (36 mg, 0.14 mmol) and 1-hydroxy-7-azabenzotriazole (12 mg, 0.087 mmol) were dissolved in dichloromethane (2.5 mL) and 1,3-diisopropylcarbodiimide (13 μL, 0.083 mmol) was added. The solution was stirred at room temperature for 20 min and added to the resin, followed by bromophenol blue (2 mM in DMF, 10 μL). The mixture was shaken at room temperature for 22 h 30 min, the solvent was filtered off, and the resin was washed with dichloromethane, DMF, and dichloromethane/MeOH (1:1; 3×4 mL of each). TFA (5% in dichloromethane, 2 mL) was added to the resin and the mixture was left at room temperature for 30 min. The resin was washed with TFA (5% in dichloromethane, 2 mL), dichloromethane (3×4 mL), and dichloromethane/MeOH (1:1, 4×4 mL). The combined filtrates were evaporated and concentrated in vacuo, to yield crude product (11 mg). After chromatography (SiO_2, dichloromethane/MeOH (5:1)), **1** (7.7 mg, 75% yield from resin **5**) was obtained: ^{1}H NMR ([D_5]pyridine): $\delta = 8.59$ (d, 1H, $J = 8.3$ Hz; NHAc), 8.42 (t, 1H, $J = 5.3$ Hz; NHAcyl), 6.74–6.54 (2×brs, 2H; OH-6, OH-4), 6.28 (brs, 1H; OH-3), 5.38 (d, 1H, $J = 3.7$ Hz; H-1), 5.30–5.22 (m, 1H; H-2), 4.52 (brs, 1H; H-4), 4.50–4.32 (m, 4H; H-3, H-5, H-6, H-6′), 3.99–3.61 (m, 4H; OCH$_2$C*H*$_2$N), 2.42 (t, 2H, $J = 7.5$ Hz; COC*H*$_2$-Alkyl), 2.17 (s, 3H; COCH$_3$), 1.82 (p, 2H, $J = 7.4$ Hz; COCH$_2$C*H*$_2$-Alkyl), 1.42–1.19 (m; Alkyl-CH$_2$), 0.88 (t, 3H, $J = 6.8$ Hz; Alkyl-CH$_3$) ppm; ^{13}C NMR ([D_5]pyridine): $\delta = 174.1$, 171.7, 99.8, 73.5, 70.8, 70.6, 68.8, 63.2, 52.1, 40.2, 37.1, 32.6, 30.4, 30.3, 30.2, 30.1, 30.1, 26.7, 23.7, 23.4, 14.7 ppm; HRMS (FAB): *m/z* calcd for $C_{26}H_{50}N_2NaO_7$: 525.3516 $[M+Na]^+$; found: 525.3536.

***N*-(2-*n*-hexadecyl-stearyl)-*O*-(*N*-acetyl-2-amino-2-deoxy-α-D-galactopyranosyl)-2-aminoethanol (2)**: Piperidine (20% in DMF, 2 mL) was added to resin **5** (114 mg, 0.025 mmol) and the mixture was shaken at room temperature for 10 min. The resin was filtered, piperidine (20% in DMF, 2 mL) was added, and the mixture was shaken for 2 h 15 min. The resin was filtered and washed with DMF and dichloromethane (3×4 mL each). 2-(*n*-hexadecyl)stearic acid (50 mg, 0.099 mmol) and 1-hydroxy-7-azabenzotriazole (14 mg, 0.11 mmol) were dissolved/suspended in dichloromethane (2.5 mL) and 1,3-diisopropylcarbodiimide (15 μL, 0.096 mmol) was added. The mixture was stirred at room temperature for 20 min and added to the resin, followed by bromophenol blue (2 mM in DMF, 10 μL). The mixture was shaken at room temperature for 20 h 30 min and the solvent was removed by filtration. The resin was washed with dichloromethane (3×4 mL) and the coupling was repeated twice for 30 h and 22 h, respectively. The solvent was filtered off, the resin was washed with dichloromethane/MeOH (1:1), DMF, THF, and dichloromethane/MeOH (1:1; 3×4 mL of each). TFA (5% in dichloromethane, 2 mL) was added to the resin and the mixture was left at room temperature for 30 min. The resin was washed with TFA (5% in dichloromethane, 2 mL), dichloromethane (4×4 mL), and dichloromethane/MeOH (1:1, 4×4 mL) and the combined organic solvents were evaporated and concentrated in vacuo, to yield crude product (21 mg). After chromatography (SiO_2, dichloromethane/MeOH (20:1→10:1)), **2** (12.4 mg, 66% yield from resin **5**) was obtained: ^{1}H NMR ([D_5]pyridine): $\delta = 8.59$–8.53 (m, 2H; NHAc, NHAcyl), 6.77 (t, 1H, $J = 4.9$ Hz; OH-6), 6.61 (d, 1H, $J = 3.8$ Hz; OH-4), 6.27 (d, 1H, $J = 6.6$ Hz; OH-3), 5.38 (d, 1H, $J = 3.6$ Hz; H-1), 5.29–5.22 (m, 1H; H-2), 4.57–4.35 (m, 5H; H-3, H-4, H-5, H-6, H-6′), 4.00–3.88 (m, 3H; OC*H*$_2$C*H*HN), 3.68–3.58 (m, 1H; OCH$_2$CH*H*N), 2.55–2.47 (m, 1H; COCH-(Alkyl)$_2$), 2.20 (s, 3H; COCH$_3$), 2.01–1.89 (m, 2H; CH(C*H*H)-(Alkyl)$_2$), 1.63–1.19 (m; Alkyl-CH$_2$), 0.88 (t, 6H, $J = 6.8$ Hz; Alkyl-CH$_3$) ppm; ^{13}C NMR ([D_5]pyridine): $\delta = 177.1$, 171.8, 100.0, 73.6, 70.9, 70.8, 69.4, 63.3, 52.1, 48.1, 40.1, 34.3, 34.2, 32.6, 30.6, 30.6, 30.5, 30.4, 30.1, 28.7, 28.6, 23.7, 23.4, 14.7 ppm; HRMS (FAB): *m/z* calcd for $C_{44}H_{86}N_2NaO_7$: 777.6333 $[M+Na]^+$; found: 777.6334.

Cell lines and mice: EL4 cells are derived from a B6 (H-2^b) thymoma.[32] β_2-Microglobulin −/− EL4 cells are derived from an EL-4 (H-2^b) lymphoma.[33] Cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 μg mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 °C in 5% CO_2. B6 and CD1d −/− B6 mice[34] were bred and maintained

in the animal house at the Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden. Female mice were used at an age of 6–12 weeks.

Generation of dendritic cells: Dendritic cells were cultured from mouse (B6 or CD1d −/− B6) bone marrow, as described previously.[35] Briefly, female mice at an age of 6–12 weeks were sacrificed and bone marrow was flushed from the large bones. Red cells were lysed with ammonium chloride. Lymphocytes and Ia-positive cells were removed with a set of monoclonal antibodies, including GK1.5 (anti-CD4), HO2.2 (anti-CD8), B21–2 (anti-Ia), and RA3–3a/6.1 (anti-B220/CD45R) (hybridomas obtained from the American Type Culture Collection, Rockville, MD), and rabbit complement (Calbiochem, Germany). The remaining population was placed in 6-well plates ($6–8\times10^6$ cells/well) in complete medium (RPMI 1640, 10% fetal bovine serum, 2 mM glutamine, penicillin (100 μg mL^{-1}), and streptomycin (100 μg mL^{-1})), supplemented with rGM-CSF (10 ng mL^{-1}; PeproTech, UK) and rIL-4 (10 ng mL^{-1}; Biosite, Sweden). The medium was changed every second day. On day 2, 75% of the medium was aspirated after gently swirling the plates and the remaining nonadherent cells, mostly in small, loosely attached aggregates, were transferred to new 6-well plates. By day 5–6, immature dendritic cells that appeared in large clusters were collected and used for experiments.

Reagents and monoclonal antibodies: α-GalCer, (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol), and a suitable vehicle were provided by the Pharmaceutical Research Laboratory, Kirin Brewery (Gunma, Japan). The glycolipid was stored in the aqueous vehicle at a concentration of 200 μg mL^{-1}. Three different anticarbohydrate monoclonal antibodies, MC2102 with specificity for galabiose,[36] p5–3 with specificity for GM3-lactam,[37] and the α-GalNAc-specific 2C4 (Dr. Bo Jansson, Bioinvent Therapeutics, Lund, Sweden) were used. The F(*ab*′)$_2$ FITC-labeled rabbit antimouse (Dakopatts, Denmark) antibody was used as the secondary antibody for flow cytometry by FACS. The FITC-labeled mouse anti-CD1d antibody was purchased from Pharmingen.

Glycolipid loading of cells and flow cytometry: Approximately one million cells were incubated with glycolipids **1**, **2, 9**, or **10** (100 μL, 5, 50, or 500 μg mL^{-1} in PBS buffer with 5% DMSO) at 26 °C for 2 h (30 min for **10**; with EL-4 and β_2-microglobulin −/− EL4 cells) or 30 min (with dendritic cells from B6 or CD1d −/− B6 mice). The cells were washed twice with PBS and stained with the primary mouse monoclonal antibody, with specificity for the corresponding carbohydrate. Subsequently, the cells were washed and incubated with a rabbit antimouse F(*ab*′)$_2$ FITC-labeled secondary antibody for an additional 30 min at 0 °C. This was followed by washing and fixation with 1% formaldehyde solution (200 μL). The cells were then analyzed by flow cytometry with a Becton Dickinson FACScan system. Forward- and side-scatter gates were set to exclude dead cells. The mean fluorescence intensity was measured. Competition experiments were carried out with EL4 cells that were first incubated with the CD1d ligand α-GalCer in the vehicle (0.002, 0.2, 10, or 20 μg mL^{-1}) or with the vehicle alone. After washing, cells were treated with the α-GalNAc lipid **2** (100 μL, 10 μg mL^{-1} in PBS buffer with 5% DMSO) as described above.

Acknowledgements

*This work was supported by the Swedish Research Council. Glycolipids **9** and **10** were a kind gift from the late Prof. Göran Magnusson, and α-galactosylceramide was kindly provided by the Kirin Brewery Pharmaceutical Division, Tokyo, Japan. We thank Prof. Jan Kihlberg for helpful comments on the manuscript.*

Keywords: antigens · glycolipids · glycosylation · immunochemistry · proteins

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Received: May 12, 2003
Revised: October 27, 2003 [F 655]

