Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells

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The CD1 family of proteins binds self and foreign glycolipids for presentation to CD1-restricted T cells. To identify previously uncharacterized active CD1 ligands, especially those of microbial origin, numerous glycolipids were synthesized and tested for their ability to stimulate mouse and human natural killer T (NKT) cells. They included analogs of the well known NKT cell agonist α -galactosyl ceramide (α -GalCer), bacterial glycolipids, and variations of the self-glycolipid, sulfatide. Bacterial glycolipids, α -galacturonosyl-ceramides from *Sphingomonas wittichii*, although structurally similar to α -GalCer, have significant differences in the sugar head group as well as the ceramide portion. The *Sphingomonas* glycosphingolipids (GSLs) and sulfatide variants were shown to activate human NKT cells as measured by IL-4 and IFN- γ secretion. Moreover, CD1d-dimer staining revealed human NKT cell reactivity toward these GSLs and to the sulfatides in a fashion comparable with α -GalCer. Because α -GalCer is a marine-sponge-derived ligand, our study here shows that bacterium-derived antigens are also able to stimulate mouse and human NKT cells.

CD1 molecules are a family of highly conserved antigen-presenting proteins that are similar in function to classic MHC molecules (1). Whereas classic MHC molecules present peptides, CD1 proteins bind and display a variety of lipids and glycolipids to T lymphocytes (2). The five known isoforms are classified into two groups, group I (CD1a, CD1b, CD1c, and CD1e in humans) and group II (CD1d in humans and mice), based on similarities between nucleotide and amino acid sequences (3).

A great diversity of lipids and glycolipids have been shown to bind specifically to each of the four isoforms. The first crystal structure of murine CD1d (mCD1d) revealed that it adopts a folded conformation closely related to major histocompatibility complex (MHC) class I proteins (4). Also, it revealed that mCD1d could accommodate long lipid tails in two hydrophobic pockets, designated A' and F', located in the binding groove. Two additional structures of hCD1b and hCD1a (5–7) confirmed this model by demonstrating that CD1, when loaded with antigenic glycolipids, binds the lipid portion in a hydrophobic groove while making the hydrophilic sugar moiety available to make contact with the T cell receptor.

Mammalian and mycobacterial lipids are known to be presented by human CD1a, CD1b, CD1c, and CD1d (1). α -Galactosyl ceramide (α -GalCer), a lipid found in the marine sponge *Agelas mauritianus* (8), has been the most extensively studied ligand for CD1d. α -GalCer, when bound to CD1d, stimulates rapid Th1 and Th2 cytokine production by V α 14i natural killer T (V α 14i NKT) cells in mice and the human homolog V α 24i NKT cells (9–13) (Fig. 1). However, its physiological significance in mammals remains unclear because it is enigmatic why an α -galactosyl ceramide of marine origin is such a potent agonist. Other known ligands, which stimulate only relatively small populations of CD1d-restricted V α 14i NKT cells, include a

Fig. 1. NKT cells are reactive to CD1d-bound α -GalCer. This stimulation results in the production of large amounts of cytokines such as IL-4 and IFN- γ .

bacterial phospholipid (PIM₄) and a tumor-derived ganglioside (GD3) (14, 15). A phosphoethanolamine, found in human tumor extracts, was shown to be recognized by 24.8.A invariant NKT cell hybridomas (16). The C-linked analog of α -GalCer (17) and α -GalCer analogs with different lipid chain lengths (18) have also been reported to have significant activities toward NKT cells.

As a part of our efforts to further understand the mechanism of CD1d-restricted T cell activation and the discovery of new glycolipid antigens, we have synthesized a number of glycolipids and tested them for NKT cell activation (Fig. 2). These included glycolipids of bacterial origin (compounds 1, 2, 5, and 6), α -GalCer analogs modified on the galactose moiety and acyl group (compounds 10–18), and variations of sulfatide (compounds 7–9), the only known promiscuous ligand for CD1 (19). The bacterial glycolipids included those isolated from the outer membrane of *Sphingomonas wittichii* (20) and glycolipids from *Borrelia burgdorferi*, the Lyme disease spirochete. CD1d-deficient (CD1d^{−/−}) mice were shown to have impaired host resistance to infection by *B. burgdorferi*, making its glycolipids attractive compounds for further study as possible natural CD1d antigens (21). The structures of its two major glycolipids were recently elucidated as cholesteryl 6-*O*-acyl- β -D-galactopyranoside, 5 (*B. burgdorferi* glycolipid 1, BbGL-I), and 1,2-di-*O*-acyl-3-*O*- α -D-galactopyranosyl-*sn*-glycerol, 6 (BbGL-II) (22). The *Sphingomonas* glycolipids, two previously uncharacterized

Abbreviations: α -GalCer, α -galactosyl-ceramide; GSL, glycosphingolipid; mCD1d, murine CD1d; NKT cell, natural killer T cell; THF, tetrahydrofuran.

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Fig. 2. Structures of glycolipids and analogs.

α -linked glycosphingolipids (GSLs), **1** and **2** (GSL-1 and GSL-2, respectively), differ most significantly from α -GalCer in the carbohydrate moiety because they contain galactosyluronic acids as the polar head group (23). However, they are more physiologically relevant as natural ligands for CD1d-mediated NKT cell activation because they originate from bacteria. Biological experiments further show that galactouronic sphingolipids stimulate IL-2 secretion in 1.2 (V α 14 V β 8.2 DN3A4) NKT cell hybridomas. An α -GalCer analog, **9**, 3''-O-sulfo-galactosylceramide (3''-O-sulfo-GalCer), also caused significant IL-2 secretion, demonstrating that V α 14i NKT cell response is less sensitive to modification at the 3''-OH position of galactose. By contrast, any modification made at the 2''-OH position of galactose abolished all biological activity. Most other synthetic analogs, however, were active. In addition, we report the conservation of reactivity of human V α 24i NKT cells to GSL-1 and GSL-2 and sulfatides **7–9**.

Materials and Methods

The synthesis of α -GalCer has been reported by this laboratory (24). Compounds **16–18** were synthesized by the same procedure with slight modifications. Intermediates **19**, **26**, and **30** are known

compounds (24–26). Experimental details for **1**, **2**, **7**, **20**, **23**, **27**, and **31** are described. The synthesis of the remaining compounds, except **8** and **9**, and their intermediates are reported separately in the supporting information, which is published on the PNAS web site.

Sphingosine Intermediate Compound 20. As shown in Fig. 3, compound **19** (3.31 g, 13.5 mmol) (**25**) was dissolved in 70 ml of dry tetrahydrofuran (THF). The solution was cooled to -40°C , and vinyl Grignard solution (31 ml of a 1 M solution in THF) was added via a dropping funnel over a period of 1 h. The temperature was kept between -20°C and -40°C . The reaction mixture was allowed to warm to room temperature and stirred for another hour. The

Fig. 3. Synthesis of sphingosine. Reagents and conditions are as follows. a, (i) $\text{C}_2\text{H}_3\text{MgBr}$, THF, Anti:Syn 3.5:1 61%; (ii) Grubbs catalyst second generation, CH_2Cl_2 , pentadecene, 71%. b, (i) BzCl , pyridine, 90%; (ii) amberlyst 15 H^+ form, MeOH, 70%.

Fig. 4. Synthesis of glycolipids. Reagents and conditions were as follows. a, **22**, TMSOTf, 67%. b, (i) TFA, DCM; (ii) HBTU, myristic acid or (*S*)-2-acetoxymyristic acid, *n*-morpholine, $\approx 92\%$, two steps. c, H_2 , 20% Pd(OH) $_2$, (ii) LiOH, $\text{H}_2\text{O}:\text{THF}:\text{MeOH}$, 38%, two steps. d, **26**, TMSOTf, 60%. e, (i) TFA, DCM, (ii) HBTU, myristic acid or (*S*)-2-acetoxymyristic acid, *n*-morpholine, $\approx 92\%$, two steps. f, (i) NaOMe, MeOH, (ii) Pd/C, H_2 , EtOH, 90%, two steps. g, **30**, TMSOTf, 62%. h, (i) TFA, DCM, (ii) HBTU, nervonic acid, *n*-morpholine, 60%, two steps. i, (i) NaOMe, MeOH, quant; (ii) Bu_2SnO , MeOH; (iii) $\text{Me}_3\text{N}\cdot\text{SO}_3$, THF, 95%. j and k, (i) LDA, TMSOOTMS, (ii) H^+ , MeOH, 30%, two steps; then LiOH, $\text{H}_2\text{O}:\text{MeOH}$, 81%; l, novozyme 435, $\text{CH}_2=\text{CHOAc}$, 54% based on *S* isomer.

reaction was quenched by addition of 60 ml of saturated $(\text{NH}_4)_2\text{SO}_4$ solution and evaporated to dryness. The residue was diluted with water and extracted with ethyl acetate (3 $\times$). The combined organic layer was extracted with brine, dried over MgSO_4 , and evaporated to give a yellow oil. Column chromatography (Hex:EtOAc 3:1) yielded the *syn* diastereomer (2.11 g, 8.2 mmol, *anti/syn* = 3.5:1) in 61% yield. Then the *syn* diastereomer (300 mg, 1.16 mmol) was dissolved in 1 ml of dry dichloromethane in a two-necked flask equipped with a reflux condenser under argon, and 486 mg (3.48 mmol) of pentadecene was added by syringe. A solution of 20 mg

(2 mol %) of Grubb's second generation catalyst (purchased from Strem Chemicals) in 1 ml of dichloromethane was added, and the solution was heated under rapid reflux for 40 h. The reaction mixture was evaporated and then directly chromatographed (Hex:EtOAc 6:1), yielding 0.82 mmol (71%) of the desired product.

Synthesis of Glycolipids. As shown in Fig. 4, a solution of trichloroacetimidate **22** (160.4 mg, 0.258 mmol) and sphingosine acceptor **21** (100 mg, 0.198 mmol) in 4 ml of anhydrous Et_2O and

Fig. 5. IL-2 secretion by murine 1.2 hybridomas when stimulated by the indicated glycolipids loaded on CD1d-coated plates. (A) IL-2 secretion profile of 3'-O-sulfo- α -GalCer compared with α -GalCer and analogs. (B) Dose-dependent secretion of IL-2 by *Sphingomonas* GSLs and analogs. CD1d molecules (10 μ g/ml in PBS) were coated in a 96-well plate by incubation for 1 h at 37°C. IL-2 release was measured after 16 h of culture in a sandwich ELISA.

Fig. 6. Human $V\alpha 24i$ NKT cells release IL-4 and IFN- γ in response to the indicated glycolipids. Flow cytometric analyses also show that $V\alpha 24i$ NKT cells bind to the various glycolipids in response to the indicated glycolipids. (A) Human $V\alpha 24i$ NKT cells respond to synthetic *Sphingomonas* and sulfatide glycolipids. IFN- γ and IL-4 release after 16 h of culture by 1×10^5 $V\alpha 24i$ human NKT cell line in response to culture with 4×10^5 autologous immature CD14 $^{+}$ dendritic cells pulsed with the indicated glycolipid antigens at 10 μ g/ml. The negative control wells contained similar numbers of NKT cells and dendritic cells, cultured without added glycolipid. Data are mean $\pm$ SD of duplicate wells. (B) Human $V\alpha 24i$ NKT cells bind to glycolipids in the context of CD1d. Flow cytometric analysis of a human $V\alpha 24i$ human NKT cell line with human CD1d dimers that were unloaded or loaded with 10 M indicated glycolipid antigen. The cells were also stained with anti-human CD3-PerCP.

2 ml of anhydrous THF was added over freshly dried 4-Å molecular sieves and cooled to -50°C . Trimethylsilylmethyl trifluoromethanesulfonate (TMSOTf) (3.33 mg, 0.0198 mmol) was added, and the mixture was stirred at -50°C for 1 h. The mixture was allowed to warm to -20°C , and another 3.33 mg of TMSOTf was added. The mixture was then slowly allowed to warm to room temperature and stirred for 3 h. The solution was then diluted with EtOAc and filtered over Celite. The organic layer was washed with saturated aqueous NaHCO_3 and brine, dried (MgSO_4), and concentrated. The residue was purified by column chromatography on silica gel (toluene:EtOAc 12:1) to give 128 mg (67.5%, 0.134 mmol) of **23**. A general procedure for transformation of **23** to **24** can be found in the supporting information.

Compound **24** (36 mg, 0.03 mmol), dissolved in 6 ml of EtOAc,

was added to 36 mg of 20% (wt) palladium hydroxide in 1 ml of EtOAc and saturated with hydrogen. The reaction vessel was purged with hydrogen, and the mixture was stirred at room temperature overnight. The reaction mixture was filtered, and the solvent was evaporated. The above hydrogenated compound was dissolved in 2 ml of THF, 1 ml of water, and 1 ml of methanol. LiOH (9 mg, 0.14 mmol) was added to the solution, and the reaction was stirred at room temperature for 4 h. We added 100 mg of Na_2CO_3 , and the mixture was stirred for 30 min. The solvent was evaporated, and the remaining residue was purified on silica gel by column chromatography (CH_2Cl_2 :MeOH 4:1) to give 7.8 mg of **1** (38%, 0.0114 mmol, two steps).

Sulfation of 32. After deprotection of compound **32** (14 mg, 0.017 mmol), Bu_2SnO (6.5 mg, 0.0259 mmol) dissolved in 1 ml of MeOH

α -GalCer but twice that induced by the other glycolipids. Surprisingly, β -linked sulfatides **7** and **8** were observed to elicit both IFN- γ and IL-4 production. In fact, the level of cytokine secretion was comparable to the GSLs. Other α -GalCer analogs with an acetyl side chain or a shortened backbone were also tested, and some activity was also observed (data not shown).

Human NKT Cell Lines Bind to Glycolipids in the Context of CD1d. Although glycolipids stimulate the NKT cell line, we cannot invariably conclude that the glycolipids are presented by CD1d molecules and are capable of triggering the V α 24i T cell receptor expressed by the NKT cells. Therefore, to confirm the glycolipid antigen reactivity to the V α 24i T cell receptor at the single-cell level, we stained a human NKT cell line with human CD1d dimers loaded with different glycolipids, using unloaded CD1d dimers as a negative control. Each glycolipid-loaded dimer nearly universally stained the human NKT cells, whereas the unloaded dimer did not stain these cells (Fig. 6B).

Computer Modeling of GSL Complexed to mCD1d. To further understand the interaction of bacterial glycolipid **1** with CD1d, a model generated by AUTODOCK 3.0 (29) of GSL **1** complexed with the crystal structure of mCD1d was shown in Fig. 7 (4). In this model, the fatty acyl chain extends into the F' pocket, and the sphingosine chain extends toward the A' pocket. This allowed the polar head group to be oriented such that it was exposed for recognition by a T cell antigen receptor. Numerous favorable contacts could be observed between mCD1d and the GSL. Among them, possible hydrogen bonding included interactions between the carboxylate of the sugar and the backbone carbonyl of Asp-80, and the amide nitrogen of the fatty-acid tail with the Asp-80 side chain.

Studies (30) have shown mCD1d to be somewhat accommodating in terms of lipid tail length on NKT cell reactivity. However, changes in the lipid length, composition, or addition of an α -hydroxyl group on the fatty acid could cause a slight shift in orientation of the sugar, thereby affecting how the CD1d/

glycolipid complex is recognized by the T cell receptor. This could also be true of the substitution of galacturonic acid for galactose. The perturbation caused by having the 6'-OH oxidized to a carboxylic acid caused only moderate changes in NKT cell reactivity.

Conclusion

In summary, a number of glycolipids were synthesized and tested for their ability to activate NKT cells. Of particular interest were sulfatides **7–9** and the *Sphingomonas* GSLs, which showed considerable activity as measured by cytokine production. Although none of the compounds were as potent as α -GalCer, specific responses were obtained to an α -GalCer analog, 3''-O-sulfo- α -GalCer, **9**, but not to any of the four compounds having a modification of the equatorial 2''-OH of the galactose. Furthermore, glycolipid/CD1d dimer staining of human V α 24i NKT cells showed reaction toward the *Sphingomonas* GSLs (GSL-1 and GSL-2) and sulfatides **7–9** when compared with α -GalCer. We were also able to demonstrate responses to the *Sphingomonas* compounds *in vivo*, and mice deficient for NKT cells have a reduced ability to clear bacteria from the liver (Y.K., D.W., G. Kim, G.-W.X., M.A.P., M.T., K. Kawahara, C.-H.W., and M.K., unpublished work).

Bacterial α -galacturonosyl ceramides have been shown to elicit activity from both mouse and human NKT cell lines. As measured by cytokine release and CD1d dimer/glycolipid staining of a human NKT cell line, 3''-O-sulfo-GalCer, **9**, had comparable potency to the exceptional agonist α -GalCer, showing that some flexibility can be accommodated at the 3''-OH position of galactose. Any modification at the 2''-OH position almost completely abolished activity. The *Sphingomonas* GSLs are reactive to the same population of human V α 24i NKT cells as α -GalCer, showing that bacterium-derived antigens are also able to activate NKT cells.

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