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Natural *Sphingomonas* Glycolipids Vary Greatly in Their Ability to Activate Natural Killer T Cells

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SUMMARY

Mouse natural killer T (NKT) cells expressing an invariant T cell antigen receptor (TCR) recognize glycosphingolipids (GSLs) from *Sphingomonas* bacteria. The synthetic antigens previously tested, however, were designed to closely resemble the potent synthetic agonist α-galactosyl ceramide (αGalCer), which contains a monosaccharide and a C18:0 sphingosine lipid. Some *Sphingomonas* bacteria, however, also have oligosaccharide-containing GSLs, and they normally synthesize several GSLs with different sphingosine chains including one with a cyclopropyl ring-containing C21:0 (C21cycl) sphingosine. Here we studied the stimulation of NKT cells with synthetic GSL antigens containing natural tetrasaccharide sugars, or the C21cycl sphingosine. Our results indicate that there is a great degree of variability in the antigenic potency of different natural *Sphingomonas* glycolipids, with the C21cycl sphingosine having intermediate potency and the oligosaccharide-containing antigens exhibiting limited or no stimulatory capacity.

INTRODUCTION

Natural killer T (NKT) cells are a sublineage of innate-like or natural memory T cells that share properties of both T lymphocytes and NK cells (Bendelac et al., 2007; Godfrey and Berzins, 2007; Kronenberg, 2005). More than 80% of mouse NKT cells express an invariant (*i*) TCRα chain with Vα14-Jα18 rearrangement. Here we refer to these cells as Vα14*i* NKT cells. Humans have a similar population that mostly expresses an invariant Vα24-Jα18 rearrangement (Vα24*i* NKT cells). We refer to these populations in mice and humans collectively as invariant NKT cells (*i*NKT cells). *i*NKT cells participate in various immune responses through the production of large amounts of cytokines. A role for these cells has been reported in the response to tumors, the regulation of autoimmunity, the pathogenesis of asthma and other inflammatory conditions, and, most relevant for our studies, the response to microbial infections (Bendelac et al., 2007; Brigl and Brenner, 2004; Godfrey and Berzins, 2007; Kronenberg, 2005; Tupin et al., 2007).

*i*NKT cells recognize glycolipid antigens (Ags) bound to or presented by CD1d, an MHC class I-like antigen-presenting molecule (Brigl and Brenner, 2004; Moody et al., 2005; Zajonc and Kronenberg, 2007). The synthetic Ag α-galactosyl ceramide (αGalCer) was the first known Ag presented by CD1d that could stimulate the invariant TCR expressed by *i*NKT cells (Kawano et al., 1997). αGalCer is a very close structural analog of several agelasphins, compounds isolated from the *Agelus* genus of marine sponge (Morita et al., 1995). An unusual feature of this potent GSL Ag is the α linkage of the sugar to the ceramide lipid, as most other GSLs in nature were known to have a β linkage. Although a number of synthetic antigens related to αGalCer were studied to decipher structure-activity relationships (Tsuji, 2006), until recently a microbial origin for *i*NKT cell antigen was not known. Subsequently, *Sphingomonas spp*. bacteria were shown also to have GSLs with α-linked hexose sugars and sphingoid bases similar to the marine sponge agelasphins (Kawahara et al., 1991, 2002, 2006). Synthetic versions of these GSLs, with α-linked monosaccharides, either galacturonic or glucuronic acid, and a C18:0 sphingosine lipid, stimulated *i*NKT cells (Kinjo et al., 2005; Mattner et al., 2005). The natural GSL Ags in *Sphingomonas spp*. are much more heterogeneous, however, as a single strain can produce more than one GSL and, furthermore, there are differences between strains (Kawahara et al., 1991, 2000, 2001, 2002, 2006). There are variations not only in the complexity and sequence of the α-linked sugars but also in the acyl chain and sphingoid base of the ceramide lipid.

This study was designed to compare the antigenic potency of the original synthetic GSL Ag we used, called GSL-1 here, with other GSLs that represent some of the natural diversity found in *Sphingomonas spp*. We synthesized and tested GSLs with two different natural tetrasaccharide sugars and one with an unusual C21 sphingoid base. In addition to antigenic stimulation, we determined whether GSL Ags with more complex carbohydrates are subject to antigen processing to generate monosaccharide-containing GSLs, and whether these Ags can activate innate immune responses by dendritic cells in the absence of *i*NKT cells.

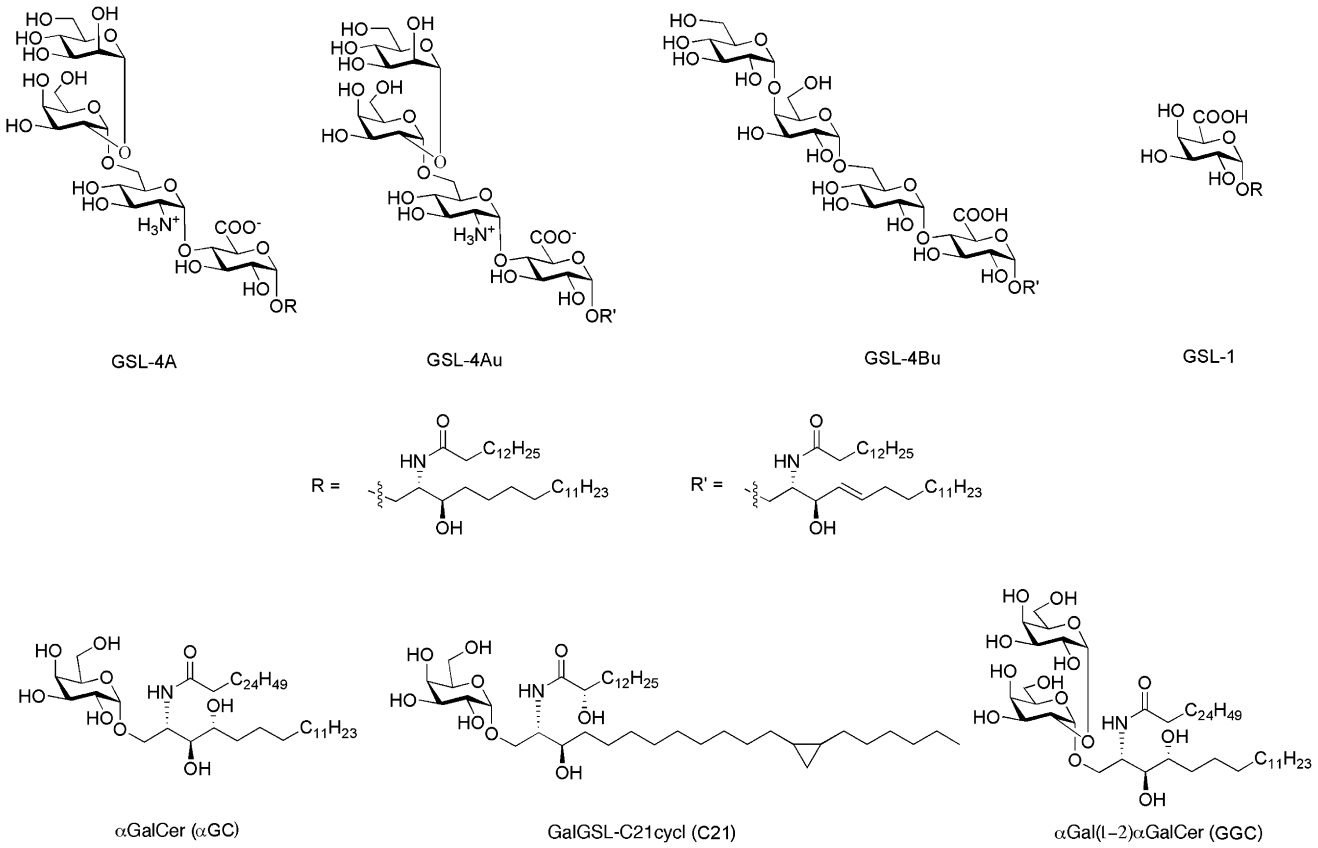


Figure 1. Glycolipid Ag Structures

Structures of the synthetic glycolipid antigens used in this study are depicted. The syntheses of GSL-4A, GSL-4Au, GSL-4Bu, and GalGSL-C21cycl(C21) are described in this paper, whereas the syntheses of GSL-1, αGalCer, and αGal(1-2)αGalCer have been described previously, as indicated in the Experimental Procedures.

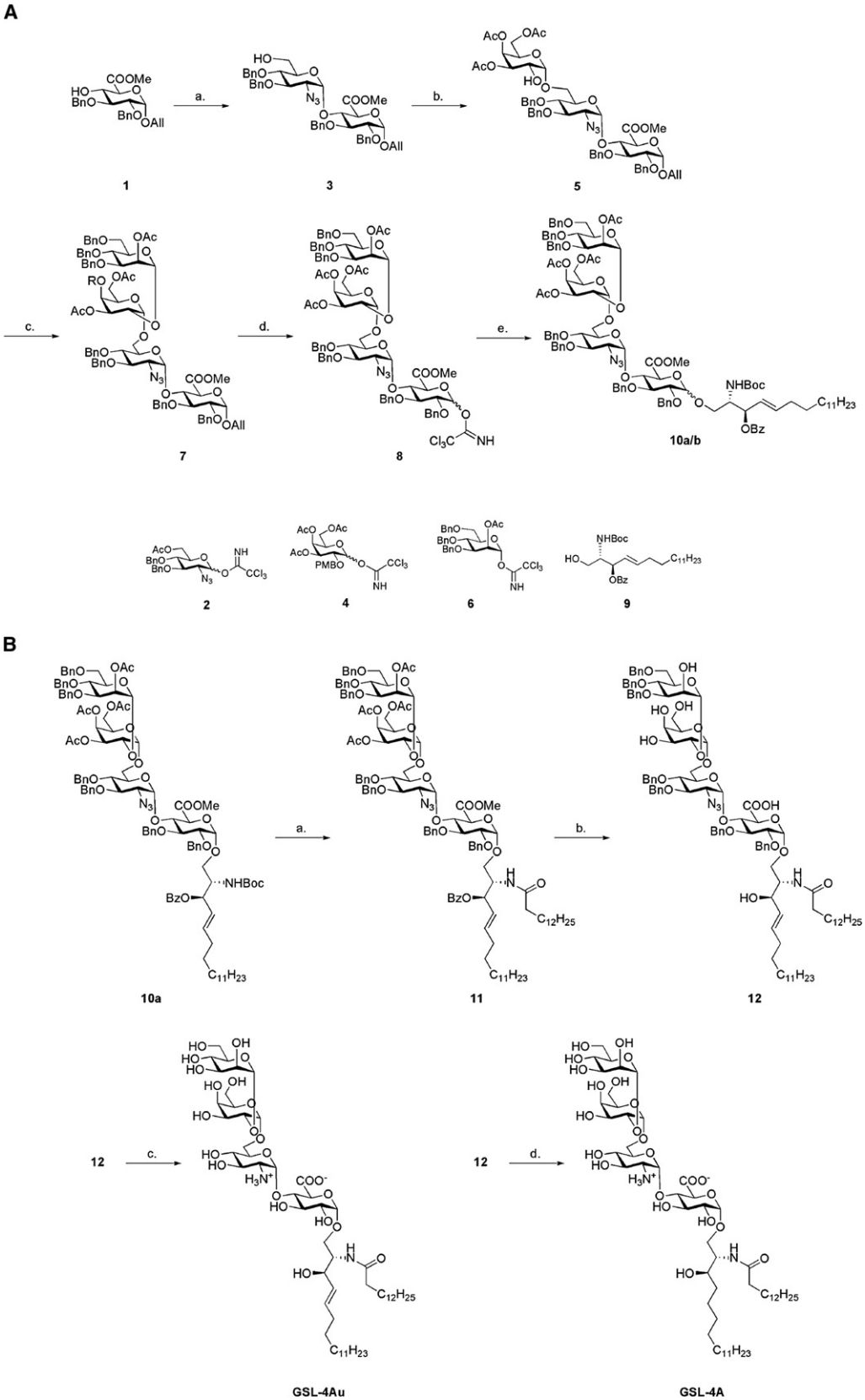
RESULTS

Glycolipid Ag Structures

The GSL structures produced by different *Sphingomonas* species are heterogeneous, and whereas some *Sphingomonas* bacteria only have GSL-1 Ags, containing monosaccharides, others synthesize more complex sugars. Table S1, available online, shows some of this antigenic diversity, including the GSLs from several species that have been recently used for studies of Vα14*i* NKT cells (Supplemental Data). To determine whether *i*NKT cells can recognize oligosaccharide-containing GSLs, we synthesized GSLs with carbohydrate moieties corresponding to the previously purified GSL-4A (Kawahara et al., 1991) and GSL-4B Ags (Kawahara et al., 2001). For the ceramide lipid, a C14 fatty acid was used for the *Sphingomonas*-related compounds, because this is the observed length of the fatty acids in the purified material. Variant forms with an unsaturated bond in the sphingosine chain were prepared, and called GSL-4Au ("u" for unsaturation) and GSL-4Bu (Figure 1). These subtle sphingosine alterations should not greatly alter the exposed carbohydrate epitope, and in fact for some experiments, the GSL-4Au antigen was more potent than its fully saturated counterpart (see below). For comparison, the structure of the highly potent phytosphingosine-containing antigen αGalCer is also shown, along with a version of this Ag containing a second galactose sugar (αGal(1-2)αGalCer) (Prigozy et al., 2001). The GSLs from *Sphingomonas* bacteria contain different sphingosine chains, including C18:0, C20:1, and a cyclopropyl-C21:0. To test how these natural sphingosine variants affect Ag potency, we synthesized a cyclopropyl-C21:0 chain containing a GSL with a single α-linked galactose sugar (GalGSL-C21cycl).

Synthesis of GSL-4A, GSL-4Au, and GSL-4Bu

The synthesis of the GSL-4 glycosphingolipids relied on the modular assembly of monosaccharide building blocks for the formation of a tetrasaccharide glycosylating agent. Glycosylation of the sphingosine building block and global deprotection steps resulted in the desired compounds. The assembly of GSL-4A commenced with the glycosylation of the glucuronic derivative **1** using the azidoglucose trichloroacetimidate **2** (Figure 2A). Galactosylating agent **4** formed an α-linkage with high stereochemical control. The *p*-methoxybenzyl group was removed by slowly raising the temperature to 0°C. This one-pot procedure furnished **5** in 57% overall yield. The last glycosylation provided fully protected tetrasaccharide **7** in excellent yield and stereoselectivity. The tetrasaccharide glycosylating agent **8** was produced in good yield via a three-step procedure, palladium-catalyzed isomerization of the allyl group and hydrolysis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11
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12
GSL-4Au
GSL-4A

of the resulting enol ether to form the hemi-acetal, followed by treatment with trichloroacetonitrile in the presence of catalytic amounts of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU). The glycosylation of sphingosine building block **9** was tackled in the subsequent step. The reaction was performed at low temperature in the presence of catalytic amounts of freshly distilled trimethylsilyl triflate (TMSOTf). The reactivity of **8** seemed low because the product was obtained in modest yield, while most of the glycosylating agent was recovered intact. Pure α anomer **10a** was isolated by standard silica gel and size-exclusion chromatography.

The fully protected GSL-4A structures were readily obtained by removal of the Boc group under acidic conditions and coupling with myristic acid in the presence of *N*,*N*,*N*′,*N*′-tetramethyl-O-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU). Compound **11** was obtained in 92% yield over two steps (Figure 2B).

Deprotection was achieved by saponification, followed either by dissolving metal reduction or by palladium-catalyzed hydrogenation. In the latter case, the removal of the benzyl groups was performed with the concomitant reduction of the olefin at the sphingosine moiety furnishing the product GSL-4Au. Birch reaction conditions were chosen in order to keep the olefin intact and obtain GSL-4A.

GSL-4Bu was generated in the same manner. The glucosylation of glucuronic acid building block **1** and deacetylation afforded disaccharide **14** in good yield (Figure 3A). Galactoside **15** proved to be an efficient glycosylating agent for the subsequent galactosylation, providing the desired trisaccharide in good yield and selectivity. The appropriately protected trisaccharide was converted to debenzoylated species **16** in excellent overall yield. Glycosylation of the axial hydroxyl of **16** with trichloroacetimidate **13** proved to be a highly selective transformation, leading to the desired α-linked product with excellent diastereocontrol. Palladium-mediated deallylation and subsequent trichloroacetimidate formation led to the desired tetrasaccharide glycosylating agent **18**. Finally, coupling of the sphingosine side chain and the tetrasaccharide in the presence of a Lewis acid proceeded with high levels of stereoinduction to furnish the desired α adduct. The modest yield of this particular transformation may be attributed to severe steric crowding in the transition state, as already observed for **10**.

Compound **19** was further transformed into the final fully protected GSL-4Bu structure, as described previously for the GSL-4A molecules. The *tert*-butoxycarbonyl group was cleaved under acidic conditions, and the liberated amine was exposed to acylation conditions with myristic acid in the presence of HATU to furnish **20** in good overall yield (Figure 3B). Hydrolysis of the esters was performed in the presence of lithium peroxide, followed by aqueous potassium hydroxide. The final benzyl ether cleavage was achieved in the presence of sodium and ammonia, affording GSL-4Bu in acceptable yield over the three steps.

Synthesis of GalGSL-C21cycl

The synthesis of C21 began with the preparation of sphingoid base **26** (Figure 4). For the *Sphingomonas* glycolipids that contain cyclopropanes in their sphingoid base, it is known that the alkyl chains are *cis*. However, the absolute stereochemistries of the cyclopropane stereogenic centers are unknown. Consequently, we decided to prepare sphingoid base **26** with the absolute stereochemistries of C2 and C3 set but with the cyclopropane having defined relative, but not absolute, stereochemistry. Starting with 10-undecyn-1-ol, silyl protection of the alcohol moiety, followed by coupling of the terminal alkyne with 1-iodohexane and hydrogenation over a Lindlar catalyst, afforded *Z*-alkene **21**. Cyclopropanation (Charette et al., 2000) and deprotection furnished cyclopropyl alcohol **22**, which was converted to Grignard reagent **23** by bromination and reaction with magnesium. The conversion of **23** to sphingoid base **26** utilized the straightforward protocol we developed for the preparation of sphinganines (So et al., 2004). Serine-derived Weinreb amide **24** (Campbell et al., 1998) was pretreated with two equivalents of a sacrificial base, followed by the addition of **23**, providing ketone **25**. Diastereoselective reduction, followed by carbamate cleavage, gave **26**. Although **26** was a mixture of diastereomers due to cyclopropane stereoisomerism (relative, but not absolute, stereochemistry set), spectroscopic characterization showed only a single set of peaks (indicating that the cyclopropane moiety was distant enough from C2 and C3 that its nonspecific stereochemistry did not affect these centers). Sphinganine **26** was coupled with activated ester **27** (the preparation of **27** is described in the Supplemental Data), prepared as a single enantiomer from tetradecene by the protocol of Murakami et al. (2005). The resultant ceramide was glycosylated with persilylated iodosugar **29** under in situ anomerization conditions recently reported by Gervay-Hague and colleagues (Du et al., 2007). The resultant α-GalCer was treated with Dowex to cleave the silyl groups. However, with repeated chromatographic purification, some ammonium salt from the glycosylation remained. Rather than lose more material, we elected to cleave the acetate group, and purification at this stage was successful, providing the C21 target.

Stimulation of Vα14*i* NKT Cell Hybridomas by Oligosaccharide GSL Ags

When presented by transfected B lymphoma cells expressing a high number of surface CD1d molecules, GSL-4A could stimulate IL-2 release from all three Vα14*i* NKT cell hybridomas tested. Each of these cells has an identical α chain of the TCR, but two have different Vβ8.2 rearrangements and one has a Vβ7 TCR (Figure 5). The responses induced by the GSL-4A compound were much weaker than those induced by GSL-1. For two hybridomas, 2C12 and 2H4, the response was stronger to the GSL-4Au variant, with an unsaturated sphingosine base, than to GSL-4A. Regardless, the data clearly establish that GSL Ags containing this natural tetrasaccharide can stimulate

Figure 2. The Synthesis of GSL-4 Compounds

(A) (a) (1) Azidoglucose **2**, TMSOTf, toluene, −25°C, 3 hr; (2) NaOMe, MeOH, rt, 15 hr, 91% (2 steps). (b) **4**, TMSOTf, toluene, −50°C to 0°C, 2 hr, 57% (2 steps). (c) **6**, TMSOTf, toluene, 0°C, 1.5 hr, 75%. (d) (1) $Pd(OAc)_2$, PPh_3, MeOH, CH_2Cl_2, rt, 8 hr, 80%; (2) Cl_3CCN, DBU, CH_2Cl_2, rt, 15 min, 76%. (e) **9**, TMSOTf, toluene, Et_2O −40°C to 0°C, 2 hr, 33% (a:b = 4:1).

(B) (a) (1) TFA, CH_2Cl_2, 0°C to rt, 2 hr; (2) myristic acid, HATU, *N*-methylmorpholine, CH_2Cl_2, 1,4-dioxane, rt, 18 hr, 92% (2 steps). (b) (1) H_2O_2, LiOH, MeOH/THF, rt, 15 hr; (2) KOH, MeOH/THF, rt, 15 hr, 99% (2 steps). (c) Na, NH_3, THF, −78°C, 45 min, 78%. (d) H_2, Pd/C, MeOH, rt, 15 hr, 98%.


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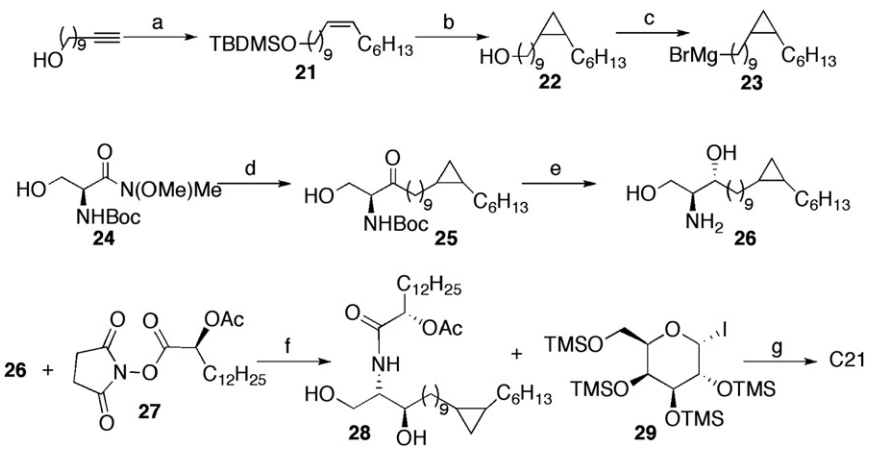

Figure 4. The Synthesis of GalGSL-C21 (C21)

(a) (1) *tert*-butyldimethylsilyl chloride, imidazole, DMF, rt, 10 hr, 92%; (2) *n*-BuLi, THF, –10°C, 10 min; hexamethylphosphoramide, iodohexane, –10°C to rt, 12 hr, 76%; (3) Pd/$BaSO_4$, quinoline, H_2, THF, rt, 2 hr, 82%. (b) (1) Et_2Zn, 2,4,6-trichlorophenol, CH_2Cl_2, –40°C, 15 min; CH_2I_2, –40°C to rt, 12 hr, 90%; (2) TBAF, THF, 0°C to rt, 2 hr, 89%. (c) (1) CBr_4, PPh_3, CH_2Cl_2, 0°C, quant.; (2) Mg^0, I_2, THF. (d) *i*-PrMgCl (2 eq.), THF; **23** in THF, –15°C to rt, 51%. (e) (1) LiAl(O*t*-Bu)$_3$H, EtOH, –78°C, 5 hr, 83%; (2) 3 M HCl, MeOH, 50°C, quant. (f.) (1) Et_3N, 80%. (g) (1) *n*-Bu_4NI, (*i*-$Pr)_2EtN$, CH_2Cl_2; Dowex; (2) NaOMe, MeOH.

immortalized Vα14*i* NKT cell hybridomas. By contrast, GSL-4B, which has a different tetrasaccharide sugar, did not stimulate any of the hybridomas.

Internalization and Lysosomal Ag Processing Are Not Required for Vα14*i* NKT Cell Stimulation by GSL-4A

Although the presentation of αGalCer by mouse CD1d (mCD1d) does not absolutely require internalization, an analog of αGalCer, αGal(1-2)αGalCer, which has an additional sugar (Figure 1), has to be internalized in order to be efficiently recognized by Vα14*i* NKT cells. Internalized αGal(1-2)αGalCer is processed in lysosomes to remove the terminal galactose, generating the monosaccharide αGalCer (Prigozy et al., 2001). Similarly, it has been reported that isoglobotrihexosyl ceramide (iGb3), an autologous GSL Ag, must be processed in lysosomes from the nonantigenic iGb4 for Vα14*i* NKT cell activation (Zhou et al., 2004). To determine whether the weak antigenic activity of the GSL-4A compounds depended upon internalization and processing, we used antigen presenting cells (APC) that express a mutant form of CD1d that does not traffic to lysosomes. The cytoplasmic tail of mCD1d has a tyrosine-based endosomal targeting motif responsible for mCD1d localization to lysosomes, where the molecules are loaded with self- or exogenous antigens. Our previous work showed that a single point mutation (I to A) in the cytoplasmic tail motif could inhibit the translocation of mCD1d to lysosomes, leading to impaired presentation of Ags such as αGal(1-2)αGalCer that require lysosomal processing (Lawton et al., 2005).

The ability of transfected A20 cells expressing the I/A cytoplasmic tail mutation of mCD1d to present the glycolipid Ag αGal(1-2)αGalCer was compared to wild-type cells. We used A20 transfectants that have similar amounts of surface mCD1d molecules (Lawton et al., 2005). Consistent with our previous work, cells expressing the I/A mutation could not stimulate IL-2 release by Vα14*i* NKT cell hybridomas in response to αGal(1-2)αGalCer, whereas wild-type mCD1d transfectants could (Figure 6A). A20 cells expressing the CD1d (I/A) mutant were comparable to cells expressing wild-type CD1d, however, in their ability to present GSL-1. This is consistent with other data indicating that monosaccharide GSL Ags have a reduced requirement for lysosomal loading onto mCD1d. Interestingly, similar to GSL-1, GSL-4Au also induced the same amount of IL-2 release with I/A mutant and wild-type mCD1d (Figure 6A). These data suggest that lysosomal antigen processing is not required for the weak stimulatory activity of a GSL Ag with a tetrasaccharide sugar. Moreover, because wild-type mCD1d was not more effective at presenting GSL-4, the data further suggest that Ag processing does not efficiently generate the more potent monosaccharide.

To confirm that GSL-4 can stimulate Vα14*i* NKT cell hybridomas without lysosomal Ag processing, we tested these compounds in an APC-free assay. Similar to the results obtained with CD1d transfectants (Figure 5), GSL-4A and GSL-4Au could stimulate Vα14*i* NKT cell hybridomas when these compounds were preincubated on mCD1d-coated microwells (Figure 6B). GSL-4A was approximately equal to GSL-4Au for each hybridoma tested, although it was slightly less potent when stimulating hybridoma 2H4. By contrast, GSL-4A was slightly more potent when stimulating hybridoma 1.2, which also reacted best to GSL-4A when presented by CD1d-transfected APC. Consistent with the results using transfected APC, GSL-4Bu could not stimulate any of these three Vα14*i* NKT cell hybridomas. These results indicate that the invariant TCR of NKT cells can recognize some tetrasaccharide-containing GSL Ags, even without lysosomal processing in APC, to generate monosaccharide-containing GSLs.

GSL-4A and GSL-4B Do Not Directly Stimulate Dendritic Cells

It was previously reported that GSL-4A purified from *Sphingomonas paucimobilis* stimulated an inflammatory cytokine

Figure 3. The Synthesis of GSL-4 Compounds

(A) (a) (1) **13**, TMSOTf, toluene, −40°C to −25°C, 1 hr, 96%; (2) NaOMe, MeOH, rt, 15 hr, 78%. (b) (1) **15**, TMSOTf, CH_2Cl_2/Et_2O, −60°C to −20°C, 1.5 hr; (2) H_2O_2, LiOH, MeOH/THF, rt, 15 hr; (3) KOH, MeOH/THF, rt, 15 hr; (4) MeI, $KHCO_3$, DMF, rt, 15 hr, 61% (4 steps). (c) **13**, TMSOTf, toluene, −40°C to 0°C, 1.5 hr, 39%. (d) (1) $Pd(OAc)_2$, PPh_3, MeOH, CH_2Cl_2, rt, 24 hr; (2) Cl_3CCN, DBU, CH_2Cl_2, rt, 1 hr, 48% (2 steps). (e) **9**, TMSOTf, toluene, Et_2O, −10°C to rt, 3 hr, 13%.
(B) (a) (1) TFA, CH_2Cl_2, 0°C to rt, 2 hr; (2) myristic acid, HATU, *N*-methylmorpholine, CH_2Cl_2, 1,4-dioxane, rt, 18 hr, 73% (2 steps). (b) (1) H_2O_2, LiOH, MeOH/THF, rt, 15 hr; (2) KOH, MeOH/THF, rt, 15 hr; (3) Na, NH_3, THF, −78°C, 45 min, 45% (3 steps).

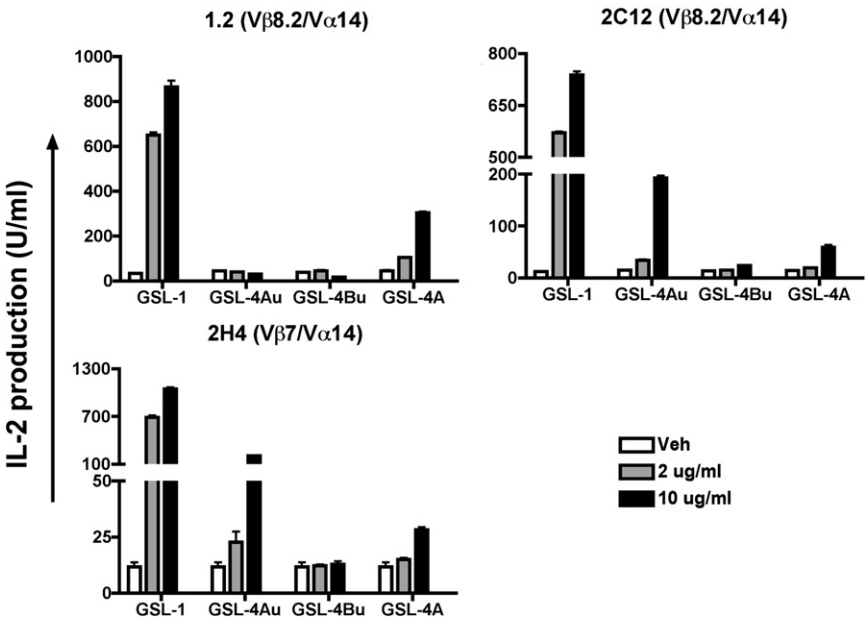

Figure 5. Stimulation of Vα14*i* NKT Cell Hybridomas by Tetrasaccharide GSL Ags

Antigen presenting cells expressing mCD1d were pulsed with the indicated amounts of synthetic GSL compounds and then cultured with Vα14*i* NKT cell hybridomas 1.2 (top left), 2C12 (top right), or 2H4 (bottom). IL-2 amounts measured in the culture supernatant are shown. The error bars indicate the SEM of triplicate measurements and the data are representative of three separate experiments.

production by human mononuclear cells (Krziwon et al., 1995). We tested whether synthetic GSL-4A and GSL-4B directly stimulate mouse bone marrow-derived dendritic cells (DCs) by stimulating toll-like receptor signaling. DCs were cultured with GSL Ags, including GSL-1, GSL-4A, GSL-4Au, and GSL-4B, or with LPS or CpG for 24 hr, and cytokines TNF (Figure S1A), IL-12p70 (Figure S1B), and IL-12p40 (data not shown) were measured in the culture supernatant. GSL-4A and GSL-4B did not induce the production of innate cytokines by DCs, although positive controls including LPS and CpG oligodeoxynucleotides were effective (Figure S1). Furthermore, the expression of CD40, CD80, and CD86 on DCs was not increased by the addition of GSL-4A or GSL-4B, although LPS and CpG did cause increased expression of these surface molecules, indicative of DC maturation (data not shown). These data show that GSL-4 compounds do not directly stimulate mouse DCs for maturation, activation, and the production of inflammatory cytokines.

Vα14*i* NKT Cell Activation by GSL with an Altered Sphingosine

We also analyzed the antigenic potency of a GSL Ag with a natural sphingosine found in many *Sphingomonas* species longer than the previously tested C18:0 sphingoid base. A synthetic GSL containing a linked galactose (GalGSL) and a ceramide with C14 fatty acid, similar to the *Sphingomonas* lipid, and a cyclopropyl-C21:0 chain sphingosine (GalGSL-C21cycl) was prepared and analyzed. Figure 7A shows GalGSL-C21cycl was antigenic for Vα14*i* NKT hybridomas 1.2 and 2H4, and the stimulation was dose dependent. Compared with αGalCer containing the identical carbohydrate moiety, however, GalGSL-C21cycl was a weaker Ag, although it was more potent than galacturonic acid-containing GSL-1.

We next tested whether GalGSL-C21cycl could stimulate Vα14*i* NKT cell hybridomas in an APC-free assay. GalGSL-C21cycl could stimulate Vα14*i* NKT cell hybridomas when this compound was preincubated on mCD1d-coated microwells (Figure 7B). These data indicate that lysosomal processing is not required for responses to GalGSL-C21cycl, similar to αGalCer and GSL-1. To determine whether GalGSL-C21cycl

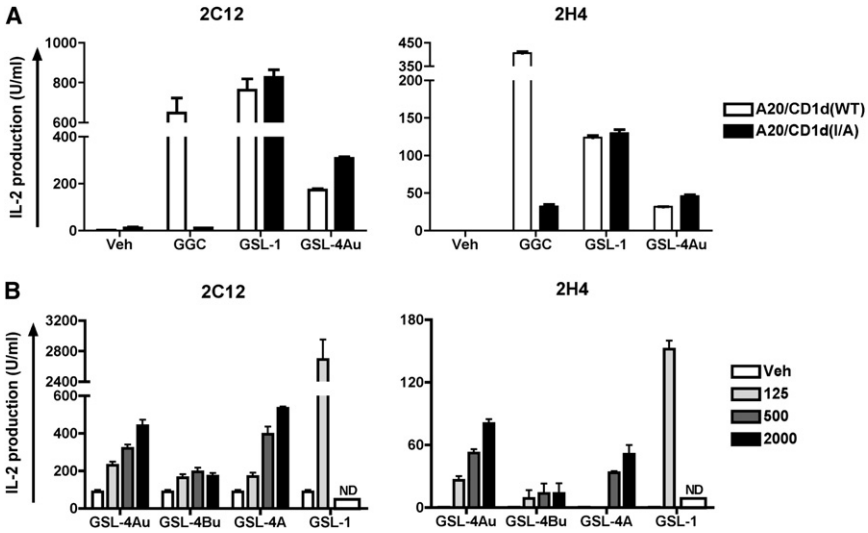

Figure 6. Internalization and Ag Processing Are Not Required for Stimulation of Vα14*i* NKT Cell Hybridomas by GSL-4A

(A) Antigen presenting cells expressing wild-type mCD1d, designated A20/CD1d(WT), or cytoplasmic tail mutant mCD1d, A20/CD1d (I/A), were pulsed with the indicated amounts of GSL compounds and cultured with Vα14*i* NKT hybridomas 2C12 (left) or 2H4 (right). GGC, αGal(1-2)αGalCer. IL-2 amounts in the culture supernatant are shown. The error bars indicate the SEM of triplicate measurements and the data shown are representative of three separate experiments.

(B) The indicated amounts (ng/well) of compounds were incubated in wells coated with mCD1d. Stimulation of Vα14*i* NKT hybridomas 2C12 (left) or 2H4 (right) was determined by the production of IL-2 in the culture supernatant. The error bars indicate the SEM of triplicate measurements and the data shown are representative of three separate experiments. ND, not done.

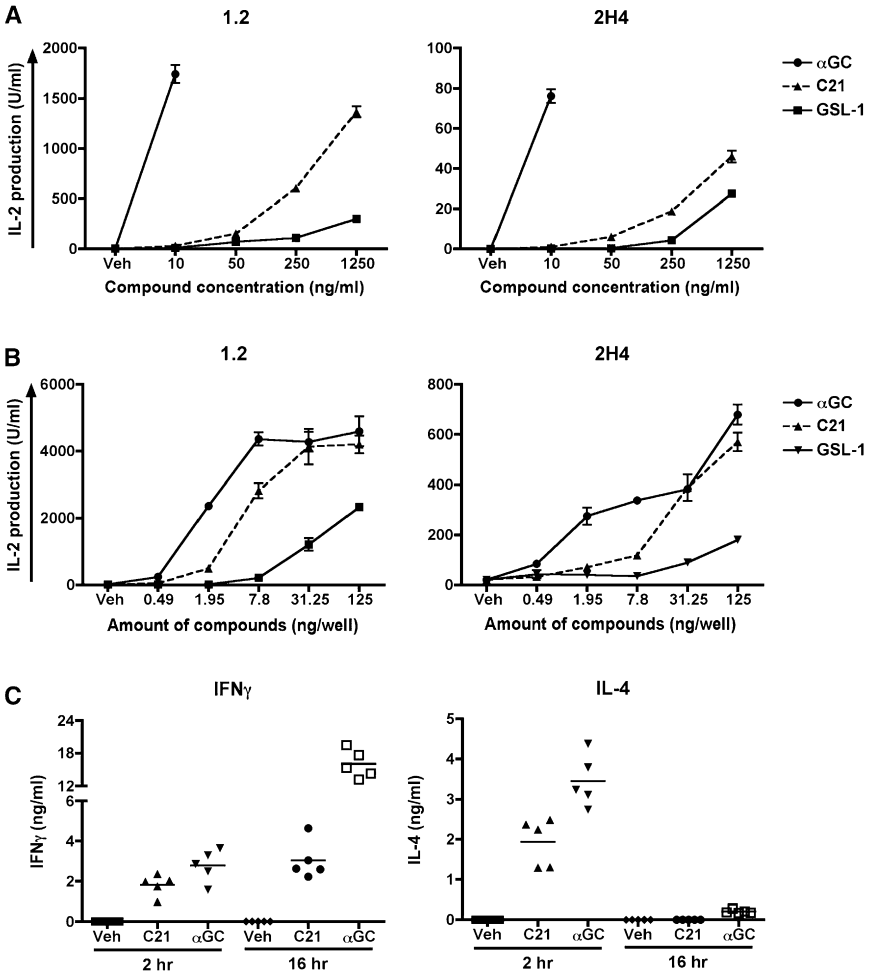

Figure 7. Antigenic Activity of GalGSL-C21cycl (C21)

(A) Comparison of the Vα14*i* NKT cell responses to αGalCer, GSL-1, and GalGSL-C21cycl (C21) by A20 transfectants expressing mCD1d. Antigen presenting cells were pulsed with vehicle or the indicated amounts of GSL compounds, and cultured with Vα14*i* NKT hybridomas 1.2 (left) or 2H4 (right). The error bars indicate the SEM of triplicate measurements.

(B) The indicated amounts (ng/well) of compounds were incubated in wells coated with mCD1d. Stimulation of Vα14*i* NKT hybridomas, 1.2 (left) or 2H4 (right), was determined by the production of IL-2 in the culture supernatant. The error bars indicate the SEM of triplicate measurements and the data shown are representative of three separate experiments.

(C) Serum IFNγ and IL-4 in mice treated with GalGSL-C21cycl. Mice (five mice/group) were injected intravenously with GalGSL-C21cycl (10 μg) or αGalCer (1 μg), and serum IFNγ and IL-4 was measured at 2 and 16 hr after injection. C21, GalGSL-C21cycl; αGC, αGalCer. The data shown are representative of two separate experiments.

can stimulate Vα14*i* NKT cells in vivo, it was injected into mice and IFNγ and IL-4 in the serum were measured. GalGSL-C21cycl could induce a substantial amount of IFNγ and IL-4 in the sera of mice by only 2 hr after injection, although this response was weaker than the response to αGalCer (Figure 7C). The appearance of cytokines in the serum is an indication of substantial antigenic potency, however, as cytokines were not observed with a number of glycolipid Ags, including GSL-4A (data not shown).

DISCUSSION

The *i*NKT cell TCR recognizes glycolipid Ags presented by CD1d. Until recently, however, the only Ags known to stimulate most *i*NKT cells were αGalCer and closely related synthetic compounds. Subsequently, it was shown that *Sphingomonas* bacteria, which are abundant throughout the environment, also have GSLs with α-linked sugars that are antigenic for *i*NKT cells. *Sphingomonas* glycolipids are heterogeneous, however, not only if different strains are compared, but in addition a single strain can produce several glycolipids with structural variations in the lipid, including the sphingosine base, as well as the sugar (Kawahara et al., 1991, 2000, 2001, 2002, 2006). The *Sphingomonas* compounds tested in the first *i*NKT cell studies included purified material, likely to contain several related GSLs, or synthetic versions of *Sphingomonas* GSLs designed for maximum similarity to αGalCer. Therefore, these synthetic compounds included only the 18 carbon sphingosine base of αGalCer, one of three sphingosine bases typical for *Sphingomonas spp*. (Kawahara et al., 1991, 2000, 2002, 2006), and monosaccharide head groups, either galacturonic or glucuronic acid (Kawahara et al., 2000, 2002, 2006). As a consequence, it remained unknown whether the diverse *Sphingomonas* glycolipids all have antigenic activity, and whether some of those with more complex sugars require carbohydrate antigen processing in order to be recognized by *i*NKT cells. To determine whether these variant GSLs from *Sphingomonas* bacteria are Ags for *i*NKT cells, we chemically synthesized antigens with tetrasaccharide head groups or with a C21 cyclopropyl sphingosine base and tested these for antigenic potency.

Here we demonstrate that synthetic versions of the *S. paucimobilis* tetrasaccharide-containing GSL-4A compounds are weak antigens for Vα14*i* NKT cell hybridomas, but a GSL-4B compound related to Ags from *S. adhaesiva* was not antigenic. Neither synthetic Ag could induce DC activation, although this had been reported earlier for purified *S. paucimobilis* GSL-4A (Kawahara et al., 2001), raising the possibility that some other components in the purified material may have been responsible for the earlier result. Whereas GSL-4A could induce IL-2 release from all three Vα14*i* NKT cell hybridomas tested, the responses induced by these compounds were much weaker than the response to the monosaccharide-containing GSL-1 antigen. IL-2 release by Vα14*i* NKT cell hybridomas was observed in an APC-free system, using CD1d-coated plates, demonstrating

that carbohydrate antigenic processing was not required. Additionally, by comparing the response to APC-expressing wild-type CD1d to the response induced by a CD1d protein bearing a single point mutation (I to A) in the cytoplasmic tail that impairs localization to lysosomes, we confirmed that carbohydrate antigen processing did not significantly enhance the response to GSL-4A, nor did it induce a response to GSL-4B.

Data from a previous report also suggested that purified *S. paucimobilis* GSL-4A could induce a weaker response from *i*NKT cells than a monosaccharide-containing GSL when fixed APC were used to stimulate *i*NKT cell hybridomas (Sriram et al., 2005). In data not shown, however, it was stated that the response to GSL-4A was almost equivalent to a GSL containing glucuronic acid as the monosaccharide head group (Sriram et al., 2005). In our studies, by contrast, using either unfixed APC, APC expressing a CD1d mutant for lysosomal trafficking, or CD1d-coated plates, GSL-4A was a much weaker Ag than GSL-1. For example, in the CD1d-coated plate assay, the lowest dose of GSL-1 tested (125 ng/well) elicited significantly more IL-2 release than 16-fold more GSL-4A. It is therefore possible that minute amounts of contaminating GSL with a monosaccharide head group could be responsible for the results published earlier. By contrast, it was recently reported that synthetic GSL-4A could not stimulate mouse or human *i*NKT cells (Long et al., 2007). We are not certain why the weak reactivity to GSL-4A was not observed, but the CD1d-coated plate assay is highly sensitive, and the A20 B cell transfectants we used express very high amounts of CD1d. Our results are consistent, however, with the finding that GSL-4A could not be processed to GSL-1 because APC lack the enzyme to cleave the *N*-glucosamine that GSL-4A contains (Long et al., 2007). GSL-4B apparently also could not be efficiently processed to generate GSL-1. GSL-4B does not contain *N*-glucosamine and, in addition to glucuronic acid, it has only glucose and galactose sugars that, theoretically, should be subject to lysosomal processing. We conclude that there must be limitations in the ability of APC to process carbohydrate antigens, besides the presence of sugars such as *N*-glucosamine. The reasons for this are not known, but it is possible that GSL-4B is not efficiently transported to lysosomes, or that it does not interact with saposins or other factors required to generate the final, processed GSL-1 product.

Although it is the hydrophilic carbohydrate head group that is exposed for TCR recognition, modifications of the lipid tails of glycolipid Ags can affect antigenic potency. For example, for αGalCer, it was shown that truncation of the sphingosine base in OCH or unsaturation of the acyl chain in C20:2 can influence not only antigenic strength but also the pattern of cytokine production (Miyamoto et al., 2001; Yu et al., 2005). Furthermore, the antigenic activity of diacylglycerol Ags from *Borrelia burgdorferi* is strongly influenced by the length of the acyl chains and the number of unsaturated bonds, as has also been observed for those *i*NKT cells that recognize phospholipid Ags (Kinjo et al., 2006). The recent structure of the trimolecular complex of the *i*NKT cell TCR interacting with a complex of αGalCer bound to human CD1d shows extensive contacts between the TCR and the top of the α helices of the CD1d molecule (Borg et al., 2007). As there is evidence that bound lipids can influence the conformation of these helices (McCarthy et al., 2007; Wu et al., 2006), it is understandable that the chemical composition of the buried lipid could influence TCR recognition. *Sphingomonas* bacteria synthesize GSL Ags with sphingosine chains that are mixtures with C20:1 and a cyclopropyl ring containing C21:0 (C21cycl), in addition to C18:0 (Kawahara et al., 1991, 2000, 2002, 2006). We synthesized a GSL containing a C21cycl sphinogsine (GalGSL-C21cycl) and tested its antigenic activity in two assays. GalGSL-C21cycl induced a substantial amount of IFNγ and IL-4 in serum of mice at 2 hr after injection, and the cytokine pattern was not too different from that induced with αGalCer, although the response to GalGSL-C21cycl was weaker and the late IFNγ in the serum was decreased proportionately somewhat more than the IL-4. GalGSL-C21cycl has a galactose head group, which provides a stronger immune stimulus than antigens containing identical lipids with galacturonic acid (Wu et al., 2006). Ags with glucuronic acid are even weaker than those with galacturonic acid (Kinjo et al., 2005). Although GalGSL-C21cycl shares the same galactose head group as αGalCer, these compounds are not directly comparable, as they have additional differences. GalGSL-C21cycl is typical of the ceramide lipids in *Sphingomonas* GSLs in that it has a C14 rather than the C26 acyl chain of αGalCer, and it lacks the 4 position hydroxyl in the sphingosine base. Whereas neither modification by itself should have a large effect on antigenic potency (Sidobre et al., 2004; Zajonc et al., 2005), the combined effects of the two changes could be highly significant. Therefore, although it is not possible to assess quantitatively the effect of the C21cycl sphingosine on antigenic potency, the results clearly show that the F′ pocket of mCD1d, which binds the sphingoid base, can accommodate a GSL that contains this type of sphingosine to form an epitope that stimulates *i*NKT cells. Structural studies will be required to determine how this longer sphingosine base fits into the F′ pocket, and how it might affect the orientation of the exposed α-linked sugar and the induced fit of CD1d that occurs when some GSL Ags bind to it (Koch et al., 2005; McCarthy et al., 2007; Zajonc et al., 2005).

SIGNIFICANCE

We have shown that the different *Sphingomonas* GSLs vary greatly in their ability to activate the invariant TCR of *i*NKT cells. As opposed to GSLs containing a single acidic sugar or galacturonic or glucuronic acid, the oligosaccharide-containing Ags we tested are either not antigenic or only weakly antigenic. It is possible that the additional sugars in α1-4 linkage to glucuronic acid provide steric hindrance for interaction with the *i*NKT cell TCR. The crystal structure of the human invariant NKT cell TCR bound to αGalCer CD1d complexes suggests that it would be difficult for the TCR to accommodate additional sugars in the 4′ position (Borg et al., 2007). Moreover, these oligosaccharides are not processed to generate the much more potent GSL-1 Ag that contains a single sugar. The reasons for this inefficient processing are unknown, but they must include factors besides the presence of a glucosamine sugar, which mammalian cells cannot efficiently cleave from GSLs. Therefore, we conclude that there must be several factors that limit the ability of antigen presenting cells to process and present more complex glycolipid structures. By contrast to the GSLs with

tetrasaccharides, a GSL containing a C21cycl sphingosine was a fairly potent Ag, indicating that the F′ pocket of mCD1d that binds the sphingosine can accommodate bases longer than C18:0. We conclude that individual *Sphingomonas* species synthesize mixtures of strongly antigenic, weakly antigenic, and nonantigenic glycolipids, based upon changes in the sugar and/or the lipid moieties. The balance of different GSLs synthesized by an individual strain reflects culture conditions and likely also reflects conditions in the environment. Based on this, we speculate that the modulation of GSL synthesis by *Sphingomonas* bacteria could serve as an immune evasion mechanism to avoid the responses of *i*NKT cells.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines

αGalCer, αGal(1-2)αGalCer, and *Sphingomonas* GSL-1 (previously also called GSL-1′ or GalAGSL) were described previously (Kawano et al., 1997; Kinjo et al., 2005; Prigozy et al., 2001; Wu et al., 2006). B cell lymphoma A20 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The generation of A20 transfectants expressing wild-type mCD1d or mCD1d with a single point mutant (I/A) in the cytoplasmic tail were characterized previously (Elewaut et al., 2003; Lawton et al., 2005), as were the Vα14*i* NKT cell hybridomas 1.2 (Vβ8.2/Vα14*i*), 2C12 (Vβ8.2/Vα14*i*; with a different CDR3β), and 2H4 (Vβ7/Vα14*i*) (Matsuda et al., 2000).

Antigen Presentation Assays

The antigen presentation assay has been described before (Lawton et al., 2005). Briefly, A20 cells transfected with wild-type or single point mutant (I/A) mCD1d were used as APC. APC (1×10^6/ml) were pulsed with vehicle or the indicated amounts of GSL Ags overnight, followed by washing with medium. APC (1×10^5 per well) were seeded in 96-well plates and cultured in the presence of 5×10^4 Vα14*i* NKT cell hybridomas for 20–24 hr. IL-2 release was evaluated in a sandwich ELISA using rat anti-mouse IL-2 mAbs (BD Biosciences, San Jose, CA, USA).

Cell-Free Antigen Presentation Assay

Stimulation of Vα14*i* NKT cell hybridomas on microwell plates coated with soluble mCD1d was carried out according to published protocols (Naidenko et al., 1999; Sidobre et al., 2004; Tupin and Kronenberg, 2006), with slight modifications. Briefly, the indicated amounts of compounds were incubated for 24 hr in microwells that had been coated with 1.0 μg of mCD1d. After washing, 5×10^4 to 1×10^5 Vα14*i* NKT hybridoma cells were cultured on the plate for 20 hr, and IL-2 in the supernatant was measured by ELISA.

Mice and In Vivo Experiments

C57BL/6 mice were from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed under specific pathogen-free conditions and the experiments were approved by the Institutional Animal Care and Use Committee of the La Jolla Institute of Allergy and Immunology. Mice were injected intravenously with 10 μg of GalGSL-C21cycl, 1 μg of αGalCer, or vehicle (0.025% Tween 20 in saline). Serum was collected 2 and 16 hr later, and IFNγ and IL-4 in serum were measured by ELISA (BD Biosciences).

SUPPLEMENTAL DATA

Supplemental Data contain one figure, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/7/654/DC1/.

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