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Effects of Lipid Chain Lengths in α-Galactosylceramides on Cytokine Release by Natural Killer T Cells

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Antigen presentation to T cells plays a central role in adaptive and innate immunity.[1] While the responses to antigen presentation can be complex, the process of antigen binding, presentation, and recognition by T cell receptors is fundamentally a series of molecular recognition events. In recent years glycolipid presentation by CD1 proteins has emerged as an important aspect of antigen recognition.[2] For example, a member of the CD1 family of proteins, CD1b, presents glycolipids from mycobacteria (e.g., *Mycobacterium tuberculosis*) to T cells,[3] which in turn release proinflammatory cytokines.[4] Another CD1 protein, CD1d, has been shown to present α-glycosylceramides (e.g., **1** in Figure 1) to a subset of T cells termed natural killer T cells (NKT cells),[5] which are primed to produce and release an array of cytokines.[6] This class of glycolipids was identified from screening of marine natural products for antitumor activity.[7] Preliminary structure−activity studies focusing on antitumor properties suggested that an acyl chain of approximately 26 carbons and a phytosphingosine base containing 18 carbons gave the most active compound.[7b]

In vitro and in vivo studies with NKT cells indicate that an array of cytokines is released in response to **1** presented by CD1d[6] including proinflammatory (e.g., interferon-γ (INF-γ)) and immunomodulatory cytokines (e.g., interleukin 4 (IL-4)). Release of proinflammatory cytokines is believed to be responsible for the antitumor,[7] antiviral,[8] antibacterial[9] effects of **1**. On the other hand, immunomodulatory cytokines produced by NKT cells in response to **1** can attenuate proinflammatory responses and thereby delay or prevent the onset of some autoimmune diseases in animal models including type 1 diabetes[10] and animal encephalomyelitis[11] (a model of multiple sclerosis). In some cases, the opposing responses of NKT cells to **1** (proinflammatory vs immunomodulatory) offset one another, resulting in no effect of **1** on disease states in animal models.[12] To fully utilize the responses of NKT cells medicinally, it would be advantageous to bias their responses to glycolipids to *either* a proinflammatory *or* an immunomodulatory response.

Little information is available about how α-galactosylceramide structure relates to the cytokine release profiles of NKT cells. It has been suggested that the length of the phytosphingosine chain can influence cytokine release.[13] Glycolipid **2b** (Figure 1) was shown to increase the amount of IL-4 relative to that of INF-γ released by NKT cells and thereby to offer additional protection against animal encephalomyelitis as compared to **1**. However, the extent to which glycolipid chain lengths influence cytokine release profiles from NKT cells has not been established.

Using an efficient synthesis of α-galactosylceramides, we have prepared a series of glycolipids in which the lipid chain lengths have been incrementally varied (**2a**−**c** and **3a**−**c**, Figure 1). The responses of NKT cells to these glycolipids have been determined,

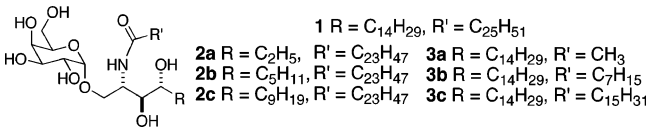
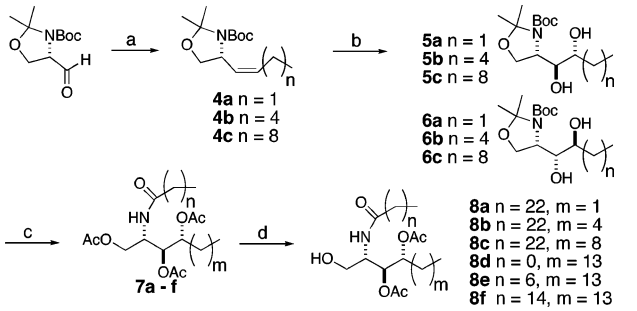


Figure 1. Structures of α-galactosyl ceramides.

Scheme 1. Synthesis of Glycosyl Acceptors **8a**−**f**[a]



[a] Reagents (yields in parentheses): a) $Ph_3P{=}CH(CH_2)_nCH_3$, THF (60−63%). b) OsO_4, NMO, *t*-BuOH, H_2O (51−72% 5, 25−28% 6). c) HCl, THF; MeOH; $HO_2C(CH_2)_nCH_3$, DCC, NHS, THF; Ac_2O, Et_3N, DMAP, THF (45−47%). d) MeONa, MeOH; TBSCl, imidazole, THF; Ac_2O, Et_3N, DMAP, THF; HF, THF (50−57%).

and we have found that further truncation of the phytosphingosine lipid chain increases the IL-4 vs INF-γ bias of released cytokines. In similar fashion, the length of the acyl chain in α-galactosylceramides influences cytokine release profiles.

We prepared α-galactosylceramides via a route that differs from those reported by other groups[7b,14] but in our hands proved to be very efficient. Phytosphingosines were derived from Garner's aldehyde (Scheme 1). Olefins **4a**−**c** were prepared as described by Berova and co-workers[15] who generated phytosphingosines from analogous alkenes using prepared phytosphingosines from alkenes analogous to **4a**−**c** using Sharpless' asymmetric dihydroxylation procedure (giving a 1-to-1 mixture of the D-*ribo* (**5**) and L-*arabino* (**6**) diols). We found that use of osmium tetraoxide with NMO alone gave a readily separable 2-to-1 mixture of diols **5a**−**c** and **6a**−**c**. Deprotection of **5a**−**c** gave the corresponding phytosphingosines, which were coupled with varied carboxylic acids followed by peracylation to give **7a**−**f**. Acylation generating **7a**−**f** was performed to simplify purification of these amphiphilic compounds. A protection−deprotection strategy was used to generate **8a**−**f**. Galactosyl donor **10** was prepared in few steps from commercially available anhydrogalactose **9** (Scheme 2). We found that use of this galactosyl donor offered advantages over perbenzylated donors in that it allowed incremental deprotection of the galactosylceramides and better purification strategies. Coupling of donor **10** with ceramides **8a**−**f** gave predominantly the α-anomers contaminated with small amounts of the β-anomers. Separation of these anomers with the full array of protecting groups intact proved to be very difficult, and removal of all of the protecting groups from **11a**−**f**

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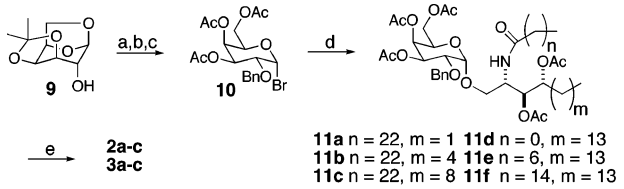
Scheme 2. Synthesis of Glycosyl Donor **10** and Coupling to Acceptors **8a**−**f** to Generate **2a**−**c** and **3a**−**c**[a]



[a] Reagents (yields in parentheses): a) NaH, BnBr, TBAI, DMF (95%). b) Ac_2O, TFA (90%). c) HBr, AcOH, CH_2Cl_2(75%). d) AgOTf, **8a**−**f**, 4Å mol sieves, CH_2Cl_2(45−62%). e) H_2, Pd/C, EtOAc, EtOH; MeONa, MeOH (84−90%).

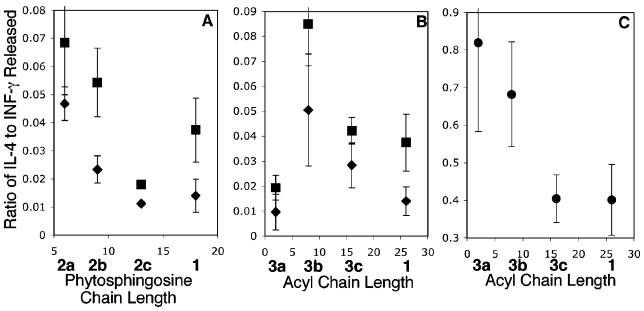


Figure 2. Ratios of cytokines released from cell populations containing NKT cells stimulated with the glycolipids indicated. Standard errors from multiple experiments are indicated. (A) Cytokines produced by spleen cells obtained from the following strains of mice: (◆) B6 and (■) BALB/c stimulated with **2a**−**c**. (B) Cytokines produced by spleen cells obtained from the following strains of mice: (◆) B6 and (■) BALB/c stimulated with **3b**−**c**. (C) (●) Cytokines produced by a human NKT cell line stimulated by **3a**−**c**.

yielded an inseparable mixture of anomers. However, deprotection of the C2″ hydroxyl group (on galactose) allowed ready removal of the trace amounts of the β-anomers. Subsequent removal of the remaining acetate groups yielded α-galactosylceramides **2a**−**b** and **3a**−**c**.

Presentation of glycolipids to NKT cells in vivo requires antigen-presenting cells that express CD1d. For one series of assays mouse spleen cells were used because this cell population contains both a relatively high number of NKT cells and antigen presenting cells. To verify that cytokine release in response to **2a**−**c** and **3a**−**c** was not strain dependent we measured cytokine release from splenocytes from two strains of mice. In addition, a human NKT cell line was used, along with antigen presenting cells,[16] to assay cytokine release. Cytokine concentrations were measured using ELISA.

The focus of the cytokine release assays was to compare relative amounts of INF-γ and IL-4 released in response to **2a**−**c** and **3a**−**c** as a measure of the bias of the NKT cells toward either proinflammatory (INF-γ) or immunomodulatory (IL-4) responses. Ratios of IL-4 to INF-γ were calculated and plotted vs phytosphingosine and acyl chain lengths (Figure 2).

Truncation of the phytosphingosine chain of **1** results in release of greater amounts of IL-4 by NKT cells in both mouse cell lines (Figure 2A). A similar effect was observed with human NKT cells (data not shown). This effect extends to relatively short chain lengths. Notably, the ratio of IL-4 to INF-γ released by NKT cells is also influenced by the length of the acyl chain (Figure 2, B and C), with shorter chain lengths increasing this ratio. The exception is with mouse NKT cells and **3a** (Figure 2B). Glycolipid **3a** caused release of relatively small amounts of both cytokines, possibly due to its inability to form a stable complex with T cell receptors (see Supporting Information). In contrast to data from mouse NKT cells, **3a** caused release of both cytokines from human NKT cells with a bias toward IL-4 (Figure 2C). Assays performed at varied glycolipid concentrations and with mouse NKT cells verified that IL-4/INF-γ ratios were independent of glycolipid concentration (see Supporting Information).

From studies of **1** and **2b**, Oki et al.[17] have suggested that truncation of the phytosphingosine chain of **1** leads to a less stable complex with CD1d and that INF-γ release by NKT cells requires longer stimulation by CD1d−glycolipid complexes than IL-4 release. The results obtained with **2a**−**c** and **3a**−**c** are consistent with this model. Furthermore, results with **3a** and mouse NKT cells suggest that loss of too much lipid results in complexes with CD1d too unstable to interact well with NKT cell receptors.

The profound influence that glycolipid-mediated stimulation of NKT cells has on the immune system has been demonstrated in the antitumor effects of **1** and the ability of **1** to inhibit the onset of autoimmunity. The fact that these are two different and presumably opposing responses to **1** suggests that **1** may not be an optimal ligand for generating proinflammatory or immunomodulatory responses. Our results suggest that there is a correlation between lipid chain length and cytokine release profiles, and the chain-shortened glycolipids bias cytokine release toward an immunomodulatory response. This property of glycolipids **2a** and **3a** may prove useful in altering immune responses and in the treatment of certain autoimmune diseases.

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Supporting Information Available: Experimental procedures for preparation of **2a**−**c** and **3a**−**c**; results from NKT cell labeling with glycolipid-CD1d tetramers; comparison of cytokine ratios from varied concentrations of glycolipids; amounts of IL-4 and INF-γ produced in response to each glycolipid. This material is available free of charge via the Internet at http://pubs.acs.org.

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