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# Structure-Based Discovery of Glycolipids for CD1d-Mediated NKT Cell Activation: Tuning the Adjuvant versus Immunosuppression Activity

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α-Galactosylceramide (α-GalCer; **1**)[1] is a derivative of a marine sponge natural product and has been shown to possess antitumor activity.[2] Further studies revealed that α-GalCer activates natural killer T (NKT) cells through CD1d-mediated antigen presentation. Unlike other T cells, NKT cells are restricted to a nonmajor histocompatibility complex (MHC) molecule, CD1d, which binds lipids and glycolipids instead of peptides.[3,4] α-GalCer, when bound to CD1d, activates NKT cells by means of T cell receptor (TCR) recognition to produce T helper 1 (Th1) and T helper 2 (Th2) cytokines, such as interferon-$\gamma$ (IFN) and interleukin 4 (IL-4), respectively.[5]

The production of Th1 cytokines is thought to correlate with the antitumor, antiviral/bacterial and adjuvant effects of α-GalCer, while Th2 cytokine production is thought to correlate with the amelioration of certain autoimmune diseases (e.g., type 1 diabetes and multiple sclerosis).[6−8] However, the efficacy of α-GalCer has been limited because of the reciprocal inhibition exhibited by Th1 and Th2 cytokines. In a Phase I study, α-GalCer was ineffective in the treatment of solid tumors possibly because the therapeutic effects of IFN-$\gamma$ were hindered by IL-4 and thus gave no net benefit.[9] Therefore, compounds which increase the selectivity toward either Th1 or Th2 cytokines responses may be more advantageous.[10,11]

Structure−activity relationship studies of α-GalCer analogues revealed that truncation of the fatty acyl or the phytosphingosine group allows for more selective Th2 response.[12,13] OCH, a phytosphingosine truncated analogue of α-GalCer, selectively induces Th2 cytokines from NKT cells.[14] Also, introduction of double bonds into the fatty acyl chain of α-GalCer, C20:1 cis/trans and C20:2 analogues, seemed to bias toward Th2 responses.[15] The C-glycoside analogue of α-GalCer seems to bias toward the Th1 response but is less potent than α-GalCer during short incubation times.[16,17]

Although numerous factors likely play a role in shifting the cytokine profile, stability of the CD1d/glycolipid complex may be a contributing cause. Time-course cytokine release profiles show that, upon Vα14 NKT-cell activation, IL-4 levels peak within 2 h while IFN-$\gamma$ levels peak 12 h after treatment with α-GalCer in mice.[15,16] IFN-$\gamma$ production requires longer TCR stimulation than IL-4.[13] Full activation of NKT cells seems to require a high-affinity TCR interaction with a long half-life.[18,19] Thus, a model to bias the cytokine profile toward a Th2 response would be to generate less stable glycolipid/CD1d complexes to shorten NKT cell stimulation times. Conversely, a biased Th1 response could be created by prolonged stimulation of TCRs on NKT cells through increasingly stable glycolipid/CD1d complexes.

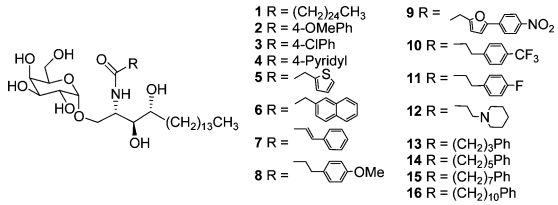


***Figure 1.*** Structure of α-GalCer **1** and fatty acyl chain analogues **2**−**16**.

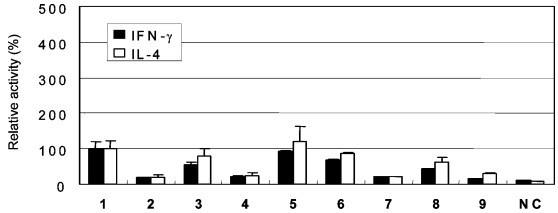


***Figure 2.*** IFN-$\gamma$ and IL-4 secretion by human NKT cell line when stimulated by 10 ng/mL of indicated glycolipids. IFN-$\gamma$ and IL-4 release was measured after 16 h of culture. Results are expressed as relative activities as mean of duplicate assays ± standard deviation. Representative data from one of three experiments are shown.

We have found that the bacterial glycolipid, GalA-GSL, activates NKT cells through CD1d-mediated antigen presentation.[20] The crystal structure of mouse CD1d/GalA-GSL[21] and activity study revealed that the 4′-OH of phytosphingosine chain and the galactose 6-OH are important in addition to the 3′-OH of phytosphingosine chain and galactose 2-OH to activate NKT cells by comparison with human CD1d/α-GalCer[22] and mCD1d/PBS-25.[23] These crystal structures confirmed that the lipid chains of α-linked glycosphingolipids were accommodated in two hydrophobic pockets. Numerous aromatic side-chain residues line the binding groove and could be utilized to make specific aromatic interactions. Specifically, Tyr73, Phe114, Phe70, and Trp114 of the A′ pocket seemed most accessible. This observation prompted us to introduce an aromatic group into the fatty acyl chain of α-GalCer with the hope of making a tighter binding glycolipid to CD1d.

In this study, we designed and synthesized a variety of fatty acyl chain analogues of α-GalCer (Figure 1) based on the available CD1d/glycolipid structures and evaluated NKT cell activation by measuring cytokine release profiles. We synthesized various fatty acyl chain analogues (Supporting Information Scheme S1 and Figure S1) and evaluated their ability to activate human Vα24NKT cells as measured by their IFN-$\gamma$ and IL-4 production relative to α-GalCer[20] (Figure 2). Shorter chain analogues **2**−**4** and **7**−**9** showed only moderate activity. However, 2-thienylacetyl analogue **5** and 2-naphthylacetyl analogue **6** demonstrated almost equal or better cytokine secretion than α-GalCer. These results suggest that the spacer chain length was too short to interact with the aromatic residues in the CD1d hydrophobic groove. These results prompted

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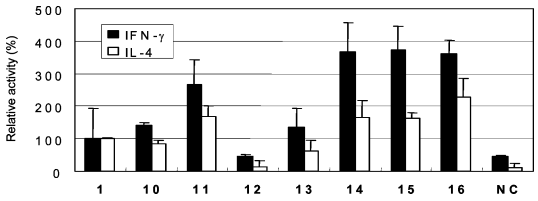


***Figure 3.*** IFN-$\gamma$ and IL-4 secretion by human NKT cell line when stimulated by 10 ng/mL of indicated glycolipids. IFN-$\gamma$ and IL-4 release was measured after 16 h of culture. Results are expressed as relative activities as mean of duplicate assays ± standard deviation. Representative data from one of three experiments are shown.

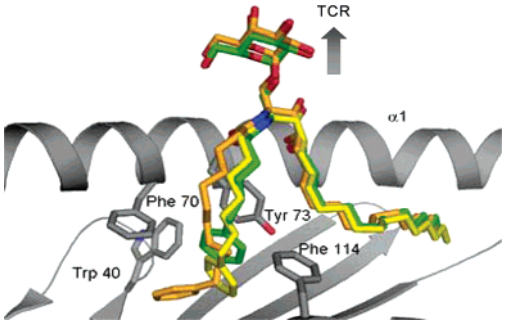


***Figure 4.*** Superimposition of the docking results of fatty acyl chain analogues **14**−**16** with hCD1d. The α2 helix is removed for clarity. **14** (green); **15** (yellow); **16** (orange).

us to evaluate analogues with a longer spacer chain (Figure 3). Elongation of the spacer chain length drastically enhanced overall cytokine production, while piperidine analogue **12** diminished their activity. 4-Fluorophenylpropionyl analogue **11** demonstrated more potent cytokine production than 4-$CF_3$ analogue **10** and 4-OMe analogue **8**. Of these compounds, the longer alkyl chain analogues **14**−**16** are 4 times more potent and biased for IFN-$\gamma$ secretion. These results show that it is possible to potentiate and tune the Th1/Th2 cytokine profile by the introduction of a terminal aromatic group with an appropriate length of spacer chain. To visualize the interactions between the designed analogues and human CD1d, Autodock 3.0[24] was utilized to model the binding of selected compounds in the hCD1d hydrophobic groove (Figure 4). **13**−**16** were individually docked, and their results did not vary significantly from the crystal structure of α-GalCer bound to hCD1d.[22] In each case, the phytosphingosine tail extended into the F′ pocket and the A′ pocket was occupied by the fatty acyl chain with the galactose headgroup presented in nearly the same configuration. Introduction of a terminal phenyl group in the α-GalCer analogues seemed to promote additional specific interactions between **14**, **15**, and the phenol ring of Tyr73 and between **16** and Trp40. Docking of compounds with a shorter spacer chain on the fatty acyl tail showed limited interaction between terminal functional groups and aromatic residues in the A′ pocket.

We also examined hCD1d binding of compounds **14**−**16** using isoelectric focusing (IEF) electrophoresis[25] (see Supporting Information). Compounds **14**−**16** demonstrated more potent inhibition of GT1b−hCD1d binding than α-GalCer and a less potent analogue **4**, supporting our hypothesis.

In conclusion, we have found that introduction of an aromatic group to the fatty acyl chain greatly enhances IFN-$\gamma$/IL-4 secretion and enables the tuning of Th1/Th2 cytokine profile, possibly through alteration of glycolipid/CD1d complex stability. Compounds **14**−**16** represent the first examples of NKT cell agonists which are more potent than α-GalCer and also exhibit a stronger Th1 cytokine response, probably due to enhanced binding to CD1d or selective interaction with $CD8^+$ versus $CD4^+$ NKT cells,[26] although this issue has been debated.[27] The origin of the enhanced potency and Th1 selectivity remains to be fully addressed. This study provides a new direction for the development of novel glycolipid-based immunotherapeutic agents which are more potent than α-GalCer and are able to exhibit greater Th1-type cytokine profiles.

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**Supporting Information Available:** Synthesis of fatty acyl chain analogues **2**−**35**; amounts of IFN-$\gamma$ and IL-4 secretion in response to these glycolipids; results from competitive binding study of hCD1d with glycolipids; complete refs 9, 15, 18, 20b, and 22. This material is available free of charge via the Internet at http://pubs.acs.org.

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