CHEMMEDCHEM

DOI: 10.1002/cmdc.200900350

The Roles of 3′ and 4′ Hydroxy Groups in α-Galactosylceramide Stimulation of Invariant Natural Killer T Cells

Chengfeng Xia,*[a, b] Wenpeng Zhang,[b] Yalong Zhang,[b] Wenlan Chen,[b] Janos Nadas,[b] Ryan Severin,[c] Robert Woodward,[b] Bin Wang,[d] Xin Wang,[d] Mitchell Kronenberg,[c] and Peng G. Wang*[b]

The marine-derived α-galactosylceramide (α-GalCer) was originally found to possess the ability to stimulate the secretion of various types of cytokines from invariant natural killer T (iNKT) cells.[1] Specifically, a major histocompatibility complex (MHC) I-like protein named CD1d presents α-GalCer on the surface of antigen-presenting cells (APC), enabling recognition by the T cell receptor (TCR) of iNKT cells.[2] Such recognition then triggers the release of a large amount of cytokines in a relatively short time by iNKT cells. These cytokines include the inflammatory T helper 1 (T_h1) cytokines (such as IFN-γ and IL-2) and immunomodulatory T_h2 cytokines (such as IL-4 and IL-10), which can regulate both the innate and adaptive immune responses. iNKT cells thus serve as the bridge between these two types of immune responses. Given these properties, α-GalCer has been extensively applied and studied in preclinical and clinical trials for treating various diseases such as cancer,[3] type I diabetes,[4] hepatitis,[5] and *Pseudomonas aeruginosa* infection.[6] There are promising results showing that α-GalCer can suppress and/or reject tumor growth in a murine model.[7]

α-GalCer differs from the mammalian glycolipids by its distinguished α-linkage between the sugar and lipid moieties; the anomeric linkage of mammalian glycolipids is in the β configuration.[8] This unique structure enables the acyl and phytosphingosine chains of α-GalCer to bind in the two deep pockets of CD1d while anchoring the sugar moiety above the surface of the protein in a position suitable for recognition by the iNKT TCR.[9] Such presentation by CD1d is stabilized by the 3-hydroxy group on phytosphingosine and the *O*-glycosidic linkage, which form H-bonding interactions with CD1d. Furthermore, the 2′-hydroxy group of galactose displays its importance by forming another hydrogen bond with CD1d (mouse Asp 153, human Asp 151). These interactions stabilize the glycolipid–CD1d complex and orient the sugar moiety in the correct position for recognition.[9a] In regards to this recognition, the crystal structure of the ternary TCR–glycolipid–CD1d complex reveals an additional crucial role for the 2′-hydroxy group of α-GalCer: interacting with TCR by forming a hydrogen bond with Gly 96α on the CDR3α loop.[9d] The conclusion from the crystal structure is consistent with experiments in which substitution of the equatorial 2′-hydroxy group with a fluoro group, hydrogen, acetamide,[10] or an axial hydroxy group (mannosylceramide)[1a] resulted in abolishment of activity. The 6′-hydroxy group, however, does not form a hydrogen bond with either CD1d or TCR,[9d] and this position was shown to tolerate some modifications without affecting the antigenicity of the glycolipid.[1a,11] Nonetheless, few conclusions have been reached for the 3′- and 4′-hydroxy groups, except some initial results that indicate the attachment of additional sugar moieties at these positions leads to elimination of activity.[11a,12] The crystal structure of the ternary complex does illustrate that the 3′- and 4′-hydroxy groups also form hydrogen bonds with the invariant TCR α-chain residues Ser 3α and Phe 29α.[9d] However, these hydrogen bonds may not be as important as that of the 2′-hydroxy group, because α-glucosylceramide (α-GlcCer), which has its hydroxy group in the equatorial instead of axial position as in α-GalCer, can still stimulate iNKT cells, albeit to a lesser extent.[1a]

Based on these observations, we hypothesized that the 4′-hydroxy group is not as important as the 2′-hydroxy group, although it contributes to the interaction with TCR, and some rational modifications can be carried out to change the profiles of the resulting derivatives. The role of the 3′-hydroxy group is ambiguous, however, thus dictating the need for a structure–activity relationship study to clarify its importance. In this study, we accordingly used a variety of analogues of α-GalCer with varying substitutions at the C3′ or C4′ positions of the galactose ring in order to elucidate the roles of these two hydroxy groups in iNKT cell stimulation.

In a variety of C3′-modified analogues, the hydroxy group was first replaced with the non-H-bonding azido group to give 3′-azido-α-GalCer **2** (Figure 1). The azido group was then reduced to an amino group, leading to an ionically charged analogue, 3′-amino-α-GalCer **3**, which was hypothesized to form a salt bridge with the TCR loops. The amino group was further converted into an acetamide group, yielding 3′-NAc-α-GalCer **4**, which should possess different preferences in H-bond formation relative to α-GalCer **1**. For the C4′ analogues, the acet-

[a] *Prof. Dr. C. Xia*[+]
State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650204 (China)
Fax: (+86) 871-522-3354
E-mail: xiachengfeng@mail.kib.ac.cn

[b] *Prof. Dr. C. Xia,*[+] *W. Zhang,*[+] *Dr. Y. Zhang, W. Chen, J. Nadas, R. Woodward, Prof. Dr. P. G. Wang*
Departments of Chemistry and Biochemistry
The Ohio State University
484 West 12th Avenue, Columbus, OH 43210 (USA)
Fax: (+1) 614-688-3106
E-mail: wang.892@osu.edu

[c] *R. Severin, Prof. Dr. M. Kronenberg*
Division of Developmental Immunology
La Jolla Institute for Allergy and Immunology
10355 Science Center Drive, San Diego, CA 92121 (USA)

[d] *Dr. B. Wang, X. Wang*
College of Pharmacy and State Key Laboratory of Element-Organic Chemistry, Nankai University, Tianjin 300071 (China)

[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200900350.

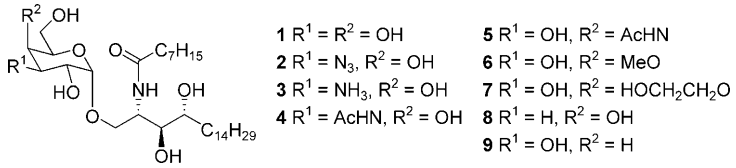


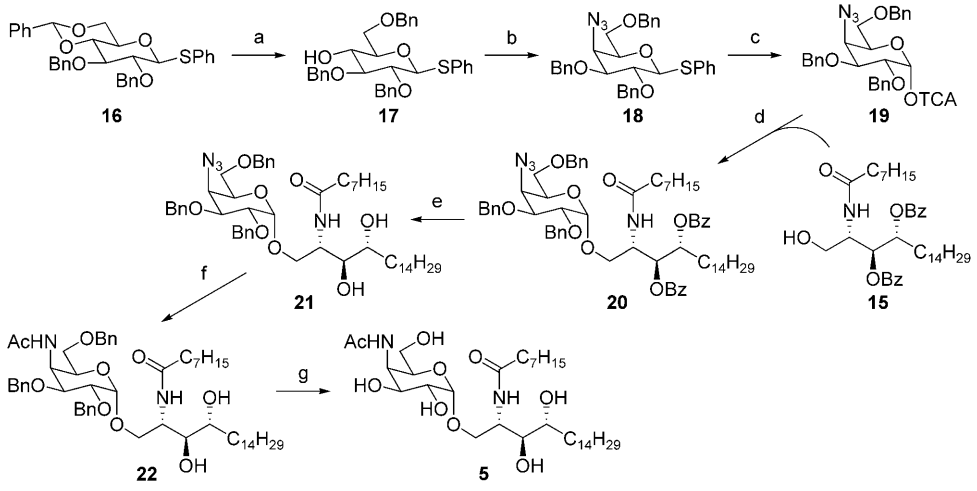
Figure 1. Structures of α-GalCer **1** and its analogues with various substituents at the C3′ or C4′ positions.

amide group was also exploited to replace the hydroxy group in the analogue 4′-NAc-α-GalCer **5**. In addition, a methyl group was introduced onto the 4′ hydroxy group so that the resulting 4′-MeO-α-GalCer **6** would only serve as H-bond acceptor, whereas a hydroxy group could act as both a donor and acceptor. To determine the importance of distance between the 4′-hydroxy group and TCR, 4′-(2-hydroxyethoxy)-α-GalCer **7** was also synthesized in order to separate the hydroxy group from the rest of the molecule by two additional methylene groups. Finally, the respective 3′- and 4′-deoxy analogues **8** and **9** were also used as control compounds.[13]

To synthesize the 4′-N-substituted analogue **5**, the corresponding donor **19** was prepared from the 4,6-benzylidene-protected glucosyl derivative **16** (Scheme 1). Selective reduction of the benzylidene under acidic conditions resulted in the 4-hydroxygalactose **17**. The newly freed hydroxy group was subjected to trifluoromethanesulfonic anhydride to form the triflic ester. This was then treated with sodium azide in DMF to introduce the azido group in the axial position, producing a pseudo-stereoisomer of the precursor. The thiophenol group was removed and converted into Schmidt's trichloroacetimide donor to increase the ratio of α-isomer product formation in the following glycosylation reaction. The glycolipid **20** was obtained in good yield with the use of TMSOTf as catalyst. We wanted to initially reduce the azido group by the Staudinger method using triphenylphosphine and water as reagents, and then to protect the amino group as acetylamide. However, the reaction only gave the triphenylphosphoimine, which was actually an intermediate of the reduction. Increasing the reaction temperature to 80 °C did not cause the intermediate to decompose. Interestingly, when the two benzoyl groups in the phytosphingosine were first hydrolyzed, and then followed by the same Staudinger reduction, the reaction went very smoothly and gave the amino product in excellent yield. A selective acetylation was performed in methanol to convert the amine group into amide **22**. This compound was then subjected to hydrogenation using palladium hydroxide as catalyst under H_2 (270 kPa) to give the 4′-NAc-GalCer **5**.

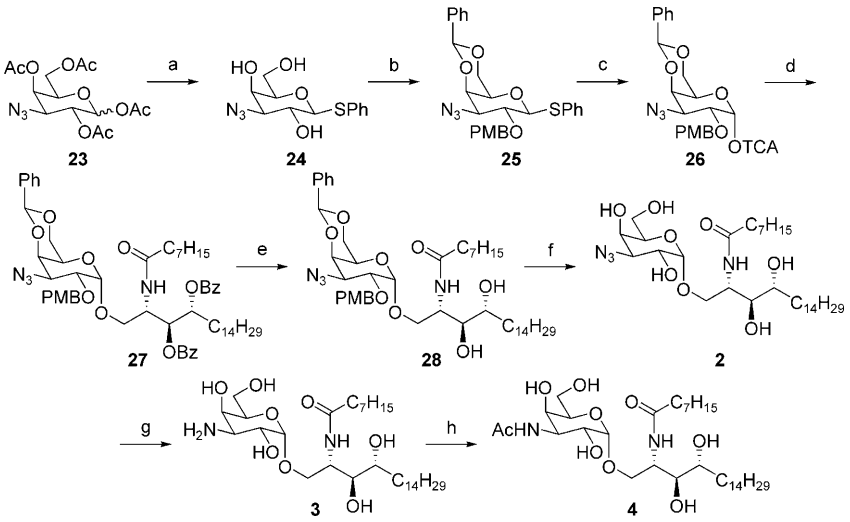
Preparation of 3′-N-substituted α-GalCer analogues **2**, **3**, and **4** took a similar approach as for **5**. The 3-azidogalactose derivative **23** was synthesized from diacetone-D-glucose in eight steps (Scheme 2).[14] After it was converted into compound **24** with the standard method, the C4- and C6-hydroxy groups were protected with benzylidene, and C2 with *p*-methoxybenzyl; both protecting groups can be removed under acidic conditions or by oxidation, without affecting the azido group. After all the protecting groups were assembled, the thiophenol group was removed and also converted into the trichloroacetimide donor. The glycosidation catalyzed by TMSOTf went smoothly and gave the product in 88% yield. The 3′-azido analogue **2** was afforded by saponification of the benzoyl groups on ceramide, and removal of the PMB and benzylidene groups was carried out by oxidation with DDQ and toluenesulfonic acid, respectively. Reduction of the azido group by hydrogenation gave the 3′-amino analogue **3**. Finally, selective acetylation of the amine group by acetic anhydride in methanol generated the 3′-NAc analogue **4**.

The 4′-O-substituted analogues **6** and **7** were synthesized by using a different method, in which the azido lipid **30**, instead of ceramide **15**, was subjected to glycosidation (Scheme 3). After glycosidation, the benzylidene was regioselectively reduced under acidic conditions by $NaCNBH_3$. The methyl or *O*-THP-protected 2-hydroxyethoxy groups were introduced by alkylation under the assistance of NaH to give **33** and **34**. Reduction the azido group and amidation of the resulting amine with octanoic NHS ester afforded the protected glycolipid. The product **6** was obtained by hydrogenation, while **7** was generated by initial treatment with HCl to remove THP and then hydrogenation.



Scheme 1. Preparation of 4′-NAc-α-GalCer analogue **5**. Reagents and conditions: a) $NaCNBH_3$, HCl, THF, 86%; b) 1. Tf_2O, pyridine, 2. NaN_3, DMF, 74%; c) 1. NBS/H_2O, 2. CCl_3CN, DBU, 79%; d) TMSOTf, Et_2O/THF, −25 °C, 67%; e) NaOMe, MeOH, 93%; f) 1. PPh_3/H_2O, 2. Ac_2O, MeOH, 84%; g) H_2, $Pd(OH)_2$, 63%.

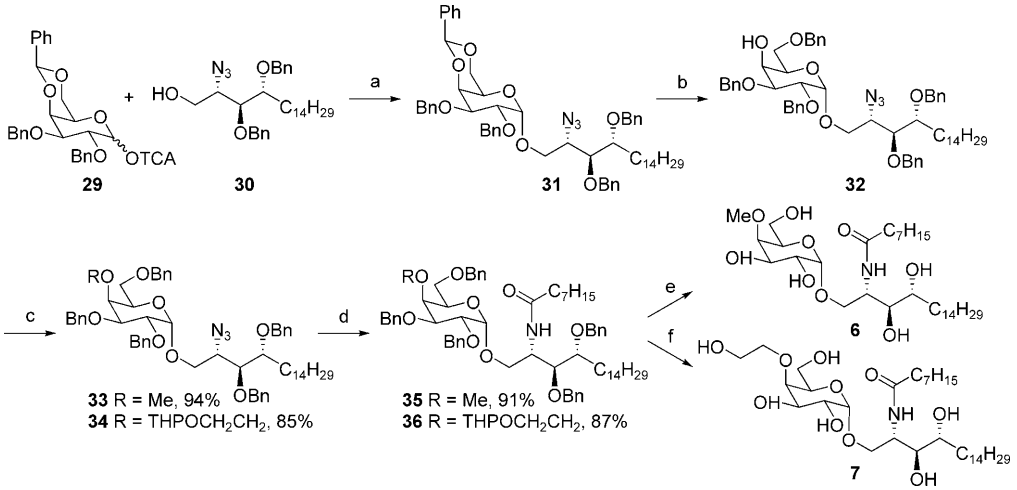
The α-GalCer analogues were first assayed with iNKT hybridoma cells (DN3A4-1.2 hybridoma, which expresses Vα14Jα281/Vβ8.2Jβ2.1 TCR).[15] CD1d-expressing A20/CD1d cells were applied to the iNKT hybridomas in order to present glycolipids to these cells. The IL-2 cytokine re-



Scheme 2. Synthesis of 3′-substituted analogues **2**, **3**, and **4**. Reagents and conditions: a) 1. PhSH, BF_3, Et_2O, 2. NaOMe, 63%; b) 1. $PhCH(OMe)_2$, *p*-TsOH, 2. PMBCl, NaH, 83%; c) 1. NBS/H_2O, 2. CCl_3CN, DBU, 89%; d) **15**, TMSOTf, −20 °C, 88%; e) NaOMe, 61%; f) 1. DDQ, 2. *p*-TsOH, 58%; g) Pd/C, H_2, quant.; h) Ac_2O, Et_3N, MeOH, 49%.

leased by stimulated iNKT hybridomas was measured to evaluate the immunogenic strength of the presented analogues. As shown in Figure 2a, all of the C3′-modified α-GalCer analogues exhibited a complete loss of iNKT cell stimulation and subsequent IL-2 cytokine release. Given that such a loss of activity corresponds to the absence of a hydroxy group, one can conclude that the hydrogen bond between the 3′-hydroxy group and the iNKT TCR and/or CD1d is very important for maintaining complex stability. Although the acetamide group of analogue **4** may actually form a hydrogen bond with the protein, the type of hydrogen bond is still different from that of the hydroxy group, thus explaining why activation of iNKT cells by this analogue is not apparent. Unlike the C3′-modified analogues, most of the C4′-modified analogues can, in fact, stimulate iNKT cells, albeit relatively weakly (Figure 2b). The activity of methyl-substituted **6** indicates that the C4′ position as H-bond donor is not required for binding with TCR. Analogue **7**, which possesses a hydroxy group at the terminus of a two-methylene-unit linker, is nearly as active as **6**, indicating that this group does not facilitate cytokine release. Notably, 4′-deoxy analogue **9** still possesses measurable iNKT cell stimulation, although it is much weaker than α-GalCer **1**. This result is quite surprising given that the α-dihexosylceramides such as α-lactosylceramide[12] and Gal-α-1,4-Gal-α-Cer,[11a] which have an additional sugar cap at the C4′ position, cannot stimulate iNKT cells unless the sugar cap is hydrolyzed by a glycosidase. A plausible explanation is that the 4′-hydroxy group contributes to the binding with iNKT TCR to some extent, but is not as important as the 2′- and 3′-hydroxy groups. Nevertheless, the very bulky sugar cap at the C4′ position blocks the TCR, inhibiting access to the glycolipid–CD1d complex, thus resulting in loss of activity. It was also observed that the 4′-NAc-α-GalCer **5** showed no activity, in contrast to the other C4′-modified analogues.

Given that the processing procedure in APC cells may have some effect on glycolipid–CD1d complex recognition, the coat-plate assay was applied to verify the above results. In this assay, glycolipid was directly presented to iNKT hybridoma cells by surface-bound purified CD1d protein to avoid the processing inside CD1d-expressing cells. N3A4-1.2 and DN3A4-1.4 hybridomas were used as iNKT cells in these assays. All of the C4′-modified α-GalCer analogues showed activity spectra similar to those in the A20/CD1d hybridoma assay, with the exception of 4′-α-NAc-GalCer **5** (Figure 3). Although **5** did not display any activity in the AC20/CD1d cell assay as mentioned



Scheme 3. Preparation of 4′-O-substituted analogues **6** and **7**. Reagents and conditions: a) TMSOTf, Et_2O, −40 °C, 67%; b) $NaCNBH_3$, HCl, THF, 91%; c) NaH, RX, DMF; d) 1. PPh_3/H_2O, 50 °C, 2. $C_7H_{15}COSu$; e) H_2, 10% Pd/C, $CHCl_3$/EtOH, 74%; f) 1. HCl, MeOH, 2. H_2, 10% Pd/C, $CHCl_3$/EtOH, 61%.

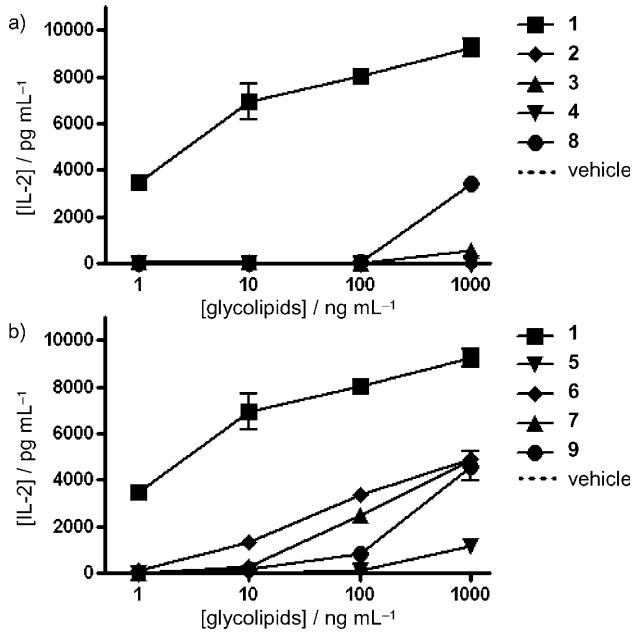


Figure 2. After stimulation by α-GalCer analogue-pulsed A20/CD1d cells, DN3A4-1.2 hybridoma cells release IL-2. Glycolipid analogues were applied at various concentrations as indicated; DMSO was used as vehicle. Shown are the IL-2 release profiles after stimulation by a) C3′- and b) C4′modified analogues. Experiments were carried out in triplicate.

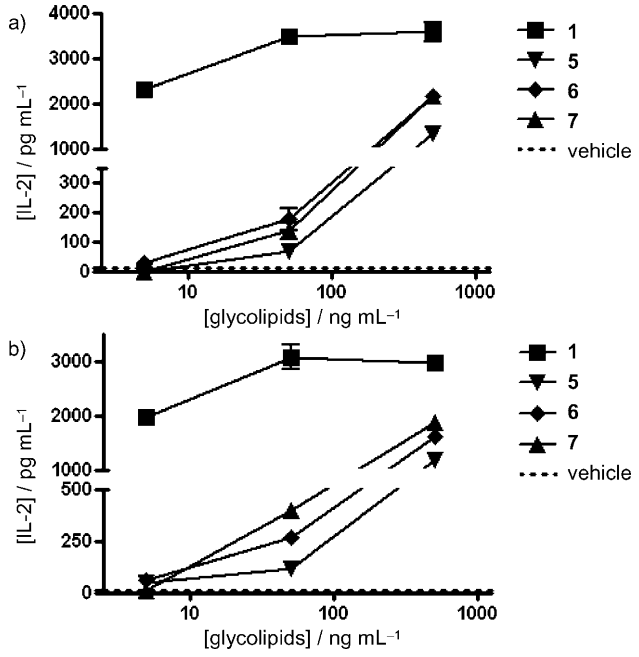


Figure 3. IL-2 release by iNKT hybridoma cell lines a) DN3A4-1.2 and b) DN3 A4-1.4 after cell stimulation by surface-bound CD1d-presented C4′-modified analogues. Glycolipid analogues were applied at various concentrations for loading onto CD1d protein as indicated; DMSO was used as vehicle. Experiments were carried out in triplicate.

above, it exhibited noticeable immunogenicity in this case. We hypothesize that such a discrepancy may arise from hydrolysis of the 4′-acetamide group in 4′-NAc-GalCer **5** by amide hydrolase(s) inside the lysosome of the AC20/CD1d cell, thus generating an -NH_3^+ group and leading to loss of activity. Additional experiments are currently in progress to verify this hypothesis.

The C4′-modified analogues were additionally subjected to an assay using a splenocyte mixture as a pseudo in vivo model. Specifically, C57BL/6 mouse spleens were ground into single-cell suspensions and stimulated by the α-GalCer analogues. Through the amount of IFN-γ and IL-4 released by the stimulated splenocytes, the T_h1/T_h2 cytokine profile of these analogues was generated. Remarkably, the C4′-substituted α-GalCer analogues were found to induce a T_h2-biased iNKT cell response (Figure 4). Although the total amount of IFN-γ is still

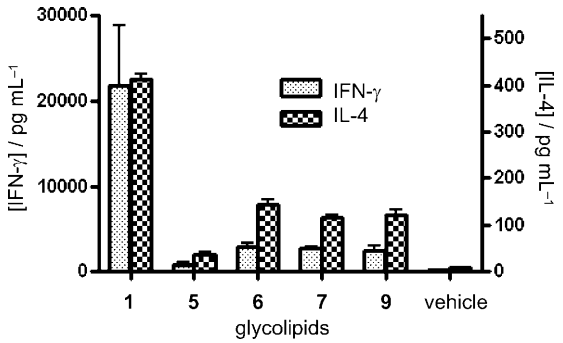


Figure 4. Splenocytes were stimulated by C4′-modified analogues, after which the released cytokines (IFN-γ and IL-4) were quantified by ELISA. Relative to α-GalCer **1**, the C4′-modified analogues **5**, **6**, **7**, and **9** stimulated more T_h2 (IL-4) cytokines than T_h1 (IFN-γ) cytokines. DMSO was used as vehicle, and experiments were performed in triplicate.

greater than that of IL-4 when NKT cells were stimulated by analogues **5**, **6**, **7**, and **9**, the quantity of IFN-γ released by NKT cells decreased more than IL-4 when these analogues were administered, relative to the original amount of these cytokines stimulated by α-GalCer **1**. Therefore, the new analogues significantly changed the relative ratio between released T_h1 and T_h2 cytokines. Such bias in the iNKT cell response was previously observed in experiments involving truncation of the length of the phytosphingosine chain (e.g., OCH)[16] or the metabolically stable α-C-GalCer.[17] Most recently, a series of 6′-derivatized α-GalCer analogues were shown to induce the T_h1-biased iNKT cell response. The observation that C4′-substituted analogues can change the profile of iNKT cell stimulation toward T_h2 cytokines is thus quite intriguing, as such immunomodulatory T_h2 cytokines can be used to ameliorate autoimmune diseases. One possible explanation for this shift in the cytokine profile may be the stability of the TCR–glycolipid–CD1d complex, as it occurred with OCH and others. However, there is a difference between human and murine NKT cell responses,[18] and thus human NKT cells will be used in the future to evaluate this biased cytokine-releasing profile.

In summary, we investigated a series of α-GalCer analogues with various substituents at the C3′ and C4′ positions of the galactose ring to evaluate the roles of the 3′- and 4′-hydroxy groups in iNKT cell stimulation. Assay results indicate that the 3′-hydroxy group is a critical feature of the TCR–glycolipid–CD1d ternary complex, and elimination of this group results in

loss of activity. In contrast, the C4′ position can tolerate some modifications without a dramatic decrease in immunogenicity. Moreover, it was observed that the C4′-substituted analogues can change the iNKT cell response profile toward a biased T_h2 cytokine release.

Experimental Section

Chemistry

Synthetic procedures along with analytical data for all compounds are given in the Supporting Information.

NKT hybridoma stimulation assays

The synthetic glycolipids were dissolved in DMSO at 1.0 mg mL^{-1} and then diluted with medium to the indicated concentration. A population of ~10^5 CD1d-transfected A20/CD1cells was pulsed by the glycolipids at concentrations of 1000, 100, 10, and 1 ng mL^{-1} in a total volume of 200 μL and then incubated overnight. After washing with medium, the pulsed A20/CD1 cells were mixed with ~50000 DN3A4-1.2 hybridoma cells and co-cultured for 24 h in a total volume of 200 μL. After another 24 h, the supernatant was collected. The amount of IL-2 released into the culture supernatant was measured by sandwich ELISA; the data are representative of three independent experiments.

For the ELISA, purified rat anti-mouse IL-2 antibody (eBioscience) was diluted 1:200 in PBS (pH 7.4), and diluted antibody (100 μL) was applied to each well and incubated overnight at 37 °C. After blocking by 10% BSA in PBS, the supernatant from in vitro stimulation (100 μL, 1:4 dilution) was applied to each ELISA well. After incubation for 2 h, the wells were washed by PBST (PBS with 0.2% *v/v* Tween-20), and the biotin-labeled second antibody (eBioscience) was applied. Finally, the HRP-conjugated streptavidin and its substrate were used as reporter system, and the absorbance of each well was measured (FlexStation 3 MicroPlate Reader, Molecular Devices). The concentration of IL-2 was calculated according to the standard wells added on each plate using SoftMax Pro (Molecular Devices).

Coat-plate hybridoma assays

The assay was carried out according to a previously published protocol.[19] First, for each well, murine CD1d protein (0.5 μg in 100 μL PBS, pH 7.4) was used to coat the surface. Glycolipid antigens with gradient concentrations (1000, 100, 10, and 1 ng mL^{-1} in a total volume of 100 μL) were incubated in the coated wells for 24 h. After washing, ~10^5 iNKT hybridoma cells, which have been well described,[20] were cultured in the treated micro-wells for 18 h. The IL-2 in the supernatant was collected, and its concentration was measured by ELISA as described above.

Splenocyte assays

Spleens from six-week-old C57BL/6 mice were grinded down to produce a single-cell suspension. The C57BL/6 mice were purchased from Jackson Laboratory (Maine, USA) and housed in the animal facility at The Ohio State University. Experimental procedures were carried out according to the protocol approved by the IACUC of The Ohio State University (Protocol 2008A0133). For each well, ~10^6 cells were cultured with glycolipid antigen at a concentration of 1000 ng mL^{-1} in a total volume of 200 μL. The mixtures were cultured for 72 h at 37 °C before the supernatants were collected. IFN-γ and IL-4 concentrations in the supernatants were measured by ELISA, as described above.

Acknowledgements

P.G.W. acknowledges the National Cancer Institute (R01 CA118208), NSF (CHE-0616892), and the Bill & Melinda Gates Foundation (51946) for financial support.

Keywords: CD1d · cytokines · glycolipids · natural killer T cells · receptors

[1] a) T. Kawano, J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, M. Taniguchi, *Science* **1997**, *278*, 1626; b) M. Kronenberg, *Annu. Rev. Immunol.* **2005**, *23*, 877.

[2] a) V. V. Parekh, M. T. Wilson, L. Van Kaer, *Crit. Rev. Immunol.* **2005**, *25*, 183; b) A. Bendelac, M. N. Rivera, S.-H. Park, J. H. Roark, *Annu. Rev. Immunol.* **1997**, *15*, 535.

[3] a) R. Nakagawa, I. Serizawa, K. Motoki, M. Sato, H. Ueno, R. Iijima, H. Nakamura, A. Shimosaka, Y. Koezuka, *Oncol. Res.* **2000**, *12*, 51; b) N. Fuji, Y. Ueda, H. Fujiwara, T. Toh, T. Yoshimura, H. Yamagishi, *Clin. Cancer Res.* **2000**, *6*, 3380; c) M. Morita, K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi, H. Fukushima, *J. Med. Chem.* **1995**, *38*, 2176; d) E. Kobayashi, K. Motoki, T. Uchida, H. Fukushima, Y. Koezuka, *Oncol. Res.* **1995**, *7*, 529.

[4] a) C. Forestier, T. Takaki, A. Molano, J. S. Im, I. Baine, E. S. Jerud, P. Illarionov, R. Ndonye, A. R. Howell, P. Santamaria, G. S. Besra, T. P. DiLorenzo, S. A. Porcelli, *J. Immunol.* **2007**, *178*, 1415; b) Y.-G. Chen, C.-M. Choisy-Rossi, T. M. Holl, H. D. Chapman, G. S. Besra, S. A. Porcelli, D. J. Shaffer, D. Roopenian, S. B. Wilson, D. V. Serreze, *J. Immunol.* **2005**, *174*, 1196; c) Y. N. Naumov, K. S. Bahjat, R. Gausling, R. Abraham, M. A. Exley, Y. Koezuka, S. B. Balk, J. L. Strominger, M. Clare-Salzer, S. B. Wilson, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 13838; d) S. Hong, M. T. Wilson, I. Serizawa, I. Wu, N. Singh, O. V. Naidenko, T. Miura, T. Haba, D. C. Scherer, J. Wei, M. Kronenberg, Y. Koezuka, L. Van Kaer, *Nature Med.* **2001**, *7*, 1052.

[5] K. Kakimi, L. G. Guidotti, Y. Koezuka, F. V. Chisari, *J. Exp. Med.* **2000**, *192*, 921.

[6] a) E. E. S. Nieuwenhuis, T. Matsumoto, M. Exley, R. A. Schleipman, J. Glickman, D. T. Bailey, N. Corazza, S. P. Colgan, A. B. Onderdonk, R. S. Blumberg, *Nature Med.* **2002**, *8*, 588; b) L. D. Hazlett, Q. Li, J. Liu, S. McClellan, W. Du, R. P. Barrett, *J. Immunol.* **2007**, *179*, 1138.

[7] a) S. M. Tahir, O. Cheng, A. Shaulov, Y. Koezuka, G. J. Bubley, S. B. Wilson, S. P. Balk, M. A. Exley, *J. Immunol.* **2001**, *167*, 4046; b) M. V. Dhodapkar, M. D. Geller, D. H. Chang, K. Shimizu, S. Fujii, K. M. Dhodapkar, J. Krasovsky, *J. Exp. Med.* **2003**, *197*, 1667.

[8] T. Natori, Y. Koezuka, T. Higa, *Tetrahedron Lett.* **1993**, *34*, 5591.

[9] a) M. Koch, V. S. Stronge, D. Shepherd, S. D. Gadola, B. Mathew, G. Ritter, A. R. Fersht, G. S. Besra, R. R. Schmidt, E. Y. Jones, V. Cerundolo, *Nature Immunol.* **2005**, *6*, 819; b) D. Wu, D. M. Zajonc, M. Fujio, B. A. Sullivan, Y. Kinjo, M. Kronenberg, I. A. Wilson, C.-H. Wong, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 3972; c) M. Zajonc Dirk, C. Cantu, 3rd, J. Mattner, D. Zhou, B. Savage Paul, A. Bendelac, A. Wilson Ian, L. Teyton, *Nature Immunol.* **2005**, *6*, 810; d) N. A. Borg, K. S. Wun, L. Kjer-Nielsen, M. C. J. Wilce, D. G. Pellicci, R. Koh, G. S. Besra, M. Bharadwaj, D. I. Godfrey, J. McCluskey, J. Rossjohn, *Nature* **2007**, *448*, 44.

[10] D. Wu, G.-W. Xing, M. A. Poles, A. Horowitz, Y. Kinjo, B. Sullivan, V. Bodmer-Narkevitch, O. Plettenburg, M. Kronenberg, M. Tsuji, D. D. Ho, C.-H. Wong, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 1351.

[11] a) T. I. Prigozy, O. Naidenko, P. Qasba, D. Elewaut, L. Brossay, A. Khurana, T. Natori, Y. Koezuka, A. Kulkarni, M. Kronenberg, *Science* **2001**, *291*, 664; b) X. T. Zhou, C. Forestier, R. D. Goff, C. Li, L. Teyton, A. Bendelac, P. B. Savage, *Org. Lett.* **2002**, *4*, 1267.

[12] W. Zhang, X. Zheng, C. Xia, R. S. Perali, Q. Yao, Y. Liu, P. Zheng, P. G. Wang, *ChemBioChem* **2008**, *9*, 1423.

[13] K. S. Wun, N. A. Borg, L. Kjer-Nielsen, T. Beddoe, R. Koh, S. K. Richardson, M. Thakur, A. R. Howell, J. P. Scott-Browne, L. Gapin, D. I. Godfrey, J. McCluskey, J. Rossjohn, *J. Exp. Med.* **2008**, *205*, 939.
[14] J. Elhalabi, K. G. Rice, *Nucleosides Nucleotides Nucleic Acids* **2004**, *23*, 195.
[15] a) N. Burdin, L. Brossay, Y. Koezuka, S. T. Smiley, M. J. Grusby, M. Gui, M. Taniguchi, K. Hayakawa, M. Kronenberg, *J. Immunol.* **1998**, *161*, 3271; b) L. Brossay, S. Tangri, M. Bix, S. Cardell, R. Locksley, M. Kronenberg, *J. Immunol.* **1998**, *160*, 3681.
[16] K. Miyamoto, S. Miyake, T. Yamamura, *Nature* **2001**, *413*, 531.
[17] G. Yang, J. Schmieg, M. Tusji, R. W. Franck, *Angew. Chem.* **2004**, *116*, 3906; *Angew. Chem. Int. Ed.* **2004**, *43*, 3818.
[18] a) M. Brigl, P. Van den Elzen, X. Chen, J. H. Meyers, D. Wu, C.-H. Wong, F. Reddington, P. A. Illarianov, G. S. Besra, M. B. Brenner, J. E. Gumperz, *J. Immunol.* **2006**, *176*, 3625; b) F. M. Spada, Y. Koezuka, S. A. Porcelli, *J. Exp. Med.* **1998**, *188*, 1529.
[19] Y. Kinjo, E. Tupin, D. Wu, M. Fujio, R. Garcia-Navarro, M. R.-E.-I. Benhnia, D. M. Zajonc, G. Ben-Menachem, G. D. Ainge, G. F. Painter, A. Khurana, K. Hoebe, S. M. Behar, B. Beutler, I. A. Wilson, M. Tsuji, T. J. Sellati, C.-H. Wong, M. Kronenberg, *Nature Immunol.* **2006**, *7*, 978.
[20] a) S. Sidobre, O. V. Naidenko, B.-C. Sim, N. R. J. Gascoigne, K. C. Garcia, M. Kronenberg, *J. Immunol.* **2002**, *169*, 1340; b) Y. Kinjo, D. Wu, G. Kim, G. W. Xing, M. A. Poles, D. D. Ho, M. Tsuji, K. Kawahara, C. H. Wong, M. Kronenberg, *Nature* **2005**, *434*, 520.

Received: August 25, 2009
Published online on September 24, 2009

