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Original article

Induction of promotive rather than suppressive immune responses from a novel NKT cell repertoire Vα19 NKT cell with α-mannosyl ceramide analogues consisting of the immunosuppressant ISP-I as the sphingosine unit

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Abstract

A 2-substituted 2-aminopropane-1,3-diol or 2-aminoethanol is the minimum structure required for the immunosuppressive activity of ISP-I, an antibiotic isolated from the culture broth of *Isaria sinclairil*. A series of α-mannosyl ceramide (α-ManCer) analogues was derived from 2-substituted 2-aminopropane-1,3-diols or 2-aminoethanols in place of sphingosine. The newly synthesized glycosides were evaluated for their effects on immune responses. In contrast to the immunosuppressive activity of the precursors, the α-ManCer analogues induced immunopromotive responses from invariant Vα19-Jα26 transgenic mouse lymphocytes more effectively than the original α-ManCer. Collectively, it is strongly suggested that the 2-substituted 2-aminopropane-1,3-diols and 2-aminoethanols mimic sphingosine in the α-ManCer analogues so that they potentially acquire specific antigenicity toward Vα19 NKT cell, a novel NKT cell subset.

Keywords: NKT cell; immunomodulation; glycosphingolipid

1. Introduction

Natural killer T (NKT) cells are defined as lymphocytes bearing both the common NK marker NKR-P1 and T cell receptors (TCRs) [1,2]. They are characterized by the expression of invariant TCR α chains such as mouse Vα14-Jα18 and human Vα24-JαQ α chains. Recently, we demonstrated the presence of a novel NKT cell repertoire (designated the Vα19 NKT cell) in mice [3]. This repertoire characteristically expressed the Vα19-Jα26 (AV19-AJ33) invariant TCR α chain that was previously found in mammalian peripheral blood [4]. Vα19 NKT cells along with the well characterized invariant Vα14-Jα18 TCR α^+ NKT (Vα14 NKT) cells [5,6] produce large amounts of both Th1- and Th2-promoting immunoregulatory cytokines, upon stimulation of the invariant TCR, and are considered to participate in the regulation of the immune system in mammalians [7,8] (M. Shimamura et al. "Invariant TCR-directed development of Vα19 NKT cell promptly producing immunoregulatory cytokines in response to TCR engagement with glycolipid antigens", manuscript to be submitted.) Therefore, the identification of specific antigens for Vα19 NKT cells is required to develop new therapies for various disorders. We have recently found that α-mannosyl ceramide (α-ManCer) in the context of one of the non-classical MHC class I molecules MR1 [9] specifically stimulates Vα19 NKT cells [8].

Previously, we found immunosuppressive activity by ISP-I [10,11], a product of *Isaria sincliairii* (ATCC 24400) which was later shown to be identical to antifungal and antibiotic

Abbreviations: α-ManCer, α-mannosyl ceramide; MNC, mononuclear cell; Vα19, NKT cell; invariant Vα19-Jα26 TCR α^+, NK1.1$^+$ T cell; Vα14, NKT cell; invariant Vα14-Jα18 TCR α^+, NK1.1$^+$ T cell.

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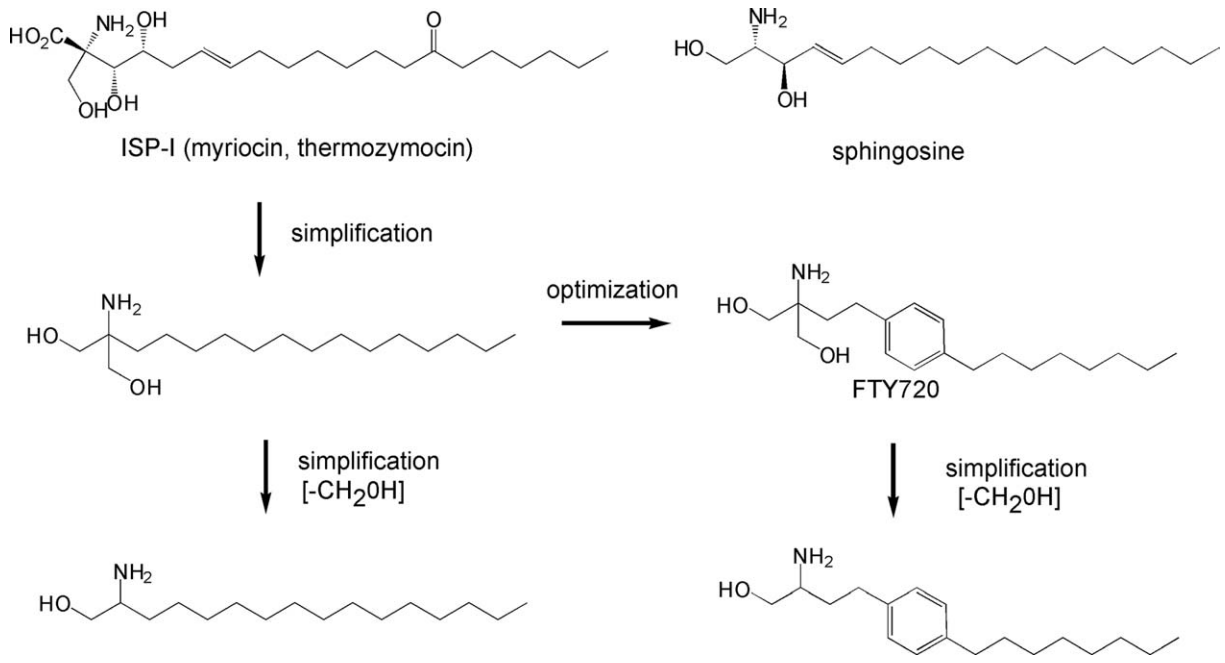


Fig. 1. Immunosuppressive ISP-I and its derivatives compared with sphingosine. ISP-I derivatives simplified and optimized in termed of the immunosuppressive activity were used for the synthesis of α-ManCer analogues.

myriocin [12] or thermzymocidin [13] (Fig. 1). A comprehensive study of the structure-activity relationship of chemically modified derivatives of ISP-I revealed that the 2-amino-1,3-propane diol, especially the 2-aminoethanol moiety, is the structure of ISP-I required for the immunosuppressive activity [10,11]. Furthermore, modification of the hydrophobic portion indicated that the 2-substituted 2-aminoethanol with a 4-octylphenyl group (FTY720) optimized the immunosuppressive potential [10,11] (Fig. 1). Loss of chirality in these compounds facilitated the synthesis. In addition, the derivatives were less cytotoxic than the original ISP-I. As suggested by the structural homology between FTY720 and sphingosine, recent studies have demonstrated that this drug targets sphingosine-1-phosphate receptors and acts as an agonist [14,15].

In the current study, we focused on a series of α-ManCer derivatives in which the sphingosine moiety was replaced with FTY720 and related aminoalcohols (Fig. 1). We found that the modified α-ManCer induced promotive rather than suppressive immune responses from Vα19 NKT cells.

2. Results and discussion

2.1. Synthesis of α-glycosyl ceramide derivatives

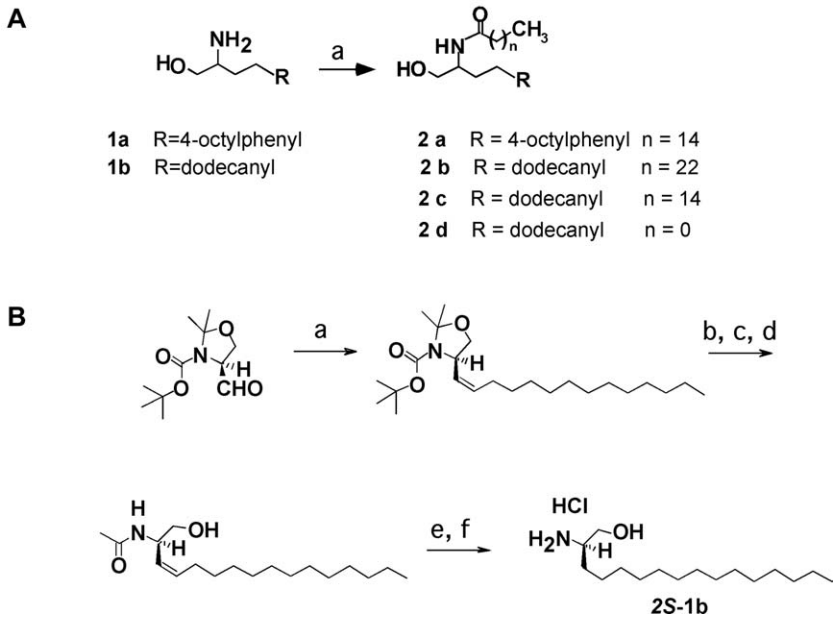
It has been found that the immunosuppressive activity of ISP-I involves the moiety of 2-amino-1,3-propanediol, especially 2-aminoethanol [10,11]. The 2-substituted derivatives depicted in Fig. 1 were used as precursors in place of sphingosine, and a series of α-glycosyl ceramide analogues were synthesized.

2-Substituted 2-aminoalcohols (**1**) were *N*-acylated with an appropriate length of acylchrolide and the resultant amides (**2**) were used as glycosylation acceptors (Scheme 1A). Similarly, glycosylation acceptors were synthesized from 2-substituted 2-amino-1,3-propanediols (**3**) (Scheme 2). One of the two hydroxymethyl groups of **3** was protected with triethyl orthoacetate to afford **4** at first. The remaining hydroxymethyl group of **4** was benzylated (**5**). Then, the oxazoline ring of **5** protecting both hydroxymethyl and amino groups was removed (**6**). The amino group was finally *N*-acylated to give **7a–d.** The resultant *O*-benzylated amides were useful as glycosylation acceptors because the benzyl group was stable during the following glycosylation reaction and was easily separable by hydrogenation afterwards simultaneously with the other benzyl groups protecting the sugar hydroxyl groups. **7a–d** were optically inactive, thus indicating that they are an equivalent mixture in terms of the chirality at the C2 of the sphingosine analogue.

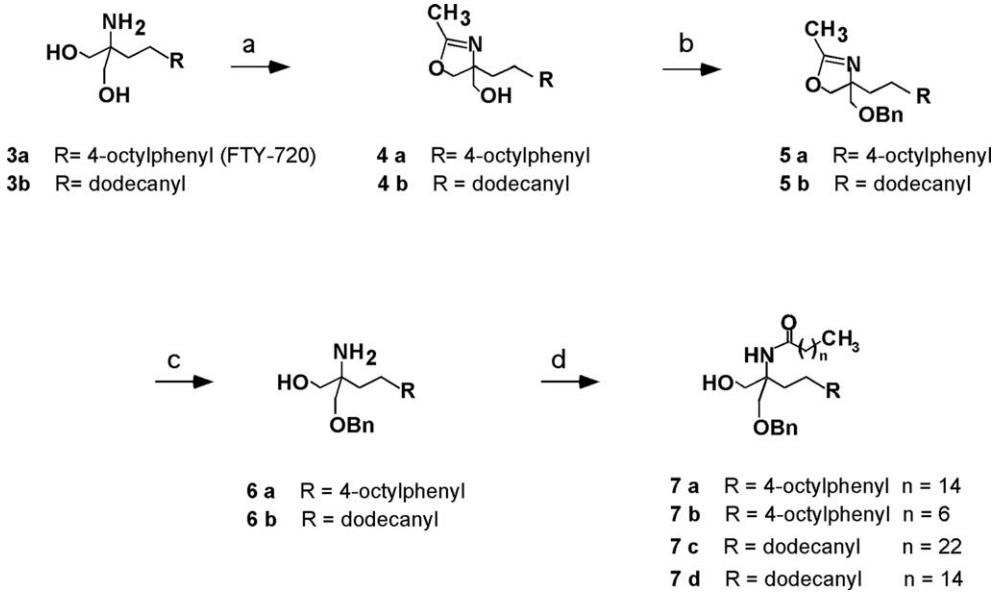
The amides **2** and **7** were next glycosylated with **8** to afford glycosides (**9**) (Scheme 3). The α-anomers were predominantly obtained when 1-fluoro 2,3,4,6-benzyl galactose was used as a glycosilation donor [16]. The α-anomers were similarly the main products when 1-*O*-acetyl 2,3,4,6-benzyl mannose was the glycosylation donor, because the β-glycosylation was hampered by the steric hindrance with the 2-axial *O*-benzyl group during the transition state of the glycosylation reaction. The predominantly formed α anomers were successfully separated from the β anomers by silica gel column chromatography at this stage. Finally, the benzyl protection groups were removed from **9** by reduction and the products (**10**) were obtained.

2.2. Immunological activity of α-glycosyl ceramide analogues

To examine whether α-glycosyl ceramide analogues derived from the immunosuppressants were immunopromotive

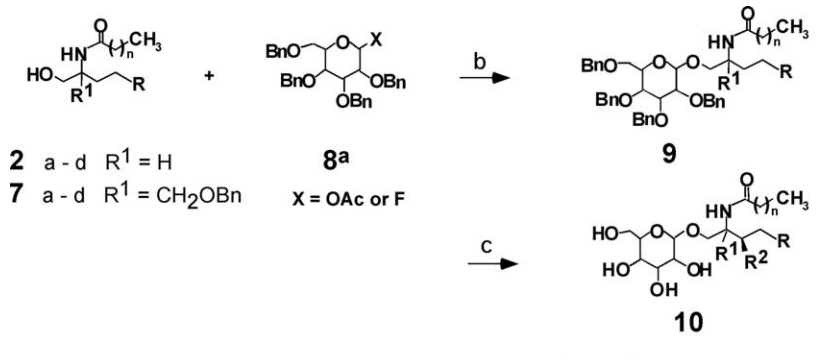


Scheme 1. (A). *N*-Acylation of 2-aminoethanol derivatives used as glycosylation acceptors. (a): Reagents and conditions, Cl C=O–$(CH_2)_n$ CH_3, TMSCl, pyridine. Yield: **2a**, 46%; **2b**, 25%; **2c**, 50%; **2d**, 41%. (B). Preparation of the diastereomers of 2-aminohexadecanol. A synthetic pathway of *2S*-**1b** is shown here. 2R-1b was similarly prepared from the corresponding diastereomer of Garner's aldehyde. (a): $[Ph_3P(CH_2)_{12}CH_3]Br$/n-BuLi/THF. (b): TFA. (c): Ac_20/pyridine. (d): 2M HCl/THF. (e): H_2. 10% Pd-C/EtOH. (f): Conc.HCl/EtOH.



Scheme 2. Synthesis of *N*-acyl 2-amino-1,3-propanediol derivatives used as glycosylation acceptors.
(a): $CH_3C(OEt)_3$, *i*PrN$(Et)_2$, Yield: 81 ~ 87 %, (b): BnBr, NaH, THF, Yield: 41 ~ 49%. (c): Conc. HCl. Yield: 88 ~ 97%. (d): (1) TMS (trimethylsilyl)Cl, pyridine, (2) ClC=O–$(CH_2)_n CH_3$, Yield: 75 ~ 85%.

or suppressive, a series of α-glycosides (Scheme 3) was tested for the effects on mouse lymphocytes. Liver mononuclear cells (MNCs) were prepared from C57BL/6 mice and Vα19-Jα26 invariant TCR α transgenic (Tg) mice [7,8] (with the TCR $\alpha^{-/-}$ background). The C57BL/6 MNCs included 30% invariant Vα14-Jα18 TCR α^+ NKT (Vα14 NKT) cells [17], whereas the Vα19 Tg^+ MNCs included 30% Vα19 NKT cells as the sole NKT cell population [7,8]. These cells were cultured as responders in the presence of the glycolipids. Immune responses were assessed by measurement of cell proliferation and IL-2 secretion in the culture supernatants (Fig. 2). α-ManCer derivatives more or less enhanced proliferation of Vα19 Tg^+ cells and the production of IL-2 irrespective of the modification in the sphingosine portion. *In addition, the derivatives induced activation of spleen cells of the Vα19 Tg+ mice previously primed with them. The spleen cells transferred to culture spontaneously secreted IL-2* (Fig. 3) *and other cytokines (data not shown). These findings strongly suggest that the α-ManCer analogues consisting of the immunosuppressive structural unit as well as intact α-ManCer are immunopromo-*



9, 10

	R	R^1	R^2	n	Hexose	Abbreviation
a	4-octylphenyl	H	H	14	α-Man	Man4PhC16
b	dodecanyl	H	H	22	α-Gal	GalC24
c	dodecanyl	H	H	14	α-Man	ManC16
d	dodecanyl	H	H	0	α-Gal	GalC2
e	4-octylphenyl	CH_2OH	H	14	α-Man	Man2HM4PhC16
f	4-octylphenyl	CH_2OH	H	14	α-Gal	Gal2HM4PhC16
g	4-octylphenyl	CH_2OH	H	6	α-Gal	Gal2HM4PhC8
h	dodecanyl	CH_2OH	H	22	α-Man	Man2HMC24
i	dodecanyl	CH_2OH	H	14	α-Man	Man2HMC16
	dodecanyl	H	OH	14	α-Man	Man3OHC16 [d]

Scheme 3. Synthesis of α-glycosyl ceramide derivatives.
(a): 1-*O*-acetyl-2,3,4,6-tetrabenzyl-D-mannose or 1-fluoro-2,3,4,6-tetrabenzyl-D-galactose. (b): BF_3-Et_2O, CH_2Cl_2, for 1-*O*-acetate. $SnCl_2$, $AgClO_4$-H_2O, molecular sieve 4A, Et_2O for 1-fluoride. Yield: **9a**, 54%; **9b**, 14%; **9c**, 66%; **9d**, 55%; **9e**, 37%; **9f**, 74%; **9g**, 37%; **9h**, 18%; **9i** 25%. *The yields of the β-anomers were less than 10%.* (c): H_2, 20%, $Pd(OH)_2$-C, EtOH. Yield: **10a**, 33%; **10b**, 33%; **10c**, 35%; **10d**, 39%; **10e**, 16%; **10f**, 76%; **10g**, 53%; **10h**, 68%; **10i** 81%. (d): Man3OHCl6 was synthesized as described previously [8].

tive to Vα19 NKT cells [8]. Above all, Man4PhC16 (**10a**) stimulated Vα19 Tg^+ cells rather intensively compared with ManCl6 (**10c**) or the intact α-ManCer (Man3OHCl6) in the same concentration (only Man3OHCl6 keeps chirality at the C2 of the sphingosine). Vα19 NKT cells recognize the mannosyl residue of α-ManCer when it is presented by the antigen-presenting molecule MRl, one of the non-polymorphic MHC class I-like molecules [8]. Thus, it is suggested that the introduction of a phenyl group in the sphingosine portion in **10a** improved the recognition of the α-mannosyl residue by the invariant Vα19 TCR, presumably arising from the augmented interaction between the modified portion of the sphingosine and the MRl antigen-presenting groove.

As found in α-ManCer [8], α-ManCer analogues had less of an effect on C57BL/6 cells than Vα19 Tg^+ cells. Combined with the finding that $NK1.1^+$ Vα19 Tg^+ cells but not $NK1.1^-$ Vα19 Tg^+ cells were responsive to the glycolipid antigens (Shimamura et al., manuscript to be submitted), it is suggested that the specific recognition of α-ManCer analogues is carried out by Vα19 NKT cells.

α-ManCer analogues synthesized here are an equivalent mixture of diastereomers in terms of the chirality at C2 of modified sphingosine as described above. To determine which form of the diastereomer is immunopromotive to Vα19 NKT cells, both *S* and *R* forms of ManC16 (**10c**) were synthesized by the same method from optically active **1b** (Scheme 1B) and assessed the activity. *2R-**10c** induced* 1.6 ± 0.3 *fold-increase of cell proliferation of Vα19Tg TCRα$^{-/-}$ liver lymphocytes, whereas 2S-**10c** caused* 1.1 ± 0.2 *fold-increase. Thus, the 2R-form diastereomer of α-ManCer analogues corresponding to the configuration of naturally occurring sphingosine are likely to primarily responsible for the immunopromotive activity.*

GalC24 (**10b**) was immunopromotive toward C57BL/6 cells. However, this glycolipid was less effective than KRN7000 (α-GalCer consisting of sphingosine-3,4-diol) [18], data not shown). In addition, Gal2HMPhC18 (**10f**) had no significant effect on C57BL/6 cells. These findings indicate a stringent structural restriction in the sphingosine portion of α-GalCer. *Presumably, KRN7000 is properly presented by CDld, so that α-GalCer is recognized as an effective antigen by invariant Vα14-Jα18 TCR$^+$ NKT cells* [18]. α-GalCer analogues with a short *N*-acyl chain (GalC2 (**10d**) and Gal2HM4PhC8 (**10g**)) suppressed the immune responses of both C57BL/6 and Vα19 Tg^+ lymphocytes, suggesting that two suitably long hydrocarbon chains are necessary for antigenic glycosyl ceramides to properly fit to the groove of CDld [19,20] or MRl.

3. Conclusion

Modified α-ManCers consisting of an immunosuppressive sphingosine analogue were promotive rather than suppressive of the immune responses by Vα19 NKT cell. Some of them were more immunopromotive than the original α-ManCer. This suggests that the structural modification in the sphingosine moiety in α-ManCer improves the space location of the α-mannosyl residue to be recognized by the invariant Vα19 TCR. More functional glycolipids should be obtained on the

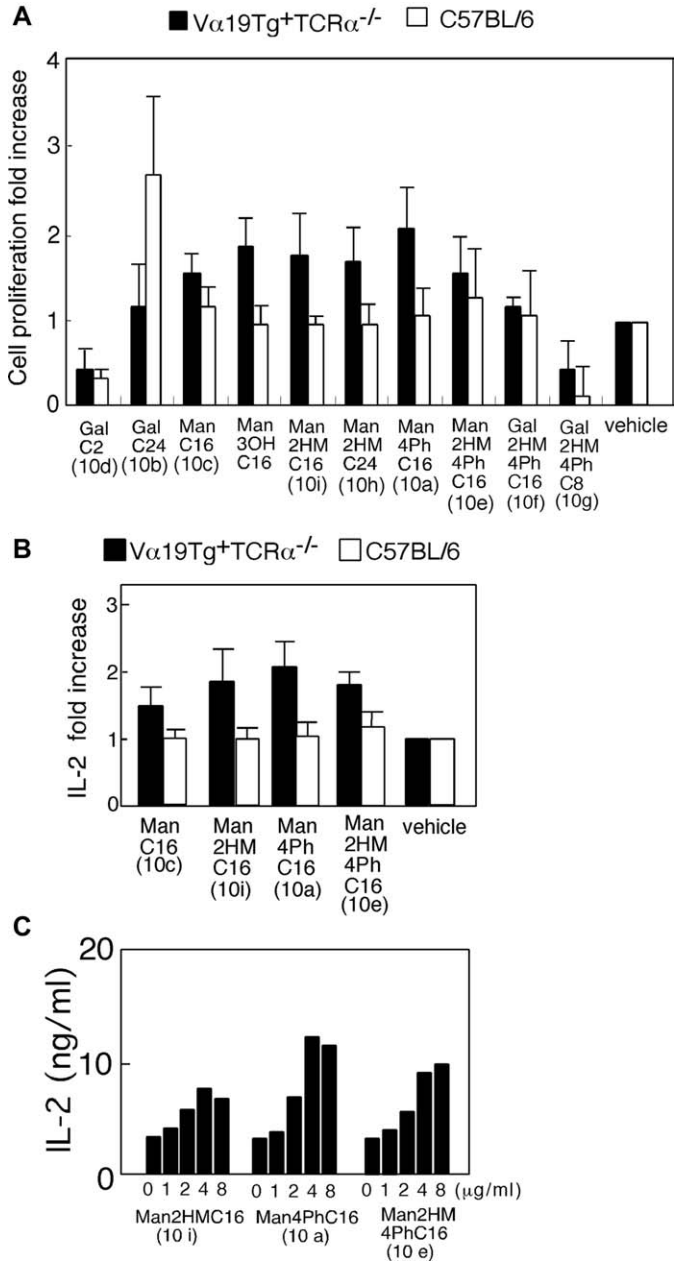


Fig. 2. Immune responses of Vα19 NKT cells in culture elicited by α-ManCer and α-GalCer derivatives. Liver MNCs from Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice were cultured in the presence or absence of glycolipids (2 μg ml^{-1}). After 2 days, the immune responses were monitored by measuring cell proliferation ([^{3}H]-thymidine incorporation for 5 h) (A) and IL-2 secretion in the culture fluid (B). Results are shown as the fold increase relative to the control culture with vehicle (1/200 v/v DMSO). *(C) Dose-dependent activation of Vα19 Tg^+ cells with α-ManCer derivatives. IL-2 production by Vα19 Tg^+ $TCR\alpha^{-/-}$ liver MNCs cells in the different concentration of α-ManCer derivatives was determined after 2 days of culture.*
Abbreviations of glycolipids are listed in Fig. 1.

examination of this structural modification. In contrast, a similar structural modification in the sphingosine moiety of α-GalCer decreased the antigenic activity toward Vα14 NKT cells presumably due to reduced interaction between α-GalCer and CDld.

Modified α-ManCers are possibly useful for developing new therapies for various immunological disorders, since they have potency to induce immune responses of Vα19 NKT cells not only in culture but also in vivo.

4. Experimental

4.1. General

Silica gel chromatography was performed on Merck Kiesegel 60. ^{1}H-NMR spectra were recorded on a JEOL α400 spectrometer (400 MHz, JEOL, Tokyo, Japan) using tetramethylsilane (TMS) as an internal standard. Mass spectra were

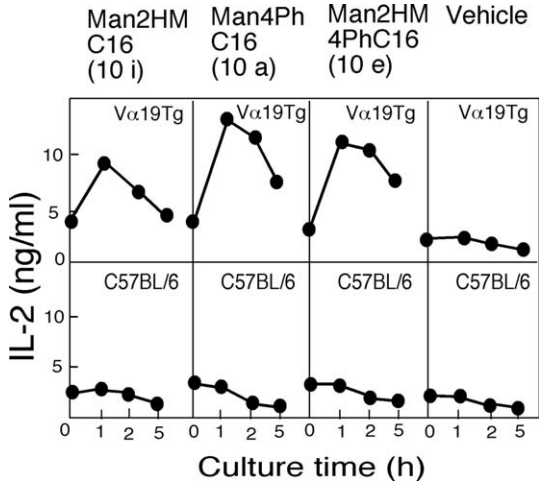


Fig. 3. Priming of Vα19 NKT cells in vivo with challenge of α-ManCer derivatives.
Spleen cells from Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice previously injected with glycolipids (20 μg per animal) or vehicle (DMSO) via the tail vein were cultured for the period indicated. Culture supernatants were harvested and tested for production of cytokines at the indicated time points.

measured on a JMS-DX300 (JEOL). High-resolution mass spectra were measured on a JMS700 (JEOL). Infrared spectra were recorded on an FT/IR-5300 spectrometer (JASCO, Tokyo, Japan). Elemental analyses were performed on a CHN coder MT-5 (Yanaco, Tokyo, Japan).

4.2. Synthesis of α-glycosyl ceramide analogues

4.2.1. Precursors for glycosyl acceptors

2-Amino-4-(4-octylphenyl)butanol (**1a**), 2-aminohexadecanol (**1b**) (Scheme 1), 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol (**3a**, FTY-720) and tetradeanyl-1,3-propanediol (**3b**) (Scheme 2) were obtained as described previously [10]. Optically active 2-aminohexadodecanol (*2S*-**1b**, *2R*-**1b**) were synthesized from each diastereomer of Garner's aldehyde (1, 1-dimethyl (*S*)- or (*R*)-5-formyl-2,2-dimethyl-3-oxazolidinecarboxylate) [21] as shown in Scheme 1B as previously described [22].

4.2.2. Glycosylation acceptors

The precursors were *N*-acylated with acyl chloride of an appropriate length (Schemes 1 and 2). For example, *N*-[1-hydroxymethyl-3-(4-octylphenyl) propyl] hexadecanamide (**2a**) was obtained as follows. 2-Amino-4-(4-octylphenyl) butanol (**1a**, 1.1 g, 4.0 mmol) was dissolved in pyridine (40 ml). To this solution was added chlorotrimethylsilane (1.1 ml, 8.7 mmol) in drops over 10 min at 5 °C. The solution was stirred at room temperature for 1 hour. Then palmitoyl chloride (1.4 ml, 4.6 mmol) was added in drops to the reaction solution over 10 min and the resulting mixture was stirred at the same temperature for 3 hours. After evaporation of the solvent in vacuo, the residue was dissolved in $CHCl_3$ and washed successively with water and brine, then the organic phase was separated and dried over anhydrous magnesium sulfate. After concentration, the residue was purified by recrystallization from AcOEt to give the product (**2a**) as yellow crystal (0.94 g, yield, 46%).

To prepare glycosylation acceptors for 2-amino-1,3-propanediol, one of the two hydroxymethyl groups of (**3**) was selectively *O*-bebzylated via **4** and **5**. Then, the products (**6**) were *N*-acylated to afford **7** as indicated in Scheme 2 by using standard procedures.

4.2.3. Glycosylation and removal of the protective groups

Glycosylation acceptors (**2a–d**, **7a–d**) were *O*-glycosylated with **8** (1-*O*-acetyl-2,3,4,6-tetrabenzyl-D-mannose [23], or 1-fluoro1-acetyl-2,3,4,6-tetrabenzyl-D-galactose [24]) by a Lewis acid promoter (BF_3 for 1-*O*-acetate and $SnCl_2$, $AgClO_4$ for 1-fluoride), and the adducts were subjected to hydrogenolysis to take away benzyl protective groups (Scheme 3). For instance, *N*-[1-(α-D-mannopyranosyl oxymethyl)-3-(4-octylphenyl) propyl]hexadecanamide (**10a,** *Man4PhC16*) was synthesized as follows. 1-Acetyl-2,3,4,6-tetrabenzyl-D-mannose (**2a**, 0.49 g, 0.84 mmol) was dissolved in dichloromethane (20 ml) under a nitrogen atmosphere. BF_3-OEt_2 (0.11 ml, 0.89 mmol) was added in drops over 2 min at 5 °C and the mixture was stirred for 10 min. Then a solution of **2a** (0.46 g, 0.89 mmol) in dichloromethane (7.0 ml) was added in drops over 4 min and stirred for 1.5 hours. A saturated ammonium chloride solution was added to this reaction mixture. The organic phase was *separated* and washed successively with brine and dried over anhydrous magnesium sulfate. After filtration and concentration, the residue was purified by silica gel column chromatography using a mixture of hexane: AcOEt (5:1) as an elution solvent. The yellow oil (0.47 g) of the tetrabenzyl derivative (**9a**) was dissolved in ethanol (20 ml) and stirred for 4 hours in the presence of 20% $Pd(OH)_2$ on charcoal under an hydrogen atmosphere. After filtration and concentration, the product (**10a**) was obtained as a colorless amorphous solid (0.10 g, yield, 21% from **2a**).

4.2.4. Preparation of optically active ManC16 *(2S- and 2R-10c)*

Diastereomers of ManC16 (**2S**- and **2R**–**10c**) were synthesized from *2S* and *2R*-diastereomers of **1b** as described for the synthesis of the racemic mixture of **10c.** The yield of the intermediate and the final products was comparable to the yield of the racemic forms *(60–70% for 2S-**9c** and 2R-**9c**)*.

4.2.5. Product data for ***10a–i***

4.2.5.1. ***10a*** *(Man4PhC16)*. Elemental analysis: calculated for $C_{40}H_{71}NO_7$ 3/2H_2O: C; 68.14, H; 10.58, N; 1.99.

Found: C; 68.45, H; 10.64, N; 2.01.

^{1}H-NMR (CD_3OD): δ 0.87–0.90 (6H, m, methyl), 1.26 (36H, br s, methylene), 1.53–1.68 (4H, m, methylene), 2.17–2.22 (2H, m, methylene), 2.54 (2H, t, $J = 7.3$ Hz, methylene), 2.55–2.68 (2H, m, methylene), 3.36–3.45 (1H, m), 3.47–3.52 (1H, m), 3.55–3.61 (1H, m), 3.63–3.70 (3H, m), 3.76–3.83

(2H, m), and 3.95–4.10 (1H, m) for mannose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.70–4.72 (1H, m, anomeric), 7.04 (2H, d, $J = 8.3$ Hz, phenyl), 7.07(2H, d, $J = 8.3$ Hz, phenyl). MS (EI), *m/z*: 677(M^+).

IR (cm^{-1}) (KBr): 3388, 2922, 2852, 2360, 1622, 1464, 1384.

Appearance: Colorless amorphous

4.2.5.2. ***10b*** *(GalC24)*. Elemental analysis: calculated for $C_{46}H_{91}NO_7 \cdot 3/4H2O$, C, 70.50; H, 11.90; N,1.79.

Found: C, 70.57; H, 11.91; N, 1.66.

^{1}H-NMR (CD_3OD): δ 0.80 (6H, t, 6.8 Hz, methyl), 1.19 (66H, brs, methylene), 1.45–1.60 (2H, m, methylene), 2.10 (2H, t, $J = 7.3$ Hz, methylene) 3.36 (1H, dd, $J = 10.3$, 5.4 Hz), 3.46–3.71 (6H, m), 3.77 (1H, d, $J = 2.0$H), and 3.90–3.94 (1H, m), for galactose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.70 (1H, brs, anomeric).

MS (EI): *m/z*: 770 (M^+).

IR(KBr): 3433,2918,2850,1646,1543,1468.

m.p. 142–144 °C.

4.2.5.3. ***10c*** *(ManC16)*. Elemental analysis: Calculated for $C_{38}H_{75}NO_7 \cdot 1/2H_2O$: C,68.39; H,11.45; N,2.14.

Found: C;68.42; H,11.48; N,2.10.

^{1}H-NMR(CD_3OD): δ 0.89 (6H, t, $J = 6.8$ Hz, methyl), 1.28 (48H, brs, methylene), 1.59–1.67(4H, m, methylene), 2.18 (2H, t, $J = 7.3$ Hz, methylene), 3.38 (1H, dd, $J = 9.8$, 5.9 Hz), 3.46–3.52 (1H, m), 3.58 (1H, d, $J = 9.3$ Hz), 3.61–3.70 (3H, m), 3.78 (1H, dd, $J = 3.4$, 1.5 Hz), 3.81 (1H, dd, $J = 11.7$, 2.0 Hz), and 3.94–4.03 (1H, m), for mannose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.72 (1H, brs, anomeric)

MS (EI): *m/z*: 658 (M^+).

m.p. 153–155 °C.

*4.2.5.4. 2S-****10c***. Elemental analysis: Calculated for $C_{38}H_{75}NO_7 \cdot 1/2H_2O$: C, 68.42; H, 11.48; N,2.10.

Found: C; 68.39; H, 11.45; N, 2.14.

^{1}H-NMR(CD_3OD): δ 89 (6H, t, $J = 6.8$ Hz, methyl), 1.28 (48H, brs, methylene), 1.51–1.81 (4H, m, methylene), 2.19 (2H, t, $J = 7.3$ Hz, methylene), 3.41 (1H, dd, $J = 9.8$, 4.4 Hz), 3.46–3.53 (1H, m), 3.57 (1H, d, $J = 9.8$ Hz), 3.58–3.71 (3H, m), 3.78 (1H, brs), 3.82 (1H, d, $J = 11.7$ Hz), and 3.99–4.08 (1H, m), for mannose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.73 (1H, brs, anomeric).

IR (KBr): 3296, 2918, 2851, 1643, 1541, 1467, 1130, 1101, 1059.

CD: $[\alpha]_D$: +19.2 (0.25, EtOH).

m.p. 147–148.

*4.2.5.5. 2R-****10c***. Elemental analysis: Calculated for $C_{38}H_{75}NO_7 \cdot 1/2H_2O$: C, 68.42; H,11.48; N, 2.10.

Found: C; 68.39; H, 11.45; N, 2.14

NMR(CD_3OD): δ 0.89(6H, t, $J = 6.8$ Hz, methyl), 1.28 (48H, brs, methylene), 1.59–1.67 (4H, m, methylene), 2.18 (2H, t, $J = 7.3$ Hz, methylene), 3.38 (1H, dd, $J = 9.8$, 5.9 Hz), 3.46–3.52 (1H, m), 3.58 (1H, d, $J = 9.3$ Hz), 3.61–3.70 (3H, m), 3.78 (1H, dd, $J = 3.4$, 1.5 Hz), 3.81 (1H, dd, $J = 11.7$, 2.0 Hz), and 3.94–4.03 (1H, m), for mannose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.72(1H, brs, anomeric).

IR(KBr): 3306, 2917, 2850, 1644, 1543, 1468, 1139, 1105, 1059.

Mass(EI): 658.

CD: $[\alpha]_D = +48.5$ (0.20, EtOH).

m.p. 157–159.

4.2.5.6. ***10d*** *(GalC2)*. Elemental analysis: Calculated for $C_{24}H_{47}NO_7 \cdot 1/4H_2O$: C, 61.84; H, 10.27; N, 3.00.

Found: C, 61.77; H, 10.38; N, 3.07.

^{1}H-NMR (400 MHz, CD_3OD): δ 0.89 (3H, t, $J = 8$ Hz, methyl), 1.28 (24H, brs, methylene), 1.44–1.52 (1H, m, methylene), 1.53–1.61 (1H, m, methylene), 1.95 (3H, s, methyl), 3.33–3.37 (1H, m), 3.42–3.51 (1H, m), 3.63–3.79 (5H, m), 3.81 (1H, brs), and 3.90–4.03 (1H, m), for galactose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.79–4.80 (1H, m, anomeric), 7.9–8.1 (1H, m, amide).

IR (cm^{-1}) (KBr): 3278, 2915, 2851, 1648, 1558, 1472.

EI-MS, *m/z*: 462 $(M + H)^+$.

4.2.5.7. ***10e*** *(Man2HM4PhC16)*. Elemental analysis: Calculated for $C_{41}H_{73}NO_8 \cdot H_2O$, C,67.82; H,10.41; N,1.93.

Found: C;67.55; H,10.33; N,1.91.

^{1}H-NMR (400 MHz, CD_3OD): δ 0.89(6H, t, $J = 6.8$ Hz, methyl), 1.27 (34H, brs, methylene), 1.53–1.65 (4H, m, methylene), 1.93–2.08 (2H, m, methylene), 2.18–2.22 (2H, m, methylene), 2.52–2.59 (4H, m, methylene), 3.52–3.57 (2H, m), 3.61–3.73 (4H, m), and 3.79–3.84 (4H, m), for mannose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.75 (1H, d, $J = 9.8$ Hz, anomeric), 7.04 (2H, d, $J = 7.8$ Hz, phenyl), 7.08 (2H, d, $J = 7.8$ Hz, phenyl), 7.30 (1H, d, $J = 11.2$ Hz, amide).

IR (KBr): 3321, 2923, 2853, 1646, 1467.

Amorphas.

4.2.5.8. ***10f*** *(Gal2HM4PhC16)*. ^{1}H-NMR (400 MHz, CD_3OD): δ 0.83–0.87 (6H, m, methyl), 1.22 (34H, brs, methylene), 1.50–1.60 (4H, m, methylene), 1.90–2.08 (2H, m, methylene), 2.13–2.20 (2H, m, methylene), 2.45–2.57 (4H, m, methylene), 3.4–4.0 (10H, m), for galactose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.78–4.85 (1H, m, anomeric), 7.00 (2H, d, $J = 8$ Hz, phenyl), 7.05 (2H, d, $J = 8$ Hz, phenyl), 7.38 (1H, d, $J = 16$ Hz, amide).

High resolution FAB-MS, calculated for $C_{41}H_{74}N_1O_8$ $(M + H)^+$ 708.5414; found 708.5435.

4.2.5.9. ***10g*** *(Gal2HM4PhC8)*. ^{1}H-NMR (400 MHz, CD_3OD): δ 0.84–0.92 (6H, m, methyl), 1.28 (18H, brs, methylene), 1.50–1.65 (4H, m, methylene), 1.90–2.10 (2H, m, methylene), 2.13–2.25 (2H, m, methylene), 2.45–2.60 (4H, m, methylene), 3.4–4.1 (10H, m), for galactose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.78–4.85 (1H, m, anomeric), 7.04 (2H, d, $J = 8$ Hz, phenyl), 7.09 (2H, d, $J = 8$ Hz, phenyl), 7.42 (1H, d, $J = 12$ Hz, amide).

High resolution FAB-MS, calculated for $C_{33}H_{58}N_1O_8$ $(M + H)^+$, 596.4162; found 596.4153.

4.2.5.10. ***10h*** *(Man2HMC24).* ^{1}H-NMR (400 MHz, CD_3OD): δ 0.89 (6H, t, $J = 6.9$ Hz, methyl), 1.30 (62H, brs, methylene), 1.55–1.62 (2H, m, methylene), 1.65–1.75 (2H, m, methylene), 2.19 (2H, t, $J = 7.4$ Hz, methylene), 3.47–4.00 (10H, m), for mannose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.72–4.74 (1H, m, anomeric), 7.2 (1H, m, amide).

High resolution ESI-MS calculated for $C_{47}H_{93}N_1O_8Na$ $(M + Na)^+$, 822.6799; found 822.6813.

4.2.5.11. ***10i*** *(Man2HMC16).* ^{1}H-NMR (400 MHz, CD_3OD): δ 0.82–0.94 (6H, m, methyl), 1.28 (48H, brs, methylene), 1.53–1.63 (2H, m, methylene), 1.63–1.78 (2H, m, methylene), 2.15–2.21 (2H, m, methylene), 3.40–3.95 (10H, m), for mannose C2, C3, C4, C5, C6a, C6b, sphingosine C1a, C1b, C2 and C3, 4.78–4.85 (1H, m, anomeric), 7.2 (1H, m, amide).

High resolution FAB-MS, calculated for $C_{39}H_{78}N_1O_8$ $(M + H)^+$, 688.5727; found 688.5733.

4.2.6 3-Hydroxy α-ManCer 3-Hydroxy α-ManCer (Man30HCl6, Scheme 3) was prepared as previously reported [8].

4.3. Bioassay of the synthesized glycolipids

Vα19-Jα26 invariant TCR Tg mice with the TCR Cα-deficient background were established as described previously [7,8]. C57BL/6 mice were obtained from Sankyo Service Co. (Tokyo, Japan) and Jackson Laboratory (Bar Harbor, ME, USA). MNCs were prepared from single cell suspensions of mouse livers by density gradient centrifugation using Percoll (Pharmacia, Uppsala, Sweden) as described previously [3]. Liver MNCs from indicated mouse strains (2–4 months of age) were cultured in 200 μl of DMEM supplemented with 10% FCS, 100 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 5×10^{-5} M 2-ME in the presence of glycolipids dissolved in DMSO (final concentration, 2 μg ml^{-1}). The concentration of cytokines in the culture fluid was determined by ELISA after 2 days (Pharmingen, San Diego, CA, USA). Cell proliferation was assessed at day 2 of culture by measuring the incorporation of [^{3}H]-thymidine (0.5 μCi ml^{-1}, Amersham, Buckinghamshire, UK) for 5 h.

Stimulation of lymphocytes in vivo was performed as previously reported by Yoshimoto et al. [25]. Vα19 Tg^+ TCR $\alpha^{-/-}$ or C57BL/6 mice (8 ~ 20 w of age) were intravenously injected with glycolipids (20 μg per 200 μl PBS) instead of anti-CD3 antibody. Spleens were removed from mice 90 min after the injection. MNCs were immediately prepared from them by density gradient centrifugation using lymphosepar II (MBL, Gunma, Japan, $d = 1.090$). They were cultured in the DMEM (10^7 cells per ml). Cytokines in the culture fluid were determined by ELISA.

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