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Minireview

Non-reducing end α-mannosylated glycolipids as potent activators for invariant Vα19 TCR-bearing natural killer T cells

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Abstract—A novel invariant Vα19-Jα33 T cell receptor α chain, first found in mammalian blood cells, was primarily expressed by natural killer T cell repertoire (Vα19 NKT cell). Attempts have been made to find specific antigens for Vα19 NKT cells. A series of α- and β-glycosyl ceramides were synthesized and tested whether they had potential to stimulate the cells isolated from invariant Vα19-Jα33 TCR transgenic mice (where the development of Vα19 NKT cells is facilitated). Comprehensive examinations revealed substantial antigenic activity in α-ManCer that was presented by MR1, one of the MHC class Ib molecules. Next, naturally occurring and synthetic α-mannosyl glycolipids were further analyzed to determine structural requirements for natural ligands for Vα19 NKT cells. As a result, α-mannosyl phosphatidyl inositols (PI) such as $(\alpha\text{-Man})_2$-PI and α-Man-α-$GlcNH_2$-PI (a partial structure of mycobacterial lipoarabinomannan and GPI-anchors) as well as α-ManCer derivatives were found to activate Vα19 NKT cells in vivo and in vitro. Thus, Vα19 NKT cells are possibly responsive to certain α-mannosyl glycolipids and may have roles in the innate and adaptative immune systems to protect against various antigens expressing α-mannosyl glycolipids and to regulate the adaptive immune system responding to the intracellular ligands.

Keywords: Glycosphingolipid; Glycosyl phosphatidylinositol; Immunotherapy

Contents

1. Introduction

Natural killer T (NKT) cells are a lymphocyte subset bearing both the common NK marker NK1.1, a product

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of a member of the NKR-P1 gene family, and T cell receptor (TCR)-CD3 complex.[1] The major component of NKT cells (Vα14 NKT cell) expresses invariant TCR α chains (mouse Vα14-Jα18, human Vα24-Jα18)[2,3] and is positively selected by the non-polymorphic major histocompatibility complex (MHC) class I-like CD1d molecule in association with β2-microglobulin (β2m).[4,5] Vα14 NKT cells are responsive to certain glycosphingolipids presented by CD1d, for example, α-galactosyl ceramide (α-GalCer)[6] isolated from marine sponge,[7] α-glucuronosyl and α-galacturonosyl ceramides from α-proteobacteria,[8,9] and α-galactosyl diacylglycerol from *Borrelia*.[10] In addition, it has been proposed that Vα14 NKT cells are positively selected by intracellular lysosomal isoglobotriaosyl ceramide in the context of CD1d.[11]

Recently, another invariant TCR α chain consisting of Vα19-Jα33 (conventionally Jα26) has been found in peripheral blood cells of human, bovine, and mouse deficient in transporter associated with antigen presentation (TAP) by quantitative PCR analysis.[12] We have demonstrated that cells expressing the Vα19-Jα33 invariant TCR α chain are mainly present as NKT cells (designated as Vα19 NKT cell in this review) in mouse.[13] Invariant Vα19-Jα33 TCR^+ cells are absent in mice lacking MR1, another non-classical MHC class I-like molecule, thus suggesting that they are positively selected by MR1.[14] It is estimated that Vα19 NKT cells represent 1% of mononuclear cells (MNCs) in the liver,[13] thus there is a considerably large population present as lymphocyte clone. Localization of the invariant Vα19-Jα33 TCR^+ cells in gut lamina propria is also reported.[14] Similar to Vα14 NKT cells,[15] Vα19 NKT cells immediately produce large amounts of both Th1- and Th2-promoting immunoregulatory cytokines in response to the engagement of the invariant TCR,[16–18] thus they are considered

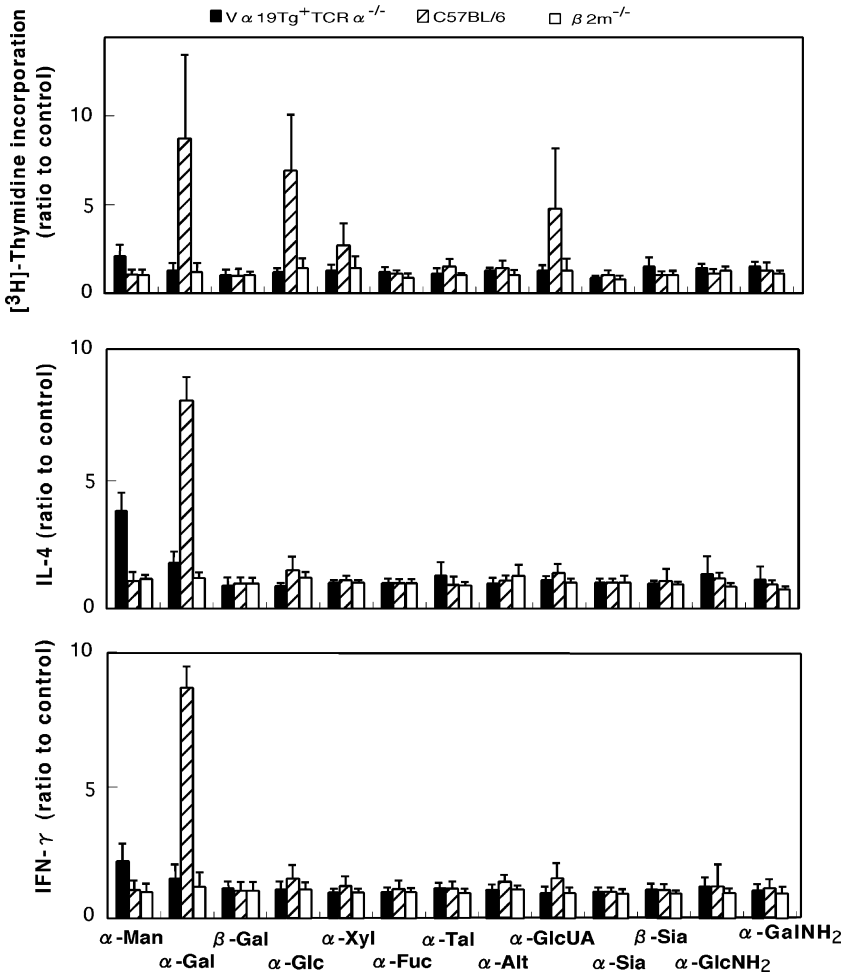


Figure 1. Activation of Vα19 Tg^+ cells with monoglycosyl sphingolipids in culture. Liver MNCs were isolated from Vα19Tg^+ TCR$\alpha^{-/-}$, C57BL/6, and $\beta2m^{-/-}$ mice by Percoll density gradient centrifugation and were cultured in the presence (1 μg/ml) or absence of glycolipids. After 2 days, the immune responses were assessed by measuring cell proliferation ([^{3}H]-thymidine incorporation for 5h), IL-4 and IFN-γ secretion in the culture supernatants. Results are shown as the fold increase relative to the control cultures with vehicle (1/200 v/v DMSO). The error bars indicate standard deviations. α-Glycosyl ceramides are abbreviated to α-Glys. β-Gal represented β-galactosyl ceramide; Sia, D-*N*-acetyl neuraminyl ceramide; Tal, D-talosyl ceramide, Alt, D-altrosyl ceramide.

to have important roles in the regulation of the immune system. The finding that over-expression of Vα19 NKT cells suppressed the progress of experimental autoimmune encephalomyelitis,[19] an animal model for multiple sclerosis, supports this notion. Therefore, the search for specific antigens for Vα19 NKT cells is quite important to develop new therapies for various immunoregulatory disorders based on the functional modulation of the lymphocyte repertoire.

The self-antigens presented by MR1 have not been identified.[20,21] Judging from the primary structure, MR1 possibly has a three-dimensional structure similar to classical MHC class I molecules that have a groove for antigen presentation. Although several key amino acid residues located at the bottom of the antigen-presenting groove and interacting with the antigens are strictly conserved in classical MHC class I molecules, those in MR1 are considerably converted.[20] Thus, it is strongly suggested that the molecular species presented by MR1 are different from those (peptides) presented by classical MHC class I molecules. The discovery of α-galactosyl glycolipids as stimulants for Vα14 NKT cells prompted us to investigate synthetic and natural glycolipids as agonists for Vα19 NKT cells.

2. Induction of immune responses from Vα19 NKT cells with synthetic α-mannosyl ceramide

In a continuous search for specific antigens for the induction of this novel NKT cell repertoire, a series of α- and β-glycosyl ceramides with a naturally occurring monosaccharide residue were synthesized and their potential to activate Vα19 NKT cells was examined.[17] The glycosphingolipids were synthesized via the glycosylation of azidosphingosines except for the synthesis of 2-aminohexosyl ceramides which were synthesized via the coupling of the 2-azido glycosyl imidate with sphingosine. Total mononuclear cells (MNCs) were prepared from the livers of invariant Vα19-Jα33 TCR transgenic (Tg) mice with TCR α chain-deficient (TCR $\alpha^{-/-}$) genetic background as responder cells. This preparation of the cells includes invariant Vα19 TCR Tg^{+} cells as the sole component of TCR^{+} cells, and it also includes the cell population with the potential to function as antigen-presenting cells. $NK1.1^{+}$ Vα19 Tg^{+} (Vα19 NKT) cells share about 30% of the total MNCs.[17] They were cultured in the presence of the synthetic glycosphingolipids, and the immune responses were assessed by measuring cytokine concentration in the culture fluid and cell proliferation (Fig. 1). Vα19 Tg^{+} $TCR\alpha^{-/-}$ cells were most efficiently induced to proliferate and produce IL-4 and IFN-γ by α-ManCer. The immune responses of Vα19 Tg^{+} $TCR\alpha^{-/-}$ cells to this glycolipid were in a dose-dependent manner (Fig. 2). In contrast, C57BL/6 cells (including about 30% of Vα14 NKT cells) were responsive to α-GalCer and to a certain extent to α-glucosyl and glucuronyl ceramide (α-GlcCer, α-GlcUACer). Both C57BL/6 and Vα19 Tg^{+} cells showed no detectable responsiveness to β-glycosyl ceramides. β2-Microblobulin-deficient ($\beta2m^{-/-}$) cells (including neither Vα19 nor Vα14 NKT cells) showed no reactivity to the glycolipids. Thus, these results suggest that Vα19 NKT cells are responsive to

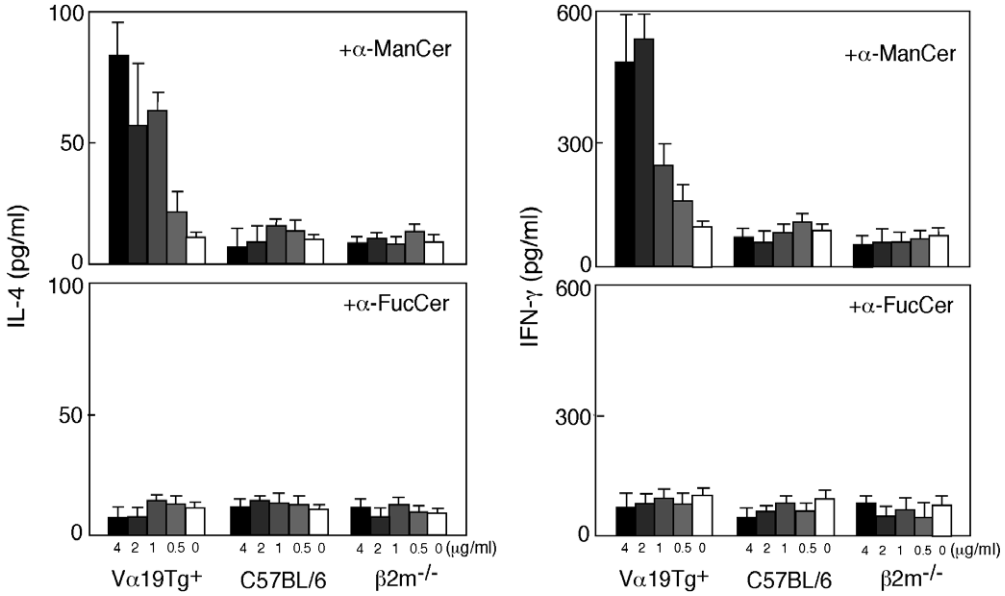
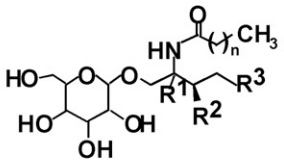


Figure 2. Dose-dependent activation of Vα19 NKT cells by α-ManCer in culture. Liver MNCs from Vα19 Tg^{+} $TCR\alpha^{-/-}$, C57BL/6, and $\beta2m^{-/-}$ mice were cultured with the indicated dose of glycolipids as described in Figure 1. The cytokine production in the culture supernatants was determined after 2 days of culture. One of the two independent experiments giving essentially the same profiles of cytokine production is shown. The error bars indicate standard deviations.

the stimulation with α-ManCer. No significant cytokine production by Vα19 NKT cells was induced in culture with either α-altrosyl ceramide or α-talosyl ceramide (both α-altrose and α-talose as well as mannose possess a 2-axial hydroxy group, but the 3-hydroxy group in α-altrose and the 4-hydroxy group in α-talose are reversed from those in α-mannose), suggesting a stringent recognition of the α-mannosyl residue by the invariant Vα19-Jα33 TCR. Since the natural occurrence of α-ManCer has not been reported yet, it is possible to spec-



Abbreviations	R^1	R^2	R^3	n	Hexose
Man4PhC16	H	H	4-octylphenyl	14	α-Man
GalC24	H	H	dodecanyl	22	α-Gal
ManC16	H	H	dodecanyl	14	α-Man
Man2HM4PhC16	CH_2OH	H	4-octylphenyl	14	α-Man
Gal2HM4PhC16	CH_2OH	H	4-octylphenyl	14	α-Gal
Man2HMC24	CH_2OH	H	dodecanyl	22	α-Man
Man2HMC16	CH_2OH	H	dodecanyl	14	α-Man
Man3OHC16	H	OH	dodecanyl	14	α-Man

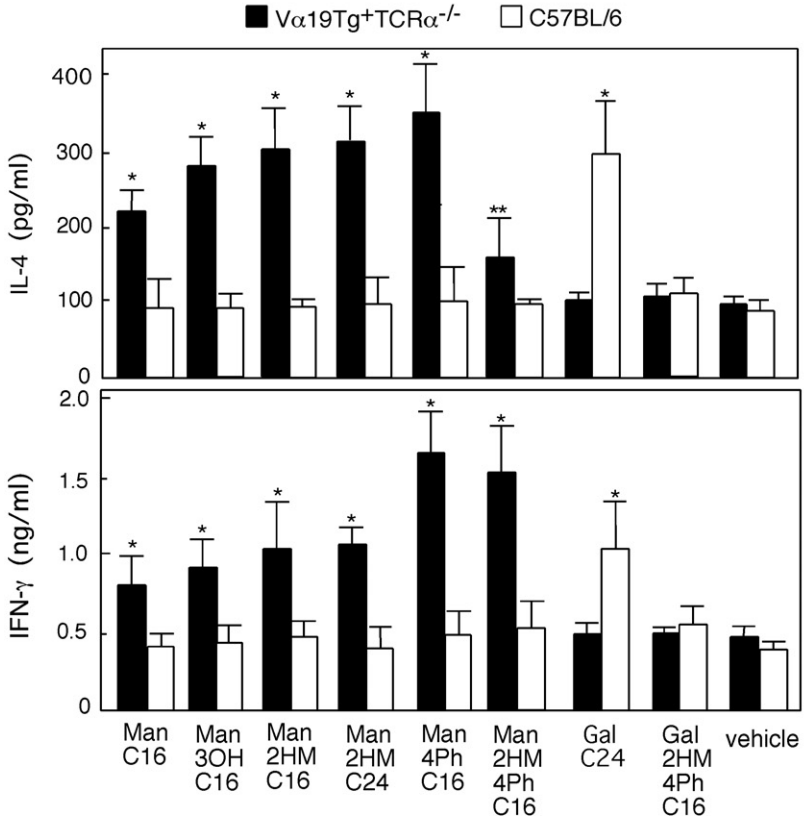


Figure 3. Immune responses of Vα19 NKT cells in culture elicited by α-ManCer and its derivatives. Liver MNCs from Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice were cultured with the addition of glycolipids dissolved in DMSO (final concentration, 2 μg/ml). After 2 days, the concentrations of IL-4 and IFN-γ in the culture fluid were determined by ELISA. The culture represented as 'vehicle' included 1/200 v/v of DMSO. The filled bars represent the culture of Vα19$Tg^+TCR\alpha^{-/-}$ cells, whereas the open bars show the results of C57BL/6 cells. The error bars indicate the standard deviation. The p values in Dunnett's multiple comparison post test are calculated in comparison with the control (cytokine levels in culture with vehicle). $*$, $p < 0.01$; and $**$, $p < 0.05$. Glycosphingolipids modified with a 2-hydroxymethyl, 3-hydroxyl, or 4-octylphenyl groups are represented as 2HM, 3OH, or 4Ph. Man3OHC16 is the original form of α-ManCer.[19] Man2HM4PhC16 is the derivative in which the sphingosine unit is replaced with FTY720.[22]

ulate that this glycolipid mimics natural ligands for the NKT cells.

3. Induction of either Th1- or Th2-dominant immune responses of Vα19 NKT cells with α-ManCer derivatives

It is possible that modification of the lipid moiety in α-ManCer will alter the interaction with the antigen-presenting molecule and improve the recognition of the α-mannose residue by the invariant TCR. Previously, we found immunosuppressive activity in ISP-I, a product of *Isaria sincliairii*.[22] FTY720 was obtained by the structural modification of ISP-I to optimize the immunosuppressive activity. As expected by the structural homology between FTY720 and sphingosine, this drug targets sphingosine-1-phosphate receptors and acts as an agonist.[23] We synthesized a series of α-ManCer derivatives in which the sphingosine moiety was replaced with FTY720 or related aminoalcohols[24] (Fig. 3). The modified α-ManCer induced promotive rather than suppressive immune responses from Vα19 NKT cells. They induced either Th1- or Th2-dominant immune responses.[25] The relative intensity of IL-4 to IFN-γ secretion by Vα19 NKT cells was dependent on the chemical structure of the stimulator. For instance, Man2HM4PhC16 (in which the sphingosine moiety is replaced with FTY720) induced IFN-γ-dominant cytokine production by Vα19 Tg^{+} cells. Presumably, manipulation of the sphingosine portion of α-ManCer alters the interaction between invariant Vα19 TCR and the α-mannosyl residue in the glycolipids, resulting in the modulation of the immune responses of Vα19 NKT cells. One of the modified α-ManCers, Man4PhC16, that has a phenyl group in the sphingosine hydrocarbon chain, induced the production of both Th1- (IFN-γ, IL-12, IL-17) and Th2- (IL-4, IL-5, IL-10) promoting cytokines more intensively than the intact α-ManCer or any other derivatives.[25]

4. Requirement of non-reducing end α-mannosyl residues for ligands for Vα19 NKT cells

To determine structural requirements for ligands for Vα19 NKT cells, naturally occurring and synthetic

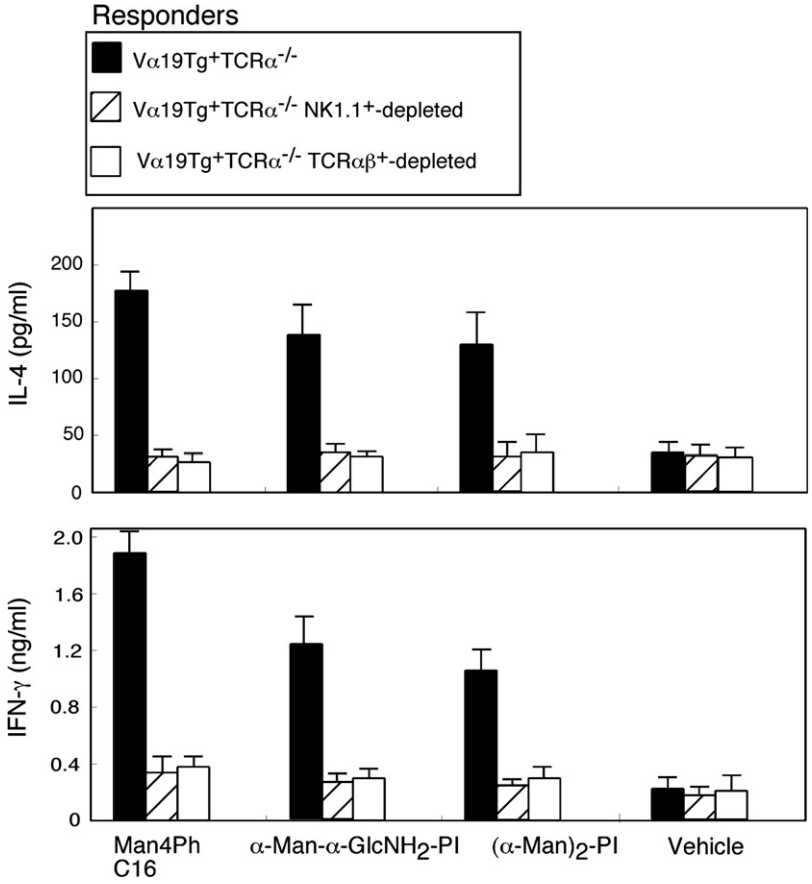


Figure 4. Determination of the cell populations in the Tg liver responding to the α-mannosyl glycolipids. Liver MNCs prepared from Vα19Tg^{+} TCRα$^{-/-}$ mice were depleted of NK1.1^{+} or TCR αβ^{+} cells using biotin-conjugated antibody and streptavidin-coated magnetic beads. Cells after depletion were cultured with the indicated glycolipids (2 μg/ml) for 3 days, and the concentration of IL-4 and IFN-γ in the supernatants was determined by ELISA. The average of triplicate cultures in one of the independent three experiments is shown. Error bars indicate standard deviations.

glycolipids were further analyzed for their potential to induce immune responses from Vα19 NKT cells. As well as α-ManCer[17] and its derivatives,[25] 2,6-di-α-mannosyl-phosphatidylinositol $(\alpha\text{-Man})_2$-PI, a partial structure of mycobacterial lipoarabinomannan (LAM)[26] and α-mannosyl-(1→4) -α-glucosamine-(1→6)-phosphatidylinositol (α-Man-α-$GlcNH_2$-PI, a partial structure of the GPI-anchor[27]) were found to be potent stimulators for Vα19 NKT cells.[28] The active glycolipids had α-mannosyl residue(s) at the non-reducing end in common. In contrast, glycolipids such as porcine blood glycosphingolipids (including β-GlcCer, LacCer, Gb3Cer, and Gb4Cer), bovine brain gangliosides (including GM3, GM2, GM1, GD1, and GT1), phospholipids (phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine), yeast glycosyl phosphoinositol ceramide mixture (α-Man-*Ino*-PO_4^-Cer etc.,[29]), LAM and its partially degraded derivatives ($(\alpha\text{-Man})_n$-PI, 40 kD),[30] β-galactosyl phytodiacylglycerol,[31] bivalve α-mannosylated trihexosyl ceramides (α-Man-Man-Glc-Cer)[32] etc. did not stimulate Vα19 Tg^+ cells up to 10 μg/ml. Taken together, it is strongly suggested that certain glycolipids with appropriately located non-reducing end α-mannosyl residue(s) have the potential to stimulate Vα19 NKT cells.

5. Immune responses of Vα19 NKT cells to α-mannosyl glycolipids presented by MR1

It was strongly suggested that the potential to respond to the α-ManCer derivatives was confined to the $NK1.1^+$ Vα19 Tg^+ (Vα19NKT) cells among the responders prepared from Vα19 Tg mice, because the depletion of the $NK1.1^+$ or $TCR\alpha\beta^+$ population from the responders reduced the responsiveness to the glycolipids[25] (Fig. 4). Presumably, lymphoid precursors bearing invariant Vα19 TCR α chains paired with appropriate TCR β chains are capable of being positively selected by the MR1-α-mannosyl-glycolipid complex and differentiated into NKT-lineage.

The stimulation of Vα19 Tg^+ cells was induced by co-culture with the cells of human B lymphoma line (Raji) transfected with the cDNA of MR1[28] (Fig. 5), while the stimulation was not found with the transfectants of any other MHC genes such as CD1, MR1, Qa2, and TL. Thus, it is likely that invariant Vα19 TCR-bearing cells are restricted by MR1 that is presenting certain endogenous antigens or chaperons. This result is in accord with the recent reports that invariant Vα19 TCR^+ cells are positively selected by MR1.[14,18]

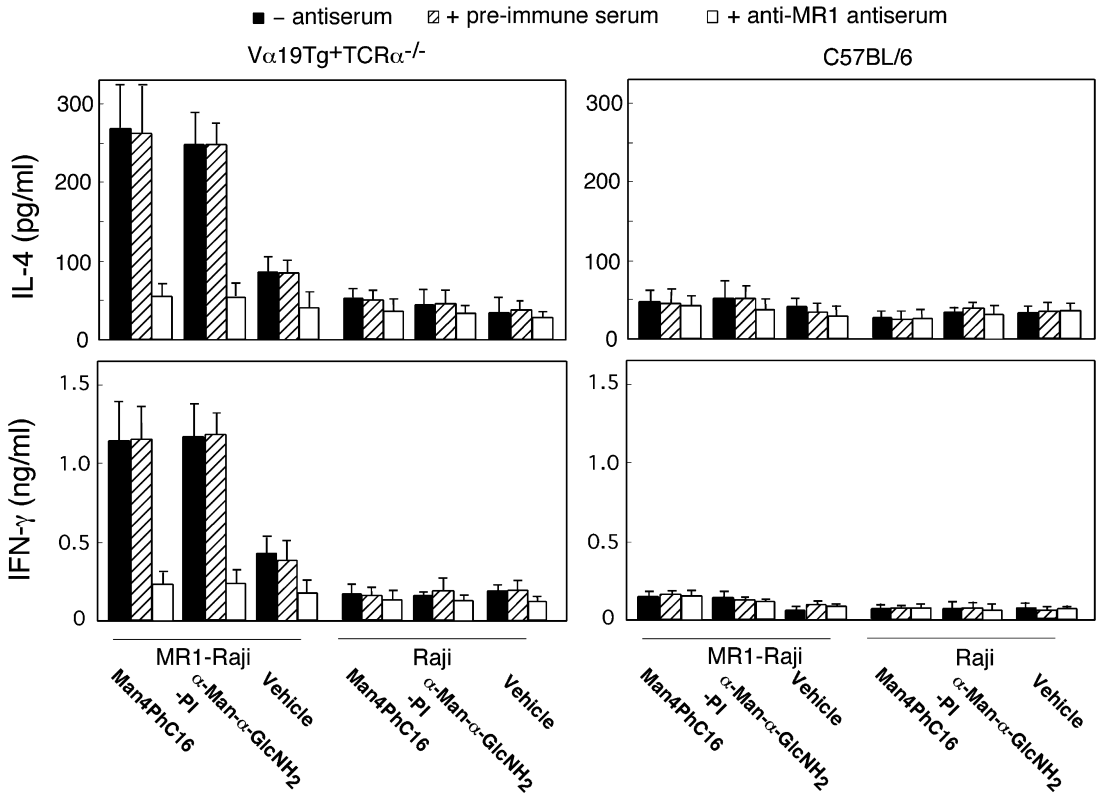


Figure 5. Stimulation of Vα19 Tg cells with glycolipid antigens in the context of MR1. MR1-transfected or non-transfected Raji cells were incubated with glycolipids (2 μg/ml) for 5 h. They were washed with medium and irradiated (3000R), then cultured with liver MNCs isolated from $V\alpha19Tg^+TCR\alpha^{-/-}$ or C57BL/6 mice for 3 d in the presence or absence of purified rabbit anti-MR1 antiserum raised against the peptide with the partial sequence of the α2 domain of MR1[28] or rabbit serum before immunization (pre-immune serum) (3 μg/ml). Cytokine concentration in the culture fluid was determined by ELISA. The averages of triplicate cultures in one of the representative two results are shown.

The immune responses of Vα19 Tg^+ cells toward MR1-transfectants were enhanced when the transfectants were previously loaded with the α-mannosyl glycolipids[28] as shown in Figure 5. Presumably, putative intracellular ligands were replaced by these glycolipids at the antigen-presenting groove in MR1 molecules. Since the truncation of the *N*-acyl group length in α-glycosyl ceramides drastically reduced the activity toward Vα19 NKT cells,[17] the lipid portion of antigenic glycolipids possibly binds to the antigen-presenting groove of MR1 leaving the sugar moiety available for the interaction with the invariant TCR. The immune responses were reduced in the presence of anti-MR1 antiserum but not pre-immune serum, thus also supporting the participation of MR1 in the antigen presentation.

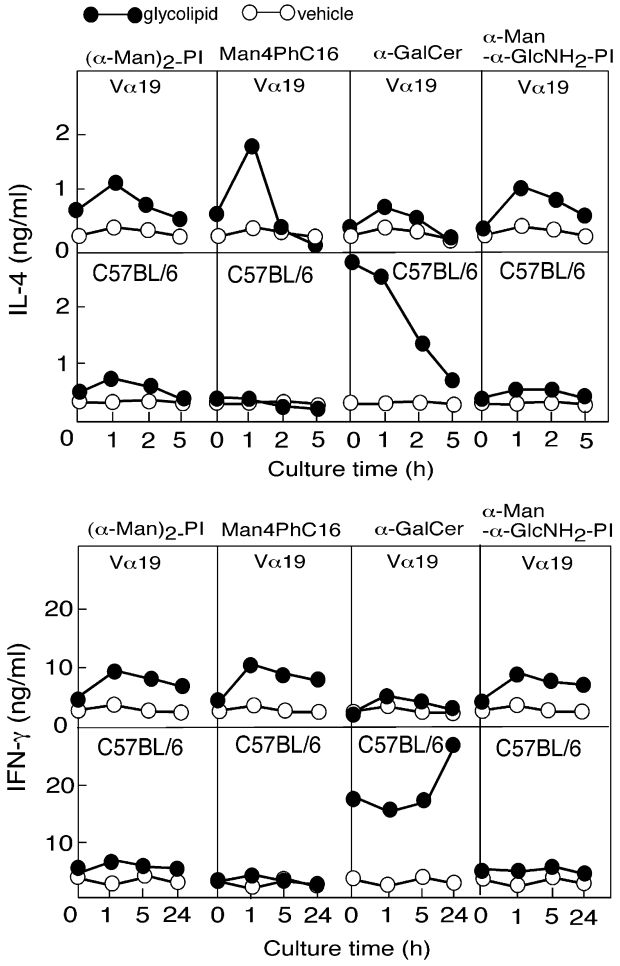


Figure 6. Immune responses of Vα19 NKT cells in Vα19 invariant TCR Tg mice injected with α-mannosyl glycolipids. Vα19 invariant TCR Tg or non-Tg mice (C57BL/6 genetic backgrounds) were intravenously injected with α-mannosyl glycolipids (10 μg/mouse dissolved in PBS). After 90 min, single cell suspension was quickly prepared from spleens, and cells were cultured for indicated hours in the DME medium (including 10% FCS) without special supplements. Cytokine concentrations in the culture supernatants were determined by ELISA.

Taken together, it is strongly suggested that invariant Vα19 NKT cells recognize α-mannosyl glycolipids that are presented by MR1.

6. Concluding remarks

The structural requirements for natural ligands for invariant Vα19 TCR^+ cells were suggested by the comprehensive examination. Certain glycolipids, each with α-mannosyl residue(s) at the non-reducing end and consisting of LAM or GPI-anchors, have been shown to be stimulus for Vα19 NKT cells when they are presented by MR1. However, the immune responses of Vα19 Tg^+ cells induced by the stimulation with α-mannosyl glycolipids were apparently less significant than those of Vα14 NKT cells induced by α-GalCer. Thus, it remains possible that glycolipids with non-reducing end α-mannosyl residue(s) are present as intracellular metabolites or in bacterial components such as endotoxins that are more immuno-competent toward Vα19 NKT cells than the glycolipids so far characterized.

Specific activators or inhibitors for Vα19 NKT cells may be important for medical applications, since specific activators for Vα14 NKT cells such as α-GalCer and its homologues have been shown to be effective in a number of animal models of disease.[33,34] The immune responses of Vα19 Tg^+ cells were induced not only in culture but also in vivo with the α-mannosyl glycolipids[25,28] (Fig. 6). In addition, the structural modification of α-ManCer enhanced either Th1- or Th2-dominant immune responses from Vα19 NKT cells.[25] Therefore, these glycolipids are prospective leading compounds to develop new therapies for immunological disorders targeting Vα19 NKT cells.

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