Glycolipid Stimulators for NKT Cells Bearing Invariant Vα19-Jα33 TCR α Chains

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Abstract: Attempts have been made to find specific antigens for a novel NKT cell subset bearing invariant Vα19-Jα33 TCR α chains (Vα19 NKT cell). Comprehensive examinations revealed substantial antigenic activity in synthetic α-mannosylceramide (ManCer) that was presented by MR1. Structural modification of the sphingosine moiety of α-ManCer improved antigenic activity to enhance either Th1 or Th2 –promoting cytokine production by Vα19 NKT cells. Such α-ManCer analogues will be useful for developing new therapies as immunomodulators.

Key Words: Glycolipid, FTY720, NKT cell, invariant TCR, immunomodulator, non-classical MHC class I.

Introduction

Natural killer T (NKT) cells are defined as lymphocytes bearing both the common NK marker NK1.1, a product of a member of the NKR-P1 gene family, and TCR-CD3 complex [1]. The major component of NKT cells (Vα14 NKT cell) [2, 3] is characterized by the expression of the invariant TCR α chain (mouse Vα14-Jα18, human Vα24-Jα18), and is positively selected by the non polymorphic MHC class I-like CD1d molecule in association with β2-microglobulin (β2m) [4, 5]. Vα14 NKT cells are responsive to certain glycosphingolipids in the context of CD1d such as α-galactosyl ceramide (α-GalCer, reference [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 [11].

Recently, another invariant TCR α chain consisting of Vα19-Jα33 (conventionally Jα26) has been found in human, bovine, and TAP-deficient mouse peripheral blood cells by quantitative PCR analysis [12]. We have demonstrated that cells expressing the Vα19-Jα33 invariant TCR α chain are mainly present as NKT cells in mouse [13]. These cells (designated as Vα19 NKT cell in this review) are absent in mice lacking the non-classical MHC class I molecule MR1, 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 they are a considerably large population as a 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 and thus are considered to have important roles in the regulation of the immune system (Shimamura, M. *et al*. Characterization of a novel NKT cell repertoire expressing an invariant Vα19-Jα26 TCRα chain using the invariant TCR transgenic mice, Abstract #4 for the 2nd International Workshop on CD1 Antigen Presentation and NKT Cells, Woods Hole, 2002, [16], [17]). Thus, Vα19 NKT cells are suggested to participate in the regulation of the immune system. The finding that overexpression of Vα19 NKT cells suppressed the progress of experimental autoimmune encephalomyelitis [18], 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 [19, 20]. MR1 possibly has a three-dimensional structure similar to classical MHC class I molecules which have a groove for antigen-presentation, but the several key amino acid residues locatied at the bottom of the antigen-presenting groove and interacting with the antigens in MR1 are quite different from those commonly conserved in classical MHC class I molecules, thus it is strongly suggested that the molecular species presented by MR1 are different from those (peptides) presented by classical MHC class I molecules [19]. 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.

SYNTHETIC α-MANNOSYL CERAMIDE AS A POTENT STIMULANT FOR Vα19 NKT CELLS

A series of α- and β-glycosyl ceramides with a naturally occurring monosaccharide residue were synthesized, and their potentials to activate Vα19 NKT cells was examined in a continuous search for specific antigens for this novel NKT cell repertoire [16]. The glycosphingolipids were synthesized *via* the glycosylation of azidosphingosines except for the synthesis of 2-aminohexosyl ceramides. The latter were synthesized *via* the coupling of the 2-azido glycosyl imidate with sphingosine. Total MNCs were prepared from livers of invariant Vα19-Jα33 (conventionally 26) TCR transgenic (Tg) mice with TCR $C\alpha^{-/-}$ genetic background as responders. This preparation of the cells includes Vα19 Tg^+ cells as the

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sole component of TCR^+ cells, and it also includes the cell population with the potential to function as antigen-presenting cells. Vα19 Tg^+ $NK1.1^+$ cells share about 30% of the cells in the total MNCs. They were cultured in the presence of the synthetic glycosphingolipids and the immune responses were monitored by measuring cytokine secretion in the culture fluid and cell proliferation (Fig. (**1**)). Vα19 Tg^+ $TCR\alpha^{-/-}$ cells were most efficiently induced to proliferate and

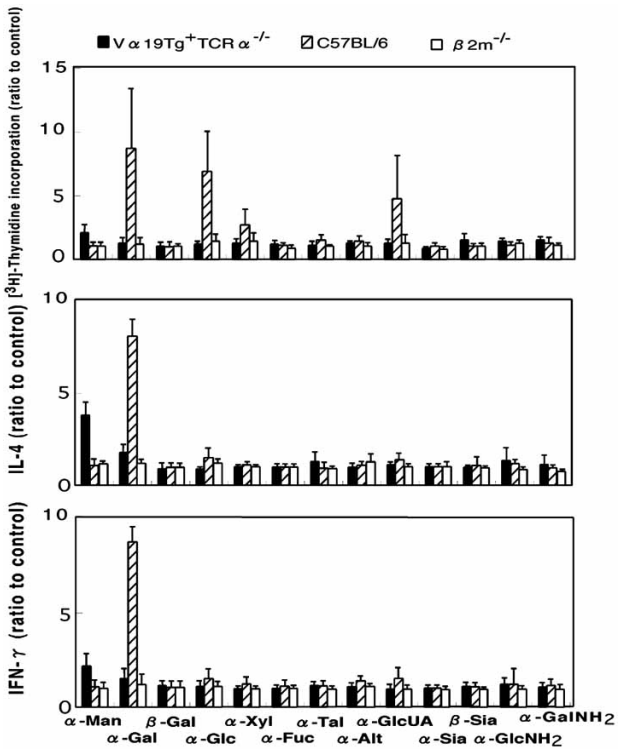

Fig. (1). Activation of Vα19 Tg^+ cells with glycolipid antigens in culture.

Liver MNCs prepared from Vα19Tg^+ $TCR\alpha^{-/-}$, C57BL/6 and $\beta 2m^{-/-}$ mice were cultured in the presence (1 μg/ml) or absence of glycolipids. After 2 days, the immune responses were monitored by measuring cell proliferation ($[^3H]$-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). Abbreviations: α-Gly, α-glycosyl ceramide; β-Gal, β-galactosyl ceramide; Sia, D-N-acetyl neuraminyl; Tal, D-talosyl, Alt, D-altrosyl.

produce IL-4 and IFN-γ by α-ManCer. 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. Thus, these results suggest that Vα19 NKT cells are responsive to the stimulation with α-ManCer. No significant immune responses of Vα19 NKT cells were 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, this glycolipid possibly mimics natural ligands for the NKT cells.

EITHER TH1 OR TH2 -DOMINANT IMMUNE RESPONSES OF Vα19 NKT CELLS TO 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, immunosuppressive activity was found in an antibiotic ISP-I, a product of *Isaria sincliairii* [21]. FTY720 was obtained by the structural modification of ISP-I to optimize the immunosuppressive activity. As suggested by the structural homology between FTY720 and sphingosine, this drug targets sphingosine-1-phosphate receptors and acts as an agonist [22]. We synthesized a series of α-ManCer derivatives [23] in which the sphingosine moiety was replaced with FTY720 or related aminoalcohols (Fig. (**2**)). The modified α-ManCer induced promotive rather than suppressive immune responses from Vα19 NKT cells. They induced either Th1- or Th2-dominant immune responses [24]. 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, Man2 HM4PhC16 (sphingosine moiety is replaced with FTY720) induced Th1-biased 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.

STRUCTURAL REQUIREMENTS FOR LIGANDS FOR Vα19 NKT CELLS

To determine structural requirements for ligands for Vα19 NKT cells, naturally occurring and synthetic glycolipids were further analyzed for potentials to induce immune responses from Vα19 NKT cells. As well as α-ManCer [16] and its derivatives [24], 2, 6-di α-mannosyl phosphatidylinositol $(\alpha\text{-Man})_2PI$, a partial structure of bacterial LAM [25]) and α-mannosyl 1-4α-glucosamine1-6-phosphatidylinositol (α-Man-$GlcNH_2$-PI, a partial structure of GPI-anchor [26]) were found as a potent stimulator for Vα19 NKT cells [27]. 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, GT1 etc.), phospholipids (phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine), yeast glycosyl phosphoinositol ceramide mixture (α-Man-Ino-PO_4^-).

Cer etc. [28], mycobacterial lipoarrabinomannan (LAM) and its partially degraded derivatives ($(\alpha\text{-Man})_n$-PI, 40kD) [29], β-galactosyl phytodiacylglycerol [30], bivalve α-man-

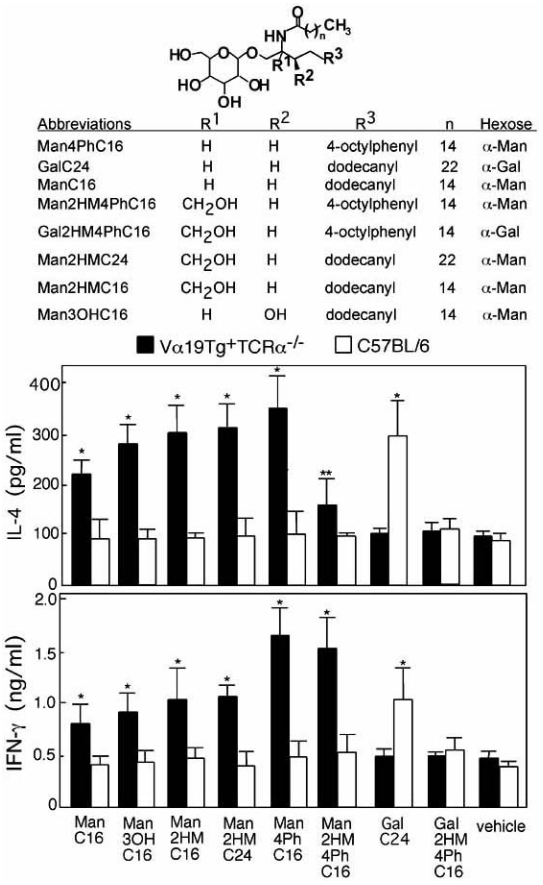

Fig. (2). Immune responses of Vα19 NKT cells in culture elicited by α-ManCer and its derivatives.

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 immune responses were monitored by measuring the concentrations of IL-4 and IFN-γ in the culture fluid (referred from the figure in the reference [24]). 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 group are represented as 2HM, 3OH or 4Ph. Man3OHC16 is the original form of α-ManCer [16]. Man2HM4PhC16 is the derivative in which the sphingosine unit is replaced with FTY720 [21].

nosylated trihexosyl ceramides (α-Man-Man-Glc-Cer) [31] etc. did not stimulate Vα19 Tg^+ cells up to 10 μg/ml . Taken together, it is strongly suggested that certain glycolipids with non-reducing end α-mannosyl residue have potentials to stimulate Vα19 NKT cells.

MR1-RESTRICTED STIMULATION OF Vα19 NKT CELLS WITH THE α-MANNOSYL GLYCOLIPIDS

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 depletion of the $NK1.1^+$ or $TCR\alpha\beta^+$ population from the responders reduced the responsiveness to the glycolipids [24] (Fig. (**3**)).

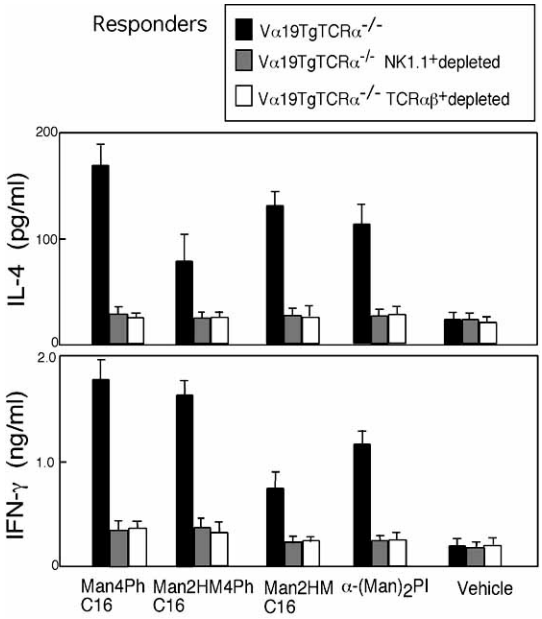

Fig. (3). Determination of the cell populations in the Tg liver responding to the α-ManCer analogues.

Liver MNCs prepared from Vα19Tg $TCR\alpha^{-/-}$ mice were depleted of $NK1.1^+$ or TCR $\alpha\beta^+$ cells using biotin-conjugated antibody and streptavidin magnetic beads. Cells were cultured with the glycolipids, 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.

Stimulation of Vα19 Tg^+ cells was induced by co-culture with cells of human B lymphoma line (Raji) transfected with the cDNA of one of the non-classical MHC class I molecules MR1 [27] (Fig. (**4**)), while it was 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, 17].

The immune responses of Vα19 Tg^+ cells toward MR1-transfectants were enhanced when the transfectants were previously loaded with the α-mannosyl glycolipids [27] as shown in Fig. **4**. Presumably, putative intracellular ligands were replaced by these glycolipids at the antigen-presenting groove in MR1 molecules. The immune responses were reduced in the presence of anti-MR1 antiserum but not pre-immune serum. Taken together, it is strongly suggested that invariant Vα19 NKT cells recognize α-mannosyl glycolipids that are presented by MR1.

CONCLUDING REMARKS

The structural requirements for natural ligands for invariant Vα19 TCR^+ cells were suggested by the comprehensive examination. Certain glycolipids, each possessing α-mannosyl residue(s) at the non-reducing end, have been shown to

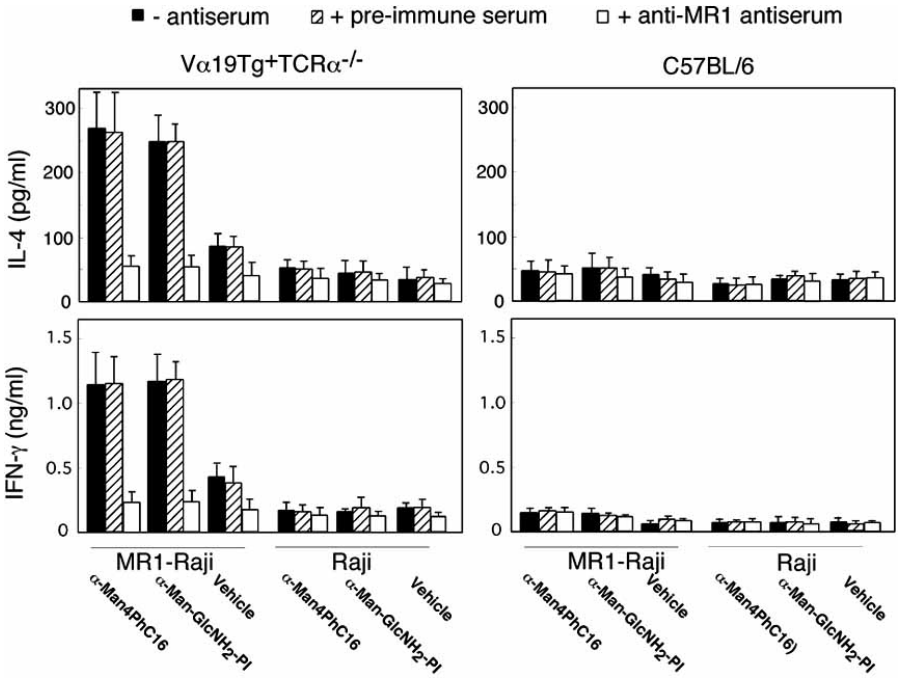

Fig. (4). Stimulation of Vα19 Tg cells with glycolipid antigens in the context of MR1.

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 Vα19Tg^{+}TCRα$^{-/-}$ mice for 3 d in the presence or absence of purified rabbit anti-MR1 antiserum or 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.

be stimulus for Vα19 NKT cells when they are presented by MR1. Since the truncation of the N-acyl group length in α-glycosyl ceramides drastically reduced the activity toward Vα19 NKT cells [16], 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. 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 brought by α-GalCer. Thus, it remains possible that glycolipids with non-reducing end α-mannosyl residue(s) are present that are more immunocompetent 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 [32, 33]. The immune responses of Vα19 Tg^{+} cells were induced not only in culture but also *in vivo* with the α-mannosyl glycolipids [24, 27]. Therefore, these glycolipids are prospective as lead compounds to develop new therapies for immunological disorders targeting Vα19 NKT cells.

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