monoclonal antibody like rituximab or block the T cell-B cell interaction by available drugs. An increase of IL-17 in the CSF also tempts us to consider therapy that modulates IL-6 or IL-23 signaling, which is involved in the generation and maintenance of Th17 cells. Because of recent advances in research, it may not take so long to establish a reasonable and more efficacious protocol for treatment of NMO.

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Conflict of interest statement

None declared.

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Synthetic Glycolipid Ligands for Human *i*NKT Cells as Potential Therapeutic Agents for Immunotherapy

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Abstract: Invariant natural killer T (*i*NKT) cells are an attractive therapeutic target in autoimmune diseases, since they play a major role in immune regulation. *i*NKT cells recognize glycolipid antigens presented by CD1d molecules that resemble the non-polymorphic MHC class I protein. α-galactosylceramide (α-GalCer) isolated from marine sponge has long been used as a prototype *i*NKT cell ligand in the laboratory. As α-GalCer is the most efficacious ligand for *i*NKT cells, its potential to treat autoimmune disease has been evaluated in animal models. Previous studies showed that α-GalCer effectively suppressed disease in some autoimmunity models, but not in others. This inconsistency may be attributed to the ability of α-GalCer to induce the production of both proinflammatory Th1 and anti-inflammatory Th2 cytokines by *i*NKT cells. To overcome this issue, we and other groups have synthesized new, unnatural glycolipids by modifying the structure of α-GalCer. These efforts have led to an identification of glycolipid compounds that provoke the production of Th2 (but not Th1) cytokines by *i*NKT cells. Among these novel ligands, an α-GalCer analogue named OCH, which contains a truncated sphingosine chain, induces a Th2 biased response by murine *i*NKT cells. Here we describe that OCH also polarizes human *i*NKT cells towards Th2, which opens up a new avenue for the clinical application of glycolipid compounds in treating of autoimmune diseases such as multiple sclerosis. The pursuit of synthetic glycolipid antigens has the great potential to lead to a better understanding of the regulatory effects of human *i*NKT cells and development of a new therapeutic agent for autoimmune diseases.

Keywords: Glycolipid, synthetic α-galacotosylceramide analogues, autoimmune disease, *i*NKT cells, Th1-Th2.

1. *i*NKT CELLS

Autoimmune diseases generate persistent tissue-specific damage and affect millions of people worldwide, leading to numerous social and economical problems. Thus, investigation of mechanisms by which autoimmunity develops and identification of novel therapeutic targets for treating autoimmune diseases are one of the major research themes in life science, as well as in pharmaceutical research. Recent research has revealed that the pathogenesis of autoimmune diseases such as multiple sclerosis (MS) may be caused by an alteration in the function of immune regulatory cells [1,2]. In fact, it was observed that the development of autoimmune diseases could be accompanied by functional changes amongst $CD25^+$ regulatory T cells [3] and invariant natural killer T (*i*NKT) cells [4,5]. Based on these data, one could argue that the restoration of regulatory cell function or the promotion of regulation by other cell types are ideal strategies for combating autoimmune diseases.

*i*NKT cells are a unique subset of T lymphocytes that display regulatory functions mainly *via* production of cytokines. They bear a distinctive T cell receptor (TCR) α chain encoded by an invariant Vα14-Jα18 rearrangement in mice or Vα24-JαQ in humans. The invariant TCRα chain pairs with a restricted repertoire of TCRβ chains, comprising Vβ8.2, Vβ7, and Vβ2 in mice or Vβ11 in humans [6,7,8]. Unlike conventional T cells that recognize peptide antigens bound to major histocompatibility complex (MHC) molecules, *i*NKT cells instead recognize glycolipid antigens bound to CD1d molecules. CD1d is a MHC class I-like molecule, which is expressed by monocytes, dendritic cells, and B cells. Optimally activated *i*NKT cells rapidly secrete large amounts of both inflammatory and anti-inflammatory cytokines and as *i*NKT cells produce such regulatory cytokines, it is supposed that they may play a critical role in the regulation of both innate and acquired immunity. Recent studies have addressed how *i*NKT cells can be activated during infectious diseases, tumor immunity, and autoimmunity: it appears that under certain conditions *i*NKT cells recognize an endogenous glycolipid bound to CD1d before secreting cytokines [9,10].

Frequencies of *i*NKT cells among peripheral lymphocytes are much lower in human than in mice [7,8]. However, human and mouse *i*NKT cells do appear to share similar characteristics in their function and activity. Human *i*NKT cells are mainly comprised of two subsets: $CD4^-CD8^-$ (double negative, DN) and $CD4^+$. Whereas the DN *i*NKT cells predominantly produce proinflammatory Th1 cytokines upon stimulation, the $CD4^+$ subset can release both Th1 and Th2 cytokines upon activation [11,12]. This unique ability to produce cytokines with antagonizing functions raises the possibility that *i*NKT cells can play an important role in the maintenance of the immune homeostasis. As *i*NKT lack TCR diversity and mount such rapid responses to antigens, one may speculate on their role in eradicating neoplasm or combating bacterial [13,14], viral [15-17], and parasite infection [18]. In addition, recent studies demonstrated that *i*NKT cells can modulate the pathogenesis of various autoimmune diseases [19-24]. However, whether *i*NKT cells play a protective or pathogenic role in autoimmunity appears to be influenced by a number of factors that require further characterization [20].

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2. GLYCOLIPID LIGANDS FOR *i*NKT CELLS

2.1. Microbial and Self Ligands

To understand the function of *i*NKT cells, the search for microbial and self ligands is essential. Recent studies have shown that glycosphingolipids (GSLs), bacterial components from the cell wall of *Sphingomonas*, are vital ligands for *i*NKT cells [13,14,25]. Among the GSLs, monoglycosylceramide (GSL-1) (Fig. **1A**) seems to act as the most potent stimulator of *i*NKT cells [26]. The GSLs may act as a substitute for lipopolysaccharide (LPS) in LPS-negative bacteria and it is accepted that the GSLs-stimulated *i*NKT cells participate in clearing such bacterial infections. *Borrelia burgdorferi* glycolipid I (BbGL-I) and II (BbGL-II) (Fig. **1A**) are also reported to activate both murine and human *i*NKT cells to elaborate IL-4 and IFN-γ [27]. In addition, glycolipids derived from *Sphingomonas wittichii* and *Borrelia burgdorferi* [28] are shown to stimulate IL-17 production by a particular subset of *i*NKT cells and *Mycobacterium bovis* (BCG) is also reported to activate a subpopulation of *i*NKT cells [29]. These results support the hypothesis that *i*NKT cells play a protective role following infection with particular bacteria that express *i*NKT cell ligands.

During investigation of *i*NKT cell ligands, glycosylphosphatidylinositol [30] and cellular phospholipids [31] were identified as possible endogenous ligands for *i*NKT cells. Recent analysis has suggested that Isoglobotrihexosylcera-

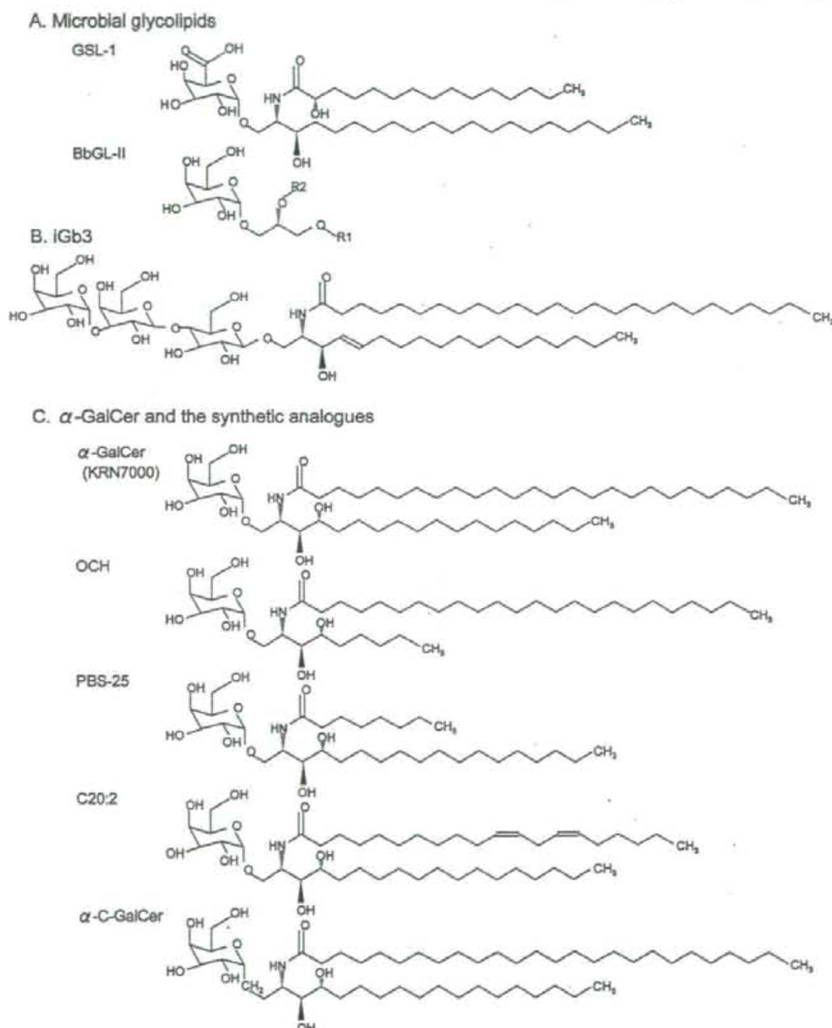

Fig. (1). Chemical structures of glycolipids and the analogues.

(**A**) GSL-1 derived from Sphingomonas bacteria, and BbGL-II derived from *Borrelia burgdorferi*. R1, *sn*-1 position of glycerol; R2, *sn*-2 position of glycerol (e.g. BbGL-IIa, R1 = C16:0, R2 = C18:1). (**B**) Endogenous ligand iGb3. (**C**) α-GalCer (KRN7000) [40] and the synthetic analogues OCH [49], PBS-25 [57], C20:2 [58], α-C-GalCer [60].

mide (iGb3) may be a natural ligand for *i*NKT cells (Fig. **1B**) [32-34] and further evidence indicates that it is likely that iGb3 functions as a physiological ligand for *i*NKT cells, which is required for their development. This hypothesis is supported by data demonstrating that a lack of β-hexoaminidase B, an enzyme which degrades iGb4 into iGb3, results in a severe impairment of thymic *i*NKT cell production in mice [35]. Furthermore, *i*NKT cell number was greatly reduced in mice that lacked a small lysosomal protein, Niemann-Pick type 2, which is involved in loading iGb3 onto CD1d [34]. However, it has recently been documented that the presence of iGb3 could not be detected in either human or murine lymphoid organs [36], casting doubt on the requirement of iGb3 for differentiation of *i*NKT cells. In addition, another research group has shown that *i*NKT cells normally develop in the thymus of iGb3 synthase-deficient mice [37]. Moreover, a defect in *i*NKT cells has been reported in multiple mouse models of lysosomal glycosphingolipids storage disease, irrespective of the specific genetic defect of lipid species stored; suggesting that storage of glycosphingolipids may disrupt the presentation of endogenous ligands by CD1d and non-specifically affect *i*NKT cell selection [38]. Therefore, it still remains unknown as to whether or not iGb3 is truly the exclusive ligand for *i*NKT cells.

2.2. α-GalCer

α-GalCer was originally isolated from glycolipid extracts isolated from marine sponge *Agelas mauritianus* during an attempt to seek substances with anti-tumor activity [39]. Studies carried out to explore the biological activity of α-GalCer against immune cells demonstrated that α-GalCer was a potent agonist for *i*NKT cells [40]. KRN7000 (Fig. **1C**), an analogue of α-GalCer modified for optimal productive condition, has been widely utilized for experimental and clinical studies. Although α-GalCer is a potent pharmacological activator of *i*NKT cells, it does not appear to be an endogenous ligand for *i*NKT cells. In fact, sugar moieties in mammalian glycolipids are essentially linked in β-anomeric position [7,40], and the presence of lipids with α-anomeric sugar such as α-GalCer has not been confirmed in mammals. Therefore, it is very likely that activation of *i*NKT cells by α-GalCer is qualitatively different from that by natural endogenous antigens with β-anomeric sugar.

Following activation with α-GalCer, *i*NKT cells release large amounts of IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α and, on a per cell basis, the amount of these cytokines secreted by activated *i*NKT cells is much greater than by conventional T cells. This rapid and abundant cytokine production by *i*NKT cells may be accounted for by the presence of pre-existing transcripts for these cytokines in naïve *i*NKT cells [41-43]. In support of this, histone hyperacetylation of IL-4 and IFN-γ promoters could take place dynamically possibly following recognition of unknown endogenous ligands, resulting in the pre-activated phenotypes of *i*NKT cells.

Cytokines released from activated *i*NKT cells can act as intercellular mediators in the entire immune network. For example, when α-GalCer is injected into mice intraperitoneally, serum levels of IFN-γ rise immediately and then persist for several hours [44,45]. Studies have revealed that not only *i*NKT cells, but also NK cells, are major producers of IFN-γ in mice injected with α-GalCer [45,46] and that the NK cell production of IFN-γ is preceded by cellular cross-talk between *i*NKT cells, dendritic cells (DCs) and NK cells. It is now known that the IFN-γ produced by *i*NKT cells plays a role in this cross-talk, although we can not ignore the roles of other molecules such as costimulatory molecules expressed by DCs, as well as IL-12 produced by DCs [9,47,48].

2.3. Synthetic α-GalCer Analogues

Although α-GalCer is a potent agonist for *i*NKT cells; stimulation of *i*NKT cells with α-GalCer induces production of both Th1 and Th2 cytokines. Given that Th1 cells principally counteract with Th2 cells *via* production of cytokines, the non-selective induction of counteracting cytokines could lower the value of α-GalCer as an immunomodulatory agent. In fact, our attempts to treat an animal model of MS, experimental autoimmune encephalomyelitis (EAE), with α-GalCer were successful only when we used mice lacking IFN-γ in which *i*NKT cells production of cytokines is biased for Th2 [44]. A selective activator of Th2 cytokines from *i*NKT cells is more preferable as a therapeutic agent for treatment of autoimmune diseases. Therefore, we screened synthetic α-GalCer analogues for their ability to activate and polarize *i*NKT cell cytokine production, and identified OCH as a first synthetic compound that could be regarded as a Th2 selective inducer (Fig. **1C**) [49]. OCH has a shorter sphingosine chain compared with α-GalCer, and provokes a relatively weak proliferative response of *i*NKT cells. Critically, OCH stimulation of *i*NKT cells *in vitro* or *in vivo* induced much lower levels of IFN-γ secretion, as compared to α-GalCer stimulation; whereas similar levels of IL-4 were produced after OCH or α-GalCer stimulation of *i*NKT cells. These data suggest that, owing to its ability to polarize *i*NKT responses towards Th2, OCH may have a greater efficacy than α-GalCer in suppressing inflammatory pathology mediated by Th1 cells. Indeed, although injecting α-GalCer did not ameliorate EAE induced in wild-type B6 mice, we demonstrated that in this disease model OCH had a significant preventive and therapeutic effect [49].

Crystal structure analysis showed that two lipid tails of α-GalCer are bound to highly hydrophobic groove of CD1d [50]. Subsequent analysis showed that the amount of IFN-γ triggered by an α-GalCer analogue was positively correlated with the length of its sphingosine chain. In contrast, there was no clear correlation between IL-4 production by *i*NKT cells and the length of sphingosine chain [51]; indicating that the shorter lipid tail may offer a structural basis for the Th2 inducing glycolipid OCH. Based on this premise, we speculated that a selective deviation towards Th2 cytokine production following OCH stimulation may correlate with an unstable association between this glycolipid and CD1d due to its shorter sphingosine chain. Consistently, α-GalCer analogues bearing a shorter sphingosine chain tended to have a shorter half-life in the binding to CD1d, indicating that TCR ligation by OCH is likely to be less persistent than that by α-GalCer. Intriguingly, TCR stimulation of *i*NKT cells with anti-CD3 antibody for a shorter time period (a few hours) induced a detectable amount of IL-4, but not of IFN-γ *in vitro*. In fact, IFN-γ production by *i*NKT cells required TCR

stimulation for a longer period of time. Taken together, we postulated that the differential cytokine profiles triggered by α-GalCer or OCH may result from a differential duration of TCR stimulation, due to the differences in length of their lipid tail that correlate with their binding stability to the CD1d molecule.

A previous study using a protein synthesis inhibitor indicated that cytokines produced by *i*NKT cells could be divided into two groups based on their dependency on *de novo* protein synthesis. IFN-γ belongs to the group of cytokines that would require *de novo* proteins synthesis, whereas IL-4 represents the group of cytokines that do not require additional synthesis of proteins. Our study has shown that protooncogene c-Rel, a member of the nuclear factor κB (NF-κB) family of transcription factors, is an important molecule in α-GalCer-induced transcription of the IFN-γ gene [51]. We have proposed a model in which the longer sphingosine chain of α-GalCer confers the ability to continuously stimulate *i*NKT cells, inducing long lasting calcium influx, which results in a sustained nuclear residence of nuclear factor of activated T-cells (NF-AT), as well as c-Rel protein synthesis. The sequence of events leads to the activation of IFN-γ promoter, enabling *i*NKT cells to produce IFN-γ. In contrast, the rather sporadic stimulation of *i*NKT cells by OCH induces a short-lived nuclear residence of NF-AT, followed by an insufficient c-Rel expression, which prohibits an efficient production of IFN-γ from *i*NKT cells. McCarthy *et al.* confirmed that shortening the phytosphingosine chain increased the rate of lipid dissociation from CD1d molecule and induced less sustained TCR signals [52]. In addition, they also demonstrated there was a decreased affinity of TCR to CD1d-bound OCH. Both the lower stability of the association of OCH and CD1d and the lower affinity of TCR and OCH/CD1d complex may contribute to the less sustained stimulation of TCR.

Activation of *i*NKT cells with α-GalCer promotes vigorous IFN-γ production by bystander cells such as NK cells. It is now thought that differential effects of α-GalCer and OCH injected *in vivo* could be partly explained by a reduced ability of OCH to stimulate production of IFN-γ from NK cells, partly due to lack of IFN-γ provided by *i*NKT cells. It is likely that the differential outcome following α-GalCer and OCH stimulation is also generated by differentially altered DCs function. In fact, we found that *in vivo* injection of OCH induces only one tenth of IL-12 in the serum of mice, as compared with α-GalCer injection [45]. Simultaneous injection of stimulating anti-CD40 antibody and IFN-γ with OCH restored IL-12 induction and administration of IL-12 together with OCH led to IFN-γ production by NK cells [45]. Therefore, the lower IL-12 production is to be linked with a lower expression of CD40L on *i*NKT cells, following OCH stimulation, although the lower levels of IFN-γ produced by *i*NKT cells and NK cells may also play a role [53–55]. In summary, OCH induces a lower amount of IFN-γ from *i*NKT cells due to the less sustained stimulation of TCR. In the context of the elaborate cellular network *in vivo*, OCH stimulation of *i*NKT cells is less efficient at cross-activating DCs and NK cells, due to less CD40L expression on the surface of *i*NKT cells and a reduced secretion of IL-12 by DCs, leading to a lower production of IFN-γ by NK cells.

An acquisition of Th2-biasing ability seems to be a general consequence of truncations in the acyl or sphingosine chains of α-GalCer [56]. PBS-25 is a synthetic compound with short of acyl chains (C8) compared with α-GalCer bearing a C26 fatty acid chain (Fig. **1C**) [57]. Thus, *i*NKT cells stimulated with this ligand would show a Th2 profile compared with those stimulated with α-GalCer. However, the mechanism of Th2-biased cytokine production mediated by PBS-25 seems different from that mediated by OCH, since, like α-GalCer, PBS-25 binds stably to CD1d [58]. Apart from the lipid chain truncation of α-GalCer, α-GalCer analogue, C20:2, which is distinguished by unsaturation of the fatty acid portion, has been reported to be a Th2 skewing ligand [59]. C20:2 contains a C20 fatty acid with cis-unsaturations at positions 11 and 14 (Fig. **1C**) [59,60]. Although C20:2 elicits a Th-2-biased response similar to those induced by OCH, the mechanisms of preferentially inducing Th2 cytokines appears to be different from OCH and remains to be clarified. Conversely, synthetic C-glycoside analogue of α-GalCer (α-C-GalCer) (Fig. **1C**) elicits Th1 type responses of *i*NKT cells even at very low concentrations and exhibits a more potent anti-malaria and anti-tumor effect compared to α-GalCer [61]. To overcome the poor solubility of α-GalCer *in vivo*, a pegylated derivative of α-GalCer (α-GalCerMPEG) has been designed and is found to be a more efficacious compound than α-GalCer [62], although the mechanisms underlying the Th1-biased response remain unclear.

3. THE EFFECT OF SYNTHETIC GLYCOLIPID ANTIGENS ON HUMAN *i*NKT CELLS

Early studies have documented that there is a reduced number of *i*NKT cells in the peripheral blood of patients suffering from systemic sclerosis [63], MS [5,64,65], and other autoimmune disease conditions [66-68]. However, inconsistent results (decreased [69,70], normal [71] or increased [72] numbers of *i*NKT cells) obtained in type 1 diabetes patients by three independent groups has led to considerable argument on the role of *i*NKT cells in autoimmunity. Moreover, *i*NKT cells derived from patients with atherosclerosis [73] appear to participate in promotion of the diseases.

The potent therapeutic efficacy of synthetic α-GalCer analogues has been reported in various autoimmune disease models: EAE [49,74-76], type 1 diabetes mellitus [77-81], arthritis [82-84], lupus [85-89] and experimental inflammatory bowel disease [90,91]. Since mouse and human *i*NKT cells share similar characteristics in function and antigen recognition, these promising results in disease models raised the possibility of the development of *i*NKT cell-mediated immunotherapy for human autoimmune diseases. However, treatment with α-GalCer sometimes exacerbated EAE [74,75] and lupus models [88,89] probably due to Th1 responses elicited by *i*NKT cell stimulation. To avoid such a deleterious effect in human, it is obvious that choice of glycolipids for clinical application requires systemic evaluation using human *i*NKT cells.

The frequency of *i*NKT cells in the peripheral blood is approximately 0.1 % in healthy human individuals (Fig. **2A**) [5,7]. Such a low cell number might raise some concern as to

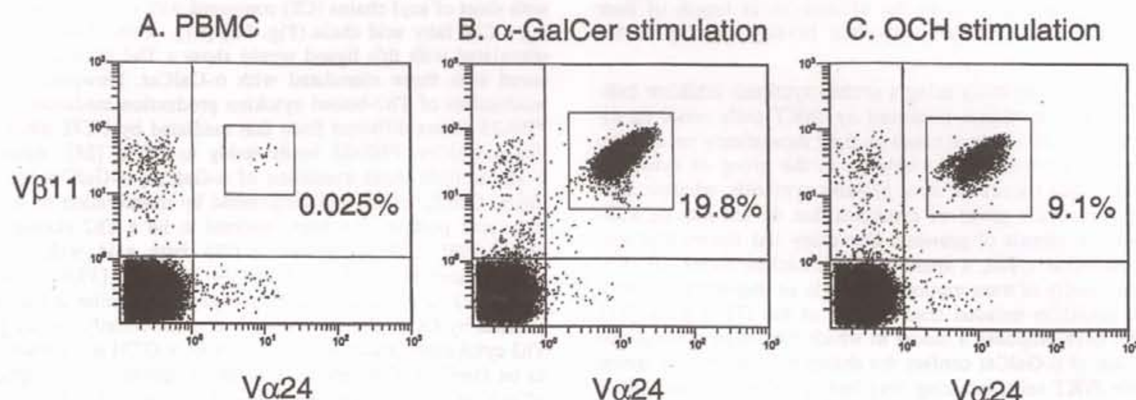

Fig. (2). Identification of Vα24$^+$Vβ11$^+$ NKT cells in PBMCs and α-GalCer/OCH-stimulated cultures.
PBMCs obtained from healthy subjects were stained with anti-Vα24 and -Vβ11 mAbs. This combination of antibodies has been widely used for identifying human *i*NKT cells . (**A**) The percentage value represents the frequency of the Vα24$^+$Vβ11$^+$ NKT cells among total lymphocytes. (**B** and **C**) α-GC- or OCH-stimulated cultures on day 7 were stained with those mAbs. The data shown here are representative out of five samples.

whether or not *i*NKT cells are relevant targets for human diseases. However, stimulating human peripheral blood mononuclear cells (PBMCs) with α-GalCer leads to a remarkable expansion of *i*NKT cells *in vitro* (Fig. **2B**). Furthermore, we have found that OCH also expands *i*NKT cells *in vitro*, although its potency to induce proliferation of human *i*NKT cells was inferior to that of α-GalCer (Fig. **2C**), suggesting that OCH could be an efficient ligand for human *i*NKT cells even if the binding affinity of OCH to human CD1d is weaker than that of α-GalCer as found in rodents. To further analyze the responsiveness of *i*NKT cells to OCH in detail, we have generated CD4$^+$ and DN *i*NKT cell clones using a single cell sorting technique. We established four CD4$^+$ and four DN *i*NKT cell clones from two healthy individuals by stimulating a single sorted CD4$^+$ NKT cell and DN NKT cell with human rIL-2, human rIL-7, and PHA-P in the presence of irradiated autologous PBMCs. All clone cells positively stained with 6B11, a specific mAb to Vα24-JαQ junction. We also confirmed that the clone cells express the invariant Vα24-JαQ rearrangement by performing DNA direct sequencing. All of the *i*NKT cell clones were found to proliferate upon stimulation with either α-GalCer or OCH in the presence of CD1d-transfected cells as APCs. As the APCs do not express conventional MHC class I or class II molecules, the clone cells should recognize glycolipid antigens in a CD1d-dependent manner. We have also generated *i*NKT cell clones using α-GalCer as a primary stimulant. These *i*NKT cell clones have maintained their reactivity to α-GalCer but were unable to generate any responses to OCH. Although not as yet proven experimentally, we speculate that an initial stimulation of *i*NKT cells with a strong agonist such as α-GalCer might heighten the threshold for signals needed to activate *i*NKT cells, and therefore, a weaker ligand like OCH may not be able to induce a detectable response upon restimulation.

When we evaluated cytokine production profiles of single cell-sorted clones, we saw a tendency for CD4$^+$ *i*NKT cell clones to produce greater amounts of cytokines than DN *i*NKT cell clones (Fig. **3A**). Following α-GalCer stimulation, CD4$^+$ *i*NKT cell clones produced large amounts of IFN-γ and TNF-α. They also produced modest amounts of IL-2, IL-4, IL-5 and IL-10. DN *i*NKT clones produced only a trace amount of these cytokines. Compared with α-GalCer, OCH stimulation induced production of lower amounts of Th1 cytokines (IFN-γ, TNF-α, and IL-2) by CD4$^+$ *i*NKT cell clones. However, the levels of Th2 cytokines (IL-4, IL-5 and IL-10) were not altered from those induced by α-GalCer. On the other hand, DN *i*NKT cells produced mainly Th1 cytokines (IFN-γ and TNF-α) when stimulated with α-GalCer. The response of DN *i*NKT cells to OCH was much lower as compared with that of CD4$^+$ *i*NKT cells. To evaluate the ability of each ligand to polarize *i*NKT cells towards Th1 or Th2, we have calculated an "OCH/α-GalCer ratio" for each cytokine by dividing quantities of cytokine produced after OCH stimulation by those following α-GalCer stimulation. Thus a higher OCH/α-GalCer ratio for a given cytokine implies that OCH is more efficacious for inducing this cytokine, as compared with α-GalCer. The results showed that the ratios for Th2 cytokines were higher than those for Th1 cytokines (Fig. **3B**), supporting that OCH stimulation also polarize human CD4$^+$ *i*NKT cells towards Th2 cytokine production. Interestingly, that Goff *et al.* also demonstrated the

A.

		IFN-γ	TNF-α	IL-2	IL-4	IL-5	IL-10
CD4$^+$-1	OCH	1600*	1972	N. D.	644	1621	259
	α-GC	6357	5949	48	887	695	290
	No Ag	78	66	N. D.	372	120	N. D.
CD4$^+$-2	OCH	5562	9155	N. D.	424	70	1122
	α-GC	19772	23121	1567	1794	62	476
	No Ag	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
CD4$^+$-3	OCH	3025	4578	N. D.	1080	158	399
	α-GC	4163	11046	2972	1764	133	125
	No Ag	104	219	N. D.	64	N. D.	N. D.
CD4$^+$-4	OCH	2570	10262	N. D.	540	3940	657
	α-GC	9708	37318	305	1448	3589	908
	No Ag	N. D.	20	N. D.	N. D.	N. D.	N. D.
DN-5	OCH	173	293	N. D.	26	1202	24
	α-GC	2167	5852	N. D.	325	9033	536
	No Ag	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
DN-6	OCH	159	22	N. D.	N. D.	23	N. D.
	α-GC	1690	85	N. D.	N. D.	259	N. D.
	No Ag	N. D.	20	N. D.	N. D.	N. D.	N. D.
DN-7	OCH	150	102	N. D.	N. D.	38	N. D.
	α-GC	728	120	N. D.	N. D.	58	N. D.
	No Ag	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
DN-8	OCH	264	38	N. D.	N. D.	45	N. D.
	α-GC	1490	54	N. D.	N. D.	179	N. D.
	No Ag	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.

*All values are expressed in pg/ml. The detection limit is 20pg/ml in this assay.
Ag: antigen, N. D.: Not detected

B.

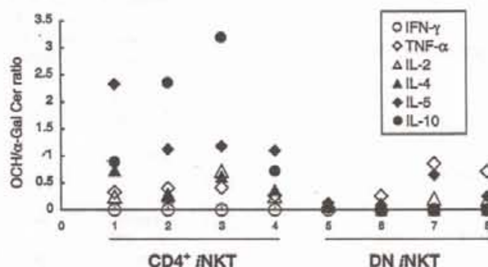

Fig. (3). Cytokine profile of CD4$^+$ and DN *i*NKT cell clones.

(A) Four CD4$^+$ *i*NKT clones and three DN *i*NKT clones were established from two donors respectively. In short, CD4$^+$ and DN Vα24$^+$ Vβ11$^+$ cell were sorted by flowcytometry into single wells of a 96-well culture plate. Each well contained a mixture of irradiated (100Gy) autologous PBMCs at 1 x 10^5 cells/well supplemented with human rIL-2 (20 units/ml), human rIL-7 (10 ng/ml) and PHA-P (1μg/ml) in complete medium. To generate *i*NKT cell clones, the culture cells were re-stimulated three times with PHA, rIL-2 and rIL-7 in the presence of irradiated APC every 18~20 days, thereafter the purity of *i*NKT cells in the culture was more than 99%. These *i*NKT cells were confirmed to bear invariant TCR consisting of Vα24/JαQ by direct DNA sequence and Vβ11 by flowcytometry. CD4$^+$ and DN *i*NKT cell clones were stimulated with α-GalCer or OCH in the presence of immature DC. Th1 cytokines (IFN-γ, TNF-α and IL-2) and Th2 cytokines (IL-4, IL-5 and IL-10) in the supernatants were examined using cytometric bead array. Four CD4$^+$ clones from two donors and four DN clones from two donors were analyzed. To discern any alteration in cytokine secretion profile following OCH stimulation, the OCH/α-GalCer ratio for each cytokine was defined according to the following equation: the value (pg/ml) in α-GalCer-stimulation/ the value (pg/ml) in α-GalCer-stimulation. (**B**) Number 1~8 represents CD4$^+$-1~DN-8 *i*NKT cell clone. The OCH/α-GalCer ratio of Th2 cytokines (IL-4, IL-5 and IL-10) are shown in filled black dots and similar ratios for Th1 cytokines (IFN-γ, TNF-α and IL-2) are depicted as open dots. CD4$^+$ *i*NKT cell clones (No. 1-4) tended towards Th2, but DN *i*NKT cell clones (No.5-8) showed no alteration of cytokine profile.

Th2-biased cytokine production from human and murine *i*NKT cells when stimulated with α-GalCer analogue with short sphingosine chain [56].

4. CONCLUDING REMARKS

Targeting a single molecule that plays a crucial role in mediating inflammatory processes is a popular strategy to develop a new drug for autoimmune diseases. Although such a highly selective drug could powerfully suppress the development of disease, it is important to note that it is potentially dangerous to suppress the self-defensive immune system, particularly when considering responses against concurrent infections and the development of malignant tumors. This concern is particularly serious in the case of autoimmune diseases, as life-long treatment with conventional drugs

could prolong life expectancy to the normal level. In contrast, targeting immune regulatory cells that are defective in autoimmune diseases is conceptually less harmful. Among known regulatory cells, *i*NKT cells are a promising target since they recognize glycolipid antigen bound to monomorphic molecule CD1d. The advantage of glycolipid over peptide is obvious in that the glycolipid drug could elicit uniform responses in highly heterogeneous populations. On the other hand, peptide therapy needs to be individually designed for each patient considering the human MHC polymorphism. Since α-GalCer was identified as an *i*NKT cell ligand in 1997, the search for alternative ligands has greatly enriched our understanding of the biology of *i*NKT cells, and has led to the identification of novel therapeutic ligands such as OCH.

ABBREVIATIONS

APC	=	Antigen presenting cell
BbGL	=	*Borrelia burgdorferi* glycolipid
CHX	=	Cyclohexamide
DC	=	Dendritic cell
DN	=	Double negative
EAE	=	Experimental autoimmune encephalomyelitis
GM-CSF	=	Granulocyte/macrophage colony stimulating factor
GSL	=	Glycosphingolipid
IFN	=	Interferon
iGb3	=	Isoglobotrihexosylceramide
IL	=	Interleukin
*i*NKT	=	Invariant Natural Killer T
LPS	=	Lipopolysaccharide
mAb	=	Monoclonal antibody
MHC	=	Major histocompatibility complex
MS	=	Multiple sclerosis
NF-AT	=	Nuclear factor of activated T-cells
NF-κB	=	Nuclear factor kappa B
NK	=	Natural killer
PBMC	=	Peripheral blood mononuclear cell
rIL	=	Recombinant interleukin
T-bet	=	T-box expressed in T cells
TCR	=	T-cell receptor
Th	=	T helper
TNF	=	Tumor necrosis factor

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