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Ⅰ．　総括研究報告

厚生労働科学研究費補助金　（こころの健康科学研究事業）
総括研究報告書

多発性硬化症の発症機構解明と治療法の開発

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研究要旨　本研究では多発性硬化症(MS)患者の予後を向上させるために、その病態解明と治療法開発に関して免疫学的基盤研究を行っている。主任研究者らは、ナチュラル・キラー（NK）細胞や NKT 細胞が MS の動物モデル実験的自己免疫性脳脊髄炎（EAE）を制御する調節細胞であることを明らかにしているが、この発見に基づき MS 患者で NK 細胞が調節細胞として働く可能性を検証した。本年度は、患者末梢血 NK 細胞除去後に抗原刺激を加える新しいアッセイを開発し、MS の寛解には NK 細胞によってかろうじて維持されている「くすぶり型寛解」と「完全寛解」のあることを示した。前者は末梢血 NK 細胞中の CD95 陽性細胞頻度の高いグループを指し、後者は同細胞頻度の低いグループ（健常者と同レベル）に相当する。MS の寛解に二つのグループが存在することは、治療方針や予後の推定において今後活用されるべき情報であると考える。EAE や自己免疫性関節炎に対して治療活性を持つ物質 OCH については、OCH と alpha-GalCer によって NKT 細胞に伝達されるシグナルの特徴が明らかになってきた。

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A. 研究目的

本研究では MS の予後向上をめざして、病態機構解明と治療法開発に関して、主に免疫学的な方法論により基盤研究を行う。MS は代表的な難治性神経疾患で、中枢神経系に炎症と脱髄病変が多発するのが特徴であるが、その本態は中枢神経抗原を標的とする自己免疫であると考えられている。ミエリン塩基性蛋白（MBP）などの自己抗原ペプチドを認識するT細胞（自己免疫性T細胞）が、炎症の引き金を引き、多くの症例で、再発と寛解を繰り返す。

MS の病態は、自己免疫性T細胞とそれに拮抗する調節細胞のせめぎ合いで理解される。自己免疫性T細胞の研究については相当の進歩が見られているが、調節細胞の研究は、まだ比較的歴史が短い。主任研究者らは、ナチュラル・キラー（NK）細胞や NKT 細胞が MS の動物モデル実験的自己免疫性脳脊髄炎（EAE）を制御する調節細胞であることを 1997 年に明らかにしている。本年度は、MS 患者で NK 細胞が調節細胞として働く可能性を新しいアッセイを用いて検証した。

B. 研究方法

1）自己抗原特異的メモリーT細胞の頻度算定：

MS 患者、対照疾患および健常者の PBMC を直接、または CD56 陽性の NK 細胞を磁気ビーズで除いてから、精製ヒトミエリン塩基性蛋白（MBP）または卵白アルブミン(OVA)で刺激した。刺激を加えてから 8 時間後に、IFN-gamma 分泌細胞の頻度を、サイトカイン分泌アッセイによりフローサイトメーターを使って評価した。

2) CD95 high MS 患者 NK 細胞の抗原特異的 T 細胞活性化に及ぼす影響の評価：CD95 high MS 患者末梢血から MBP で繰り返し刺激することにより、MBP 特異的 T 細胞クローン（TCC）を樹立した。得られた TCC を、CD95 high 患者または HLA DR の一致した CD95 low 患者の末梢血 PBMC を抗原提示細胞として MBP で刺激し、T 細胞増殖反応、およびサイトカイン産生能を ELISA、CBA で測定した。また、CD95 high PBMC から CD56 NK 細胞を除去した細胞も抗原提示細胞として利用し、その効果を評価した。

C. 研究結果

1) MS 寛解期における CD95 陽性 NK 細胞頻度：

これまでに NK 細胞中の CD95 陽性細胞の頻度が、MS 寛解期では対照に比べて有意に増加していることを報告した（Takahashi K et al. The Journal of Clinical Investigation 107: R23-R29, 2001)。MS 寛解期患者をさらに詳細に検討したところ、CD95 陽性 NK 細胞の頻度が高いグループ（CD95 high）と低いグループ（CD95 low）に分かれることがわかった。健常者で得られる値の Mean＋3SD を境界とした場合、CD95 high は寛解期患者のおよそ 4 分の 3 を占めた。この興味深い所見をさらに掘り下げるために、MBP 特異的 T 細胞の頻度を上述の新しい方法によって算定した。

2) "くすぶり型寛解"の提唱：

8 時間の MBP 刺激に反応して IFN-gamma を産生する T 細胞の頻度を、新しいアッセイにより検定した。このような短時間でサイトカインを産生する細胞は、MBP に既に感作されたメモリー T 細胞と考えられる。

健常者または MS 患者由来の PBMC を直接 MBP で刺激したところ、本アッセイでは有意な T 細胞反応を検出できなった。つぎに、NK 細胞を除去してから MBP 刺激を加えたところ、健常者ではやはり MBP に対する反応が見られなかったが、寛解期の MS 患者の相当数で有意な反応が見られた。興味あることに、MBP に対する反応の得られたのは、すべて CD95 high 患者で、CD95 low や健常者では MBP に反応する T 細胞は検出できなかった。また、OVA 特異的な T 細胞反応は、いずれのサンプルにおいても検出できなかった。これらの結果から、CD95 high では MBP に即座に反応するメモリー T 細胞の頻度が高いが、CD95 陽性 NK 細胞が調節的に機能して、メモリー T 細胞の活性化を抑制していることが推測された。

つぎに、CD95 high 患者末梢血における NK 細胞の機能を評価するために、MBP 特異的 TCC を抗原刺激する実験系を確立した。CD95 high、CD95 low あるいは NK 細胞を除去した CD95 high 由来の PBMC を抗原提示細胞として利用したが、TCC の細胞増殖反応については、いずれの細胞を抗原提示細胞として使った場合にも、有意な差は見られなかった。一方、サイトカイン産生を指標にした場合には、CD95 high の PBMC を抗原提示細胞として使った場合に IFN-γ産生がもっとも低いことがわかった。

D. 考察

以上の結果は、CD95 high 患者では、NK 細胞が寛解の維持に積極的に関与することを意味する。すなわち、MBP 反応

性メモリーT細胞が活性化しようとすると、CD95+ NK 細胞が、T細胞の IFN-γ産生を抑制し、再発症状の顕在化を阻止するものと考えられる。一方、CD95 low 患者では、MBP 反応性メモリーT細胞の頻度が低く、NK 細胞が機能を発揮する必要がない。主任研究者と共同研究者の高橋和也は、このような考察を経て、CD95 high 患者の病態を“くすぶり型寛解”、CD95 low 患者の病態を"完全寛解"と定義することを提唱した。“くすぶり型寛解”では、長期的な予後を考慮した積極的な治療が必要である（論文投稿中）。

E. 結論

MS の寛解期には、自己反応性メモリーT細胞の頻度が健常者のレベルにまで低下した場合と、その頻度が高い場合がある。後者においては、その活性化を抑止するために、NK 細胞が重要な役割を果たす（くずぶり型寛解）。この場合に主に貢献するのは、CD95+ NK 細胞である。逆に CD95+ NK 細胞の頻度を算定することによって、MS 寛解期の状態が客観的に評価できることを意味する。今後、治療方針の決定や長期予後推測の指標における役割が期待される重要な情報と考えている。

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G. 知的所有権の取得状況

1. 特許取得

（申請中案件）

新規な糖脂質及びこれを有効成分とする自己免疫疾患治療薬；出願番号特願2001-247055；出願人株式会社ジェノックス創薬研究所；発明者　山村　隆、三宅　幸子

PCT国際出願　新規な糖脂質及びこれを有効成分とする自己免疫疾患治療薬；国際出願番号　PCT/JP02/08280；出願人株式会社ジェノックス創薬研究所；発明者　山村　隆、三宅　幸子

糖脂質誘導体及びその製造法並びにそれらの合成中間体及びその製造法; 出願番号特願 2003-037397；出願人　第一サントリーファーマ株式会社、国立精神・神経センター総長　高橋　清久；発明者　山村　隆 他

2. 実用新案登録

なし

3. その他

なし

資　　料

NKT Cell Stimulating Synthetic Glycolipids as Potential Therapeutics for Autoimmune Disease

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(in press)

List of abbreviations

α-GalCer:	α-galactosylceramide
AGL:	altered glycolipid ligand
APL:	altered peptide ligand
B6:	C57BL/6
CNS:	central nervous system
EAE:	experimental autoimmune encephalomyelitis
GPI:	glycosylphosphatidylinositol
IFN:	interferon
MBP:	myelin basic protein
MHC:	major histocompatibility complex
MOG:	myelin oligodendrocyte glycoprotein
MS:	multiple sclerosis
NK:	natural killer
PLP:	proteolipid protein
TCR:	T cell receptor
Th1:	T helper type 1
TNF:	tumor necrosis factor

Abstract

Although T cells were previously believed to recognize only peptide antigen associated with the major histocompatibility complex (MHC), recent studies have shown that there are unique T cells specialized for recognition of lipid or glycolipid antigens bound to the MHC class I-like CD1 molecules (CD1a, b, c or d). Among these lipid-specific T cells, CD1d-restricted T cells, also referred to as natural killer (NK) T cells, are of special interest as a target of drug development, since their role in immunoregulation has been indicated in various physiological or disease conditions including autoimmunity. They are unique in their homogeneous ligand specificity for α-glycosylated sphingolipid and secrete large amounts of regulatory cytokines shortly after T cell receptor (TCR) engagement. The first glycolipid identified as an NKT cell ligand was α-galactosylceramide (α-GalCer) derived from marine sponges. α-GalCer exhibits significant immunomodulatory effects by stimulating NKT cells. However, we found that an altered analogue of α -GalCer with a shorter sphingosine chain (OCH), is more useful than α -GalCer for treatment of autoimmune disease models, because of its ability to selectively induce IL-4, a key cytokine for control of autoimmunity. As such, altered glycolipid ligands (AGL) of α-GalCer appear to be promising reagents for treatment of human autoimmune diseases.

Introduction

Autoimmune diseases such as multiple sclerosis (MS) and type I diabetes mellitus remain a major health problem in the 21st century that provide a fundamental challenge for drug development. The list of currently available drugs for MS includes interferon-β and copolymer 1 (Cop 1) for long-term management of the disease [1,2]. However, these drugs have only limited value at best, as they cannot halt the progression of neurological disability in a majority of the patients with MS.

Understanding the immunological mechanisms underlying MS has been greatly facilitated in the past two decades due to studies on an animal model for MS, experimental autoimmune encephalomyelitis (EAE) [3,4]. In addition, clinical trials of cytokines [5] and synthetic peptides [6] have given us deep insights into the pathogenesis of MS. A large body of evidence now supports the view that MS is an autoimmune disease, in which autoimmune T cells play a central role in mediating the inflammatory process within the central nervous system (CNS) [7,8]. The pathogenic autoimmune T cells involved in EAE/MS are known to produce interferon-γ (IFN-γ), IL-2 and tumor necrosis factor (TNF)-α when properly activated

with antigen. This pattern of cytokine production would define the pathogenic autoimmune T cells as being T helper type 1 (Th1) cells. Of note is that Th1 cells and Th2 cells (the latter secreting IL-4, IL-5, IL-10 and IL-13) cross-regulate each other via secreting cytokines and that the cytokine milieu is critical for inducing Th1 or Th2 subsets from naive T cells. A previous observation that IFN-γ treatment induced exacerbation of MS [5] is now interpreted as a strong evidence for the role of Th1 cells in MS, given that IFN-γ would augment the activity of this T cell subset.

Regarding the target antigen for the Th1 cells mediating MS pathology, myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) have been explored as the prime candidates [9,10,11]. Immunization of susceptible laboratory animals with these antigens induces development of EAE, characterized by ascending limb paralysis with inflammatory demyelinating lesions in the CNS. In a recent clinical trial, a peptide analogue of the immunodominant sequence of MBP has caused clinical exacerbation in a proportion of the patients [6], although this peptide was expected to halt disease progression. This unsuccessful trial proved that T cells responding to the MBP mimic, that are most probably MBP-reactive autoimmune T cells, play an important role in these patients.

To control pathogenic Th1 cells by inducing Th2 bias, infusion of Th2 cytokines could be considered for clinical use. However, clinical trials of recombinant cytokines, except for IFN-β, have mostly failed because of accompanying side effects. Given a physiological role of each cytokine *in vivo*, cytokine therapy should work if an optimal amount of the cytokine is delivered selectively to the inflammatory lesions. To this end, non-pathogenic autoimmune T cells that can accumulate in the inflammatory lesions are being considered as a potential vehicle for cytokine delivery. Indeed, studies have documented that autoimmune T cells transfected with the genes encoding anti-inflammatory cytokines can suppress autoimmune inflammation [12,13]. However, this strategy seems to be impractical unless major technical advances in culturing autoimmune T cells in vitro take place.

Regulatory cells as targets for drug development

Although self-reactive T cells are eliminated in the selection process in the thymus, it is now established that the negative selection for the potentially dangerous T cells (central tolerance) is not perfect; in fact autoimmune T cells represent a normal component of the T cell repertoire [14]. To maintain good health and to avoid development of autoimmune diseases, the autoimmune T cells exported from the

thymus have to be properly controlled in the periphery by a mechanism that would protect against tissue injury mediated by autoimmune attack. Such control is referred to as peripheral tolerance. It is now widely accepted that regulatory cells play essential roles in peripheral tolerance. Natural killer (NK) T cells [15,16] on which we will focus in this review are within the up-dated list of regulatory cells, along with $CD25^+CD4^+$ T cells [17], and NK cells [18]. Because regulatory cells would control harmful autoimmunity in a highly sophisticated manner mainly via producing cytokines, it is an attractive therapeutic strategy to induce or strengthen the regulatory cells and let them produce cytokines in the relevant sites. Peptides have been exploited for inducing regulatory T cells in EAE and MS, which proves the feasibility but has also revealed the potential problems for clinical use [6, 19].

Properties and roles of NKT cells

NKT cells are a minor subset of lymphocytes that were classically defined as cells expressing both T cell receptor (TCR) and NK cell markers (such as NKRP1). Studies have revealed that the majority of the NKT cells are reactive to α-galactosylceramide (α-GalCer) bound to CD1d molecule. α-GalCer is a glycosylceramide containing an α-anomeric sugar with a longer fatty acyl chain (C_{26}) and sphingosine base (C_{18}) (Fig 1). The α-GalCer-reactive NKT cells have been most intensively studied in the past decade [reviewed in 15,16]. Here we focus on the glycolipid-specific NKT cells and the term "NKT cells" will be used for this cell type below.

As reflected by the name, NKT cells have unique properties that are intermediate between those of innate and acquired immunity. Here we point out just two of these: the semi-invariant TCR expression and the rapid production of large amounts of cytokines. The TCR of NKT cells is composed of the invariant α-chain [Vα14-Jα281 in mice; Vα24-JαQ in human] and the β-chain that is heterogeneous but uses a selective gene segment [Vβ8.2 or Vβ7 segment in mice; Vβ11 in humans]. This restricted TCR expression is consistent with their homogeneous specificity for glycosylceramide bound to the non-polymorphic CD1d molecule. Upon TCR engagement, the NKT cells would rapidly produce large amounts of IL-4 and IFN-γ. The immunological role of NKT cells has been evaluated intensively, making use of NKT cell-deficient mice (CD1d knockout or TCR Jα281 knockout) or NKT cell TCR transgenic mice. Nowadays, it is widely recognized that they play a critical role in tumor rejection, regulation of autoimmune diseases, protection against infection, and tolerance induction [15,16]. Of interest, the number of NKT cells are greatly reduced in human

autoimmune diseases, such as type I diabetes and MS [20,21], suggesting that NKT cells can be a target for treatment of autoimmune diseases.

Glycolipid ligands for NKT cells

α-GalCer, the representative ligand for NKT cells, was first isolated from the marine sponge *Agelas Mauritanius* [22]. Synthetic α-GalCer [(2S, 3S, 4R)-1-*O*-(α-D-galactopyranosyl)–*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol] and its derivatives were later used to study glycolipid recognition by NKT cells (Fig 1). In the pioneering work by Taniguchi and his colleagues [23], it was shown first that ceramide itself or β-galactosylceramide (β–GalCer) do not induce proliferation of mouse NKT cells, indicating that α-anomeric conformation of the sugar moiety is essential for the glycolipid to act as an efficient ligand for NKT cells. It is of note that α–linked glycosphingolipids have not been found in mammalian cells and therefore, α-GalCer and its derivatives are not natural ligands for NKT cells. Among α-liked glycosphingolipids examined, α-GlcCer [(2S, 3S, 4R)-1-*O*-(α-D-glucopyranosyl)–*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol] was stimulatory for NKT cell proliferation, but α-ManCer [(2S, 3S, 4R)-2-amino-*N*-hexacosanoyl-1-*O*-(α-D-mannopyranosyl)-1,3,4-octadecanetriol] was not. This indicates that although the 4-hydroxyl configuration of the sugar may not be important, the 2-hydroxyl group is probably critical for contact with the TCR. Taniguchi *et al.* also proved that 3,4-hydroxyl groups of the phytosphingosine are important. NKT cells also recognized diglycosylated ceramides such as Galα1-6Galα1-1'Cer. Of note, the α-anomeric configuration of the inner sugar was critical for NKT cell recognition, but that of the outer sugar was not.

Comparison of α-GalCer with its derivatives having a shorter hydrophobic chain demonstrated that truncation of the fatty acyl chain as well as that of the sphingosine base reduces the stimulatory activity of the sphingolipid [23]. In contrast, Brossay *et al.* reported that an α–GalCer derivative with a very short fatty acyl chain (C_2) was still efficient in stimulating mouse NKT cell hybridomas [24]. The discrepancy in the results can be explained by the differences in the properties of hybridoma cells used by Brossay *et al.* [24] and the freshly isolated NKT cells from NKT transgenic mice used in the prior study [23]. Shortly after α-GalCer was identified as a ligand for mouse NKT cells, it was found that human Vα24 NKT cells would also recognize α-GalCer bound to CD1d [25,26]. This striking conservation of human and mouse NKT cells in recognition of the glycolipid/CD1d complex may indicate the essential role of NKT cells in mammalian species.

Besides α-GalCer and its derivatives, natural glycosylphosphatidylinositols (GPI) [27] and phospholipids [28] have been reported to stimulate NKT cells. It is interesting to postulate that GPI recognition by NKT cells may help trigger antibody production and contribute to eradicating parasite infection. However, there are still controversies regarding the NKT recognition of GPI or phospholipids. We need to explore these possibilities more intensively in the future.

Treatment of EAE with α–GalCer

EAE is the prototype Th1 autoimmune disease model that helps evaluation of new therapeutic compounds designed for autoimmune disease. Given the property of NKT cells to produce IL-4, we speculated that α–GalCer might protect against development of EAE by inducing IL-4 production by NKT cells. To evaluate preventive effects of α-GalCer on EAE, we induced EAE in C57BL/6 (B6) mice by immunizing with MOG 35-55 peptide [29]. Although we tried protocols with varying doses of α-GalCer or different timing of injection, we did not observe any significant effect of the synthetic glycolipid on the clinical course of EAE. Of note, α-GalCer did strongly stimulate NKT cells and induced cell proliferation as well as IL-4 production. However, it also induced IFN-γ production by NKT cells (Fig 2, middle). We postulated that α-GalCer could not prevent EAE because the therapeutic effect of IL-4 was neutralized by the IFN-γ simultaneously produced by NKT cells. We showed several lines of evidence supporting this idea [29]. First, we found that α-GalCer would inhibit EAE induced in IFN-γ knockout mice whose NKT cells are unable to produce IFN-γ but could produce IL-4. Secondly, α-GalCer was found to augment the clinical signs of EAE induced in IL-4 knockout mice, whose NKT cells would produce IFN-γ but not IL-4. Thirdly, we showed that stimulation of NKT cells with α-GalCer in the absence of CD28/B7.2 co-stimulation would lead to selective IL-4 production. Injection of α-GalCer-pulsed spleen cells whose B7.2 expression was blocked by antibody led to the suppression of EAE in wild-type mice. As such, EAE could be prevented when ligand stimulation would lead to selective production of IL-4 by NKT cells *in vitro* (Table).

Altered glycolipid ligands and their immunomodulatory effects

Conventional T cells are known to change their pattern of cytokine production, when they are triggered with a suitably altered ligand. Such a ligand, referred to as altered peptide ligand (APL), generally has an alternative residue at a critical site(s)

responsible for TCR contact. Studies showed that APLs of MBP could change MBP-reactive Th1 T cells into harmless and disease-protective Th2 T cells [6,30]. Given this information, we hypothesized that there might exist an altered form of α-GalCer, or an altered glycolipid ligand (AGL), that would change the cytokine profile of NKT cells from Th0 type (producing both IFN-γ and IL-4) to Th2 type (predominantly producing IL-4). Such a ligand could be an ideal therapeutic for EAE.

One may postulate that a modification at the TCR contact site of α–GalCer is appropriate for obtaining such an analogue. Regarding the lipid antigen recognition by T cells, it is currently believed that the hydrophilic cap of the sugar moiety contacts the TCR of NKT cells and hydrophobic aliphatic chains bind to the CD1 molecule expressed by antigen presenting cells [31]. Previous studies have identified the critical parts of α–GalCer for inducing NKT cell proliferation [23,24]. We thus synthesized AGLs of α-GalCer with modification at the known critical sites or in the length of the CD1d-binding aliphatic chains (Fig 1). We have so far examined three of these AGLs in depth for their abilities to stimulate NKT cells and to modulate clinical course of EAE.

We first noticed that the AGLs NH and 3,4D were unable to induce a proliferative response by NKT cells *in vitro*. In contrast, the third AGL, OCH, possessing a shorter sphingosine chain could induce a significant proliferation of spleen NKT cells, although the response was about five- to tenfold lower than that induced with α-GalCer [32]. We also measured the amounts of IFN-γ and IL-4 in the culture supernatant. We could not detect these cytokines in the supernatant of spleen NKT cells stimulated with NH or 3,4D. Stimulation with α-GalCer and OCH were found to induce both IFN-γ and IL-4. Of interest, OCH induced less IFN–γ but more IL-4 *in vitro* compared with α-GalCer. In parallel, we measured the serum levels of the cytokines after intraperitoneal injection of the glycolipids into wild-type B6 mice (Fig 3A). Injection of α-GalCer induced a rapid elevation of IL-4 with the peak value at 2 h and a delayed and prolonged elevation of IFN-γ in the mice. While NH was non-stimulatory, injection of 3,4D induced a lower production of both IL-4 and IFN-γ than did α–GalCer injection. Most interestingly, OCH injection dissociated the production of IL-4 and IFN-γ : production of IL-4 was unaffected but IFN-γ was much lower (Fig 3A; right panel). Injection of OCH into mice deficient for NKT cells did not induce an elevation of serum cytokines, indicating that NKT cells mediated the robust cytokine responses in the wild-type mice.

Given that OCH would induce predominant IL-4 production by NKT cells, we postulated that this glycolipid might prevent development of EAE by inducing Th2 bias of NKT cells. In support of this postulate, intraperitoneal or oral administration

of OCH on the day of sensitization with MOG 35-55 peptide was found to prevent development of EAE in wild-type mice in both clinical and pathological parameters (Fig 3B; left panel). We interpreted that the effect of OCH on EAE was mediated by IL-4 produced by NKT cells, because OCH was not effective in EAE induced in NKT cell-deficient or IL-4 knockout mice (Fig 3B; right) or when it was co-injected with neutralizing antibody against IL-4 (Fig 3B; middle). Furthermore, IgG1 antibody against MOG 35-55 peptide (the hallmark of Th2 response) was elevated in the mice treated with OCH, demonstrating the Th2 bias of autoimmune T cells by OCH treatment.

Since OCH induced a weaker proliferation of NKT cells than did α-GalCer, it was theoretically possible that α-GalCer, given at lower doses, might induce selective induction of IL-4 by NKT cells. However, we experimentally ruled out this possibility: lower doses of α-GalCer injected into wild-type mice induced elevation of both IL-4 and IFN-γ and did not alter the ratio of serum IFN-γ to IL-4 at their peak [32].

α–GalCer therapy for other autoimmune disease models

While we were studying the preventive effect of OCH on EAE, other laboratories were interested to know if α–GalCer might be preventive against development of autoimmune type 1 diabetes in NOD mice [33,34,35]. The results of these independent studies were in basic agreement that multiple injections of α-GalCer (twice/week) would induce Th2 bias of NKT cells and significantly inhibit diabetes development. We have confirmed that multiple injections of α-GalCer as well as of OCH would suppress diabetes in NOD mice (Miyake *et al.* unpublished). These results demonstrate that spontaneous autoimmune disease such as NOD diabetes can be treated by repeated stimulation of NKT cells with glycolipid ligands. Given the unique property of OCH as inducing Th2 bias of NKT cells, it is of interest to know if OCH is more efficacious than α-GalCer in promoting disease protection. We would propose that the dynamic changes of NKT cells after repeated stimulation need to be further characterized, given that α–GalCer injection would induce a short-term depletion of NKT cells *in vitro* .

The preventive effect of α-GalCer on EAE has recently been reported with protocols different from ours [36,37]. In these studies, α-GalCer was injected prior to sensitization with peptide [36] or a mixture of α-GalCer with encephalitogenic peptide was co-immunized [37]. We have tried to reproduce these results, but to date have been unsuccessful for unknown reasons. In any event, it seems that a single