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-

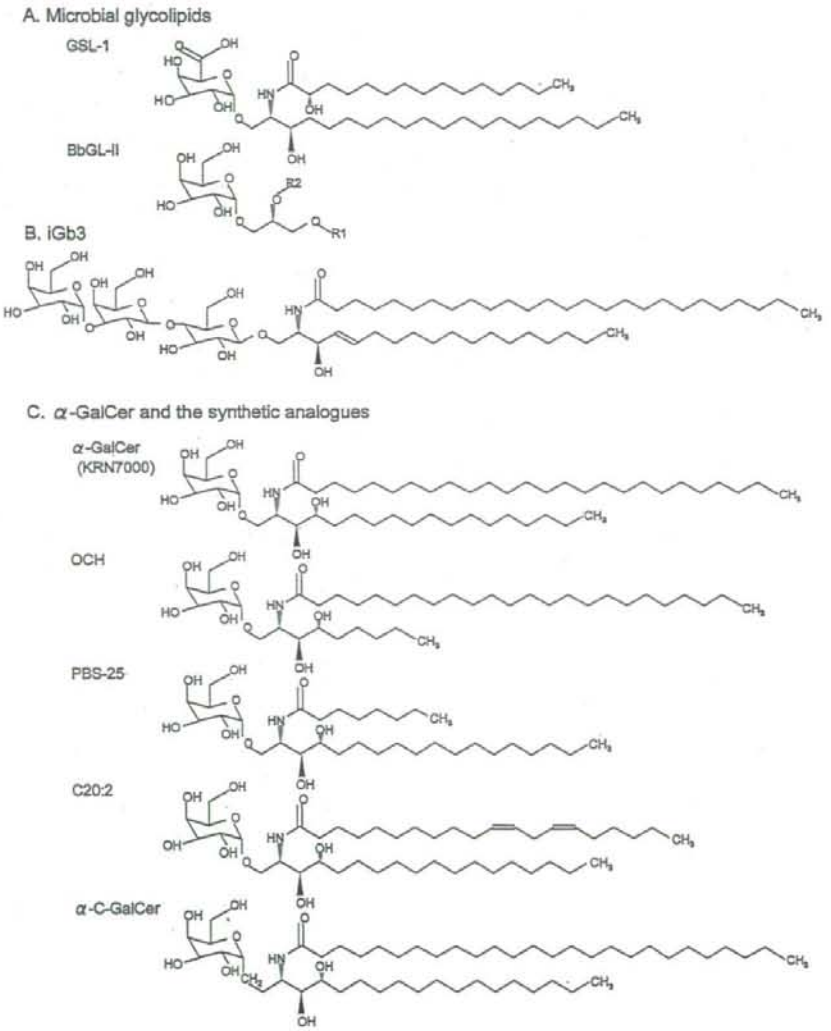

Fig. (1). Chemical structures of glycolipids and the analogues.

(**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

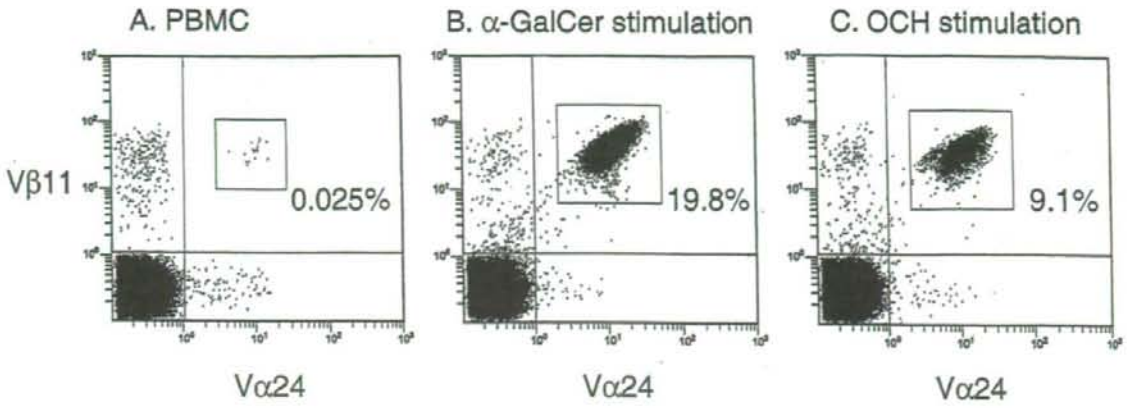

Fig. (2). Identification of Vα24$^+$Vβ11$^+$ NKT cells in PBMCs and α-GalCer/OCH-stimulated cultures.

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	1557	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.

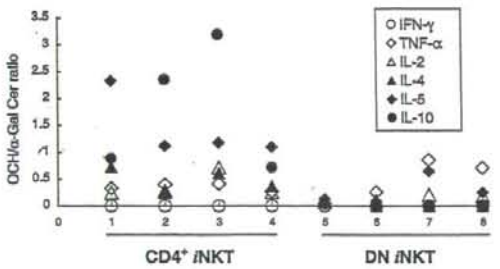

Fig. (3). Cytokine profile of CD4⁺ and DN *i*NKT cell clones.

(**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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Altered production of immunoregulatory cytokines by invariant Vα19 TCR-bearing cells dependent on the duration and intensity of TCR engagement

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Abstract

Cells bearing invariant Vα19-Jα33 TCR α chains are believed to participate in the regulation of inflammatory autoimmune diseases. In this study, the potential to produce immunoregulatory cytokines by these cells was characterized in order to find the mechanism underlying their immunoregulatory functions. Serum levels of IL-4, IL-10, transforming growth factor-β, IFN-γ and IL-17 increased in mice over-expressing an invariant Vα19-Jα33 TCR α transgene (Vα19 Tg) in response to anti-CD3 antibody injection. NK1.1$^+$ Vα19 Tg$^+$, but not NK1.1$^-$ Vα19 Tg$^+$ cells, promptly produced immunoregulatory IL-4, IFN-γ and IL-17 upon invariant TCR engagement with immobilized anti-CD3 antibody in culture. The activation of Vα19 Tg$^+$ cells then triggered the production of pro-inflammatory cytokines by bystander cells. Interestingly, the ratio of T_h2 cytokines such as IL-4, IL-5 and IL-10, but not pro-inflammatory IL-17, to IFN-γ was increased when the intensity of the stimulation to invariant TCR was attenuated. Collectively, these findings suggest that invariant Vα19 TCR$^+$ cells have the potential to participate in the regulation of inflammatory autoimmunity by producing T_h2-biased cytokines in certain circumstances.

Introduction

In adaptive immunity, antigen receptors expressed by T and B lymphocytes exhibit extensive diversity in their responses to different antigens. Besides those lymphocytes expressing diverse antigen receptors, several subsets of lymphocytes are characterized by a limited antigen receptor diversity, for instance, B1 B cells (1), certain γδ T cell sub-populations (2) and NK1.1$^+$ Vα14-Jα18 invariant TCRα$^+$ (Vα14 NKT) cells (3, 4).

The essential requirement of invariant Vα14-Jα18 TCR α chain expression for the development of Vα14 NKT cells is demonstrated in the invariant TCR transgenic (Tg) mice, where Tg$^+$ Vα14 NKT cells are similar to native NKT cells in TCR β composition, antigen recognition and cytokine production (5).

The presence of another invariant TCR α chain [Vα19-Jα33 (conventionally Jα26)] was shown by quantitative PCR analyses in mouse, human and bovine lymphoid cells (6, 7). We demonstrated that this invariant TCR α chain was preferentially expressed in NKT cells but not conventional T cells and that more than half of the hybrid cell lines produced from NKT cells of CD1-deficient livers expressed this invariant TCR α chain (8). Recently, the localization of the invariant Vα19 TCR$^+$ cells in gut lamina propria was demonstrated (9). These cells [designated as mucosal-associated invariant T (MAIT) cells] are suggested to control IgA production in the intestine (9). They are under restriction by one of the evolutionarily conserved MHC class Ib molecules, MHC-related protein 1 (MR1) (10). It is suggested that MAIT cell activation is ligand dependent, but the natural ligand associated with MR1 is not determined (11). It has been also reported that cells bearing invariant Vα7.2-Jα33 TCR α chains (corresponding to mouse Vα19-Jα33) but not Vα24-JαQ TCR α chains (corresponding to mouse Vα14-Jα18) accumulated in the lesions of multiple sclerosis patients (12).

Mice over-expressing an invariant Vα19-Jα33 TCR α Tg with a natural TCR α promoter generated invariant Vα19 Tg$^+$ cells in gut lamina propria and other lymphoid organs including the liver (13–16). Invariant Vα19 TCR Tg$^+$ cells

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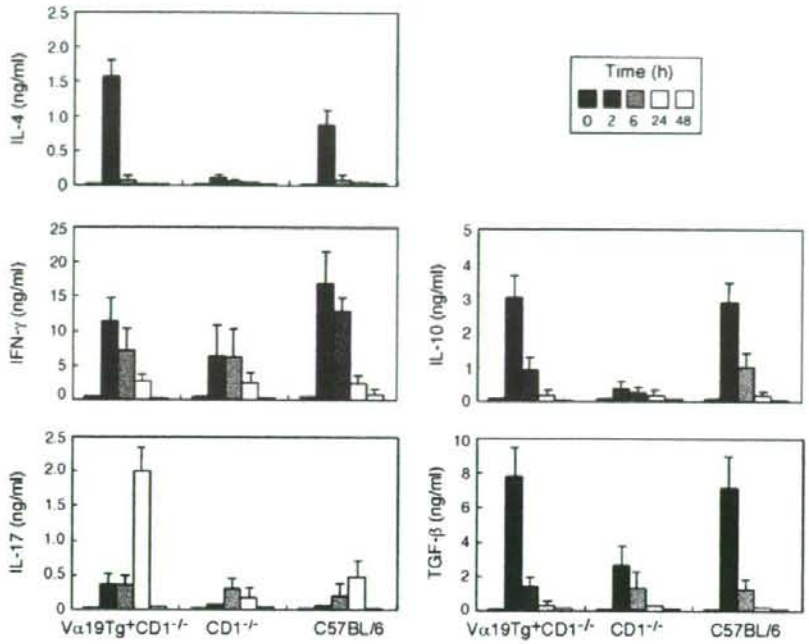

Fig. 1. Cytokine production by Tg and non-Tg mice in response to *in vivo* treatment with anti-CD3 antibody. Vα19 Tg$^+$ CD1$^{-/-}$, CD1$^{-/-}$ and C57BL/6 mice were injected with anti-CD3 antibody (2C11), and serum cytokine levels were determined at various points after the injection by ELISA. The average ± SD of three mice from each strain is shown. Experiments were repeated three times, and essentially the same results were obtained. Note that serum IL-17 levels in 129/Sv and BALB/c mice (less than a few percentage of these genetic backgrounds were included in that of CD1$^{-/-}$ mice) were comparable to the levels in C57BL/6 mice (data not shown).

produce immunoregulatory cytokines in response to TCR engagement (13–16). These cells are thought to regulate inflammatory autoimmune diseases (14). In the current study, cytokine production by Vα19 Tg$^+$ cells was characterized in detail to find how these cells participate in the regulation of immune responses.

Materials and methods

Mice

C57BL/6 mice were purchased from Sankyo Service Co. (Tokyo, Japan). Beta2m-deficient mice, backcrossed with C57BL/6 mice for six generations, were obtained from Jackson Laboratory (Bar Harbor, ME, USA). CD1-deficient mice were provided by M. J. Grusby (Harvard University) (17). They were backcrossed with C57BL/6 mice for six generations, and mice with phenotypes H-2^b, NK1.1$^+$ and CD1$^{-/-}$ were selected. TCR Cα-deficient mice, backcrossed with C57BL/6 mice for 10 generations (18), were provided by H. Ishikawa (Keio University) and M. Nanno (Yakult Co.).

Establishment of Vα19 Tg mice

A Vα19-Jα 33 Tg with the endogenous TCR α promoter and enhancer was injected into C57BL/6 and TCR Cα-deficient fertilized eggs, and Tg mouse lines were established (15). A Vα19Tg$^+$ CD1$^{-/-}$ mouse line was established from one of the three Tg lines with the C57BL/6 background by crossing it with CD1-deficient mice. Vα19 Tg mice were compared with non-Tg mice in the same litter or with non-Tg mice with an appropriate genetic background (C57BL/6, 129/Sv or BALB/c) possibly included in each Tg line.

The experiments using mice were permitted by the Animal Experimental Committee of Mitsubishi Kagaku Institute of Life Sciences and performed along the guidelines of the committee.

In vivo stimulation of Vα19 Tg lymphocytes by TCR engagement

Mice of Vα19Tg$^+$ CD1$^{-/-}$ and CD1$^{-/-}$ from the same litter and C57BL/6 genetic background (8 weeks of age) were intravenously injected with anti-CD3 antibody (2C11; PharMingen, San Diego, CA, USA; 1.5 μg per mouse) in 200 μl PBS. They were bled at various times after the injection, and the cytokine levels in serum were determined by ELISA. Cytokines in the supernatants were determined by ELISA.

In vitro stimulation of Vα19 Tg cells through TCR

Plastic culture plates were pre-coated with anti-CD3 antibody (2C11, PharMingen) at the indicated concentration in PBS, at 4°C for 16 h, and washed three times with DMEM. Mononuclear cells (MNCs) were isolated from the spleens and livers of mice (8–10 weeks of age) by density gradient centrifugation using Lymphosepar II (IBL, Gunma, Japan; d = 1.090) or Percoll (Pharmacia, Uppsala, Sweden) as described previously (19). They were cultured on the plates in DMEM (10% FCS), and cytokines in the culture supernatants

were analyzed by ELISA. In some experiments, MNCs were separated according to the expression of NK1.1 and/or TCR αβ by a cell sorter (EPICS-Altra, Coulter Co., Hialeah, FL, USA) before culture. The purity of NK1.1$^+$, TCR αβ$^+$ and NK1.1$^-$, TCR αβ$^+$ fractions were in the range of 91–95% and 92–96%, respectively.

Intracellular cytokine staining

Spleen MNCs were cultured on a plate pre-coated with anti-CD3 antibody for 1–2 days. They were stimulated with phorbol myristate acetate (50 ng ml^{-1}, Sigma) and ionomycin (750 ng ml^{-1}, Sigma) in the presence of Golgi plug at the recommended concentration (PharMingen) for the next 5 h. Cells were stained with anti-NK1.1 and anti-TCR Cβ antibodies, permeabilized and fixed with Cytofix/Cytoperm Plus kits (PharMingen) according to the manufacturer's protocol. Then they were stained with anti-cytokine antibody.

Cytokine determination by ELISA

Cytokines were determined by ELISA using antibodies from PharMingen.

Results

Prompt T_h2-biased cytokine production and the subsequent pro-inflammatory cytokine production by Vα19 Tg cells upon TCR engagement

An invariant Vα19-Jα33 TCR gene segment, isolated from a hybrid line (7), was cloned into a TCR α vector containing the TCR α endogenous promoter and enhancer, and Tg mice (Vα19 Tg mice) were produced (16). The development of NK1.1$^+$ Tg$^+$ [NK1.1$^+$ Vα19-Jα33 invariant TCR α$^+$ cell (Vα19 NKT cell)] cells predominated in the liver and other lymphoid organs. For instance, Vα19 NKT cells share 29.5, 7.5 and 3.6% of MNCs in the liver, bone marrow and spleen of Vα19 Tg mice with TCR α-deficient genetic background. Cells from these mice were used to characterize cytokine production by Vα19 Tg cells.

Serum levels of cytokines in Vα19 Tg$^+$ and non-Tg mice were compared upon stimulation with anti-TCR antibody to identify the immunoregulatory cytokines in activated Vα19 TCR$^+$ cells (Fig. 1). Vα19 Tg$^+$ CD1$^{-/-}$, CD1-deficient (both lacking Vα14 NKT cells) and normal mice were injected with anti-CD3 antibody and bled at various time points. Production of T_h2-biased cytokines (IL-4, transforming growth factor-β and IL-10) was found in the C57BL/6 mice at 2 h after antibody injection, while this prompt cytokine production was reduced in CD1$^{-/-}$ mice. The reduction was eliminated by the introduction of Vα19 Tgs, as demonstrated by the cytokine levels in Vα19 Tg$^+$ CD1$^{-/-}$ mice. In these mice, the restoration of T_h2 cytokine levels was more significant than that of IFN-γ levels. In addition, the kinetics of the decline in T_h2 cytokine levels was faster than that of IFN-γ levels in Vα19 Tg$^+$ CD1$^{-/-}$ serum. Thus, over-generated Vα19 Tg$^+$ cells in Tg mice may take the place of Vα14 NKT cells in C57BL/6 mice and are responsible for the prompt production of T_h2-biased immunoregulatory cytokines in response to TCR engagement.

The T_h2 cytokine production found in CD1$^{-/-}$ mice may be partially attributable to invariant Vα19 TCR$^+$ cells.

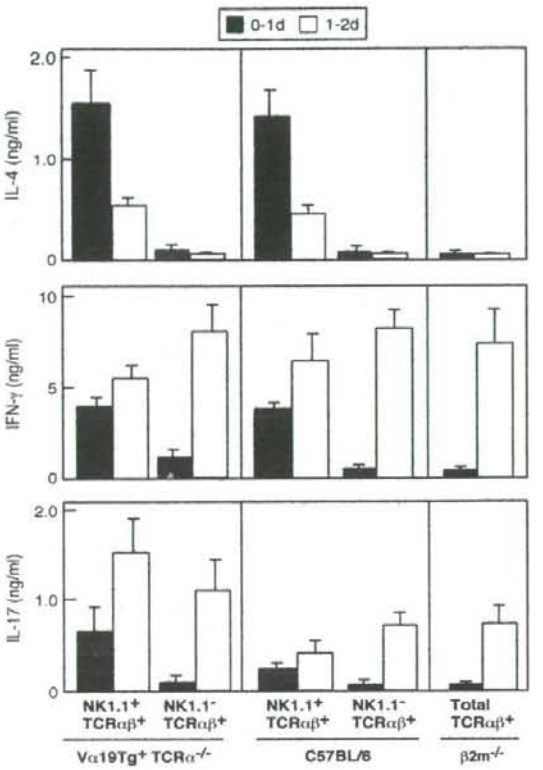

Fig. 2. Cytokine production by Tg and non-Tg cells in culture upon TCR engagement. Liver MNCs were prepared from Vα19 Tg$^+$ TCR α$^{-/-}$, C57BL/6 and β2m$^{-/-}$ mice. NK1.1$^+$ TCRαβ$^+$ and NK1.1$^-$ TCRαβ$^+$ cells were sorted using a cell sorter. Cells in both fractions were cultured in wells (2×10^5 ml^{-1}) pre-coated with anti-CD3 antibody (10 μg ml^{-1}). The culture medium was exchanged daily. The concentration of cytokines in the supernatants was determined by ELISA. One of the two independent experiments, which gave essentially the same cytokine profiles, is shown.

Prompt T_h2-biased cytokine production by Vα19 Tg$^+$ cells was followed by pro-inflammatory-biased cytokine production as shown by the serum levels of IFN-γ and IL-17 at 6 h and the later time points. The rise in IL-17 levels was more significant in Vα19 Tg than in non-Tg mice.

The temporal change in the cytokine profiles of Vα19 Tg$^+$ cells was further demonstrated in the analysis of those cells *in vitro* upon TCR cross-linking (Fig. 2). NK1.1$^+$ and NK1.1$^-$ T cells prepared from Vα19 Tg$^+$ TCR α$^{-/-}$ and normal mouse livers accompanied with total TCR αβ$^+$ cells from β2m$^{-/-}$ livers (lacking both Vα19 and Vα14 NKT cell population) were cultured in wells pre-coated with anti-CD3 antibody (10 μg ml^{-1}) and the cytokine production by them was determined. NK1.1$^+$ T cells, but not NK1.1$^-$ T cells, isolated from both strains of mice promptly produced a significant amount of IL-4, while the cells in each fraction continuously produced IFN-γ until the second day of culture. Similar profiles of cytokine production were observed in the culture of NK1.1$^+$ and

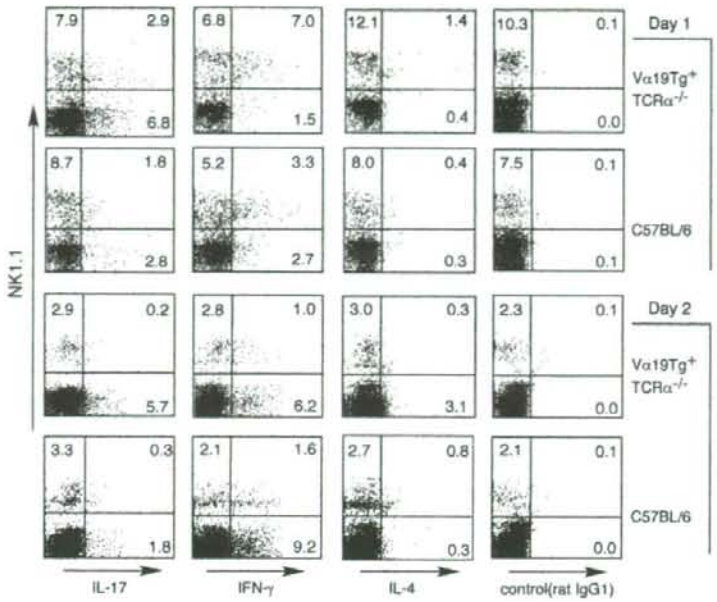

Fig. 3. Prompt production of cytokines by Vα19 NKT cells in response to TCR engagement. Spleen MNC cells from C57BL/6 and Vα19 Tg$^+$ TCR $\alpha^{-/-}$ mice were cultured in wells pre-coated with anti-CD3 antibody for 1–2 days and then stimulated with phorbol myristate acetate and ionomycin for 5 h. Intracellular cytokines were analyzed by flow cytometry. Plots are gated on TCR $\alpha\beta^+$ cells. Data are representative of three independent experiments.

NK1.1$^-$ spleen cells from Vα19 Tg$^+$ and non-Tg mice (data not shown). These observations strongly suggest that NK1.1$^+$ invariant Vα19 TCR$^+$ cells in Tg mice as well as Vα14 NKT cells in wild-type mice (20) are responsible for early-phase T_h2-dominant and subsequent pro-inflammatory cytokine secretion following antigenic stimulation.

Next, Tg and non-Tg cells were analyzed for intracellular cytokine production to identify the producer of each cytokine after TCR engagement. Intracellular cytokine staining patterns in spleen TCR $\alpha\beta^+$ cells of Tg and non-Tg mice are shown in Fig. 3. IL-4, IL-17 and IFN-γ were mainly produced by NK1.1$^+$ cells on day 1 of culture, whereas these cytokines were produced by NK1.1$^-$ cells on day 2 of culture in both Tg and non-Tg cells. These findings support the findings in Fig. 2 that T_h2-dominant cytokine production by the TCR engaged NK1.1$^+$ Vα19 TCR$^+$ (Vα19 NKT) cells in Tg mice or presumably Vα14 NKT cells in non-Tg mice is followed by the pro-inflammatory cytokine secretion by conventional T cells.

Altered profiles of cytokine production by Vα19 Tg$^+$ cells depending on the intensity of TCR engagement

Although the potent immunoregulatory function of Vα19 Tg$^+$ cells toward inflammatory diseases is accounted for by their early-phase T_h2-biased cytokine production, it is not in accord with the subsequent pro-inflammatory-dominant cytokine production. To address this question, we examined cytokine production of Vα19 Tg$^+$ cells during different intensities of TCR engagement. MNCs were isolated from the livers of Vα19 Tg$^+$ TCR $\alpha^{-/-}$, C57BL/6 and $\beta2m^{-/-}$ mice (NK1.1$^+$ TCR$\alpha\beta^+$ cells share ca. 30, 26 and 0.5% and NK1.1$^-$ TCR$\alpha\beta^+$ cells share 19, 20 and 25% of liver MNCs of these mice) (16). These cells were cultured on a plate pre-coated with different concentrations of anti-CD3 antibody, and the cytokine production by these cells was determined. The profiles of cytokine production dependent on the dose of anti-CD3 antibody were observed (Fig. 4A). The Tg$^+$ and non-Tg cells, but not the $\beta2m^{-/-}$ cells, produced a significant amount of IL-4 on day 1 of culture, while the cells of all the strains produced larger amounts of cytokines other than IL-4 on the second day of culture. These observations are well in accord with those found in Fig. 2 and suggest that the major cytokine producers in both Tg$^+$ and non-Tg cells are NKT cells and T cells on day 1 and day 2 of culture, respectively. In addition, the Tg$^+$ and non-Tg cells continuously produced T_h2 cytokines (IL-4, IL-5 and IL-10) more than $\beta2m^{-/-}$ cells until day 2 of culture; the production was saturated before reaching the maximum dose of anti-CD3 antibody. The relative production of each cytokine by Vα19 Tg$^+$ and B6 cells to the production by $\beta2m^{-/-}$ cells was plotted along with the dose of anti-CD3 antibody to emboss the contribution by Vα19 and Vα14 NKT cells (Fig. 4B). In addition to the prompt IL-4 production proportional to the intensity of TCR engagement by Vα19 as well as Vα14 NKT cells, it is also suggested that both NKT cells, especially Vα19 NKT cells, have the potential to continuously produce T_h2-dominant cytokines after TCR engagement within an appropriate range of intensity.

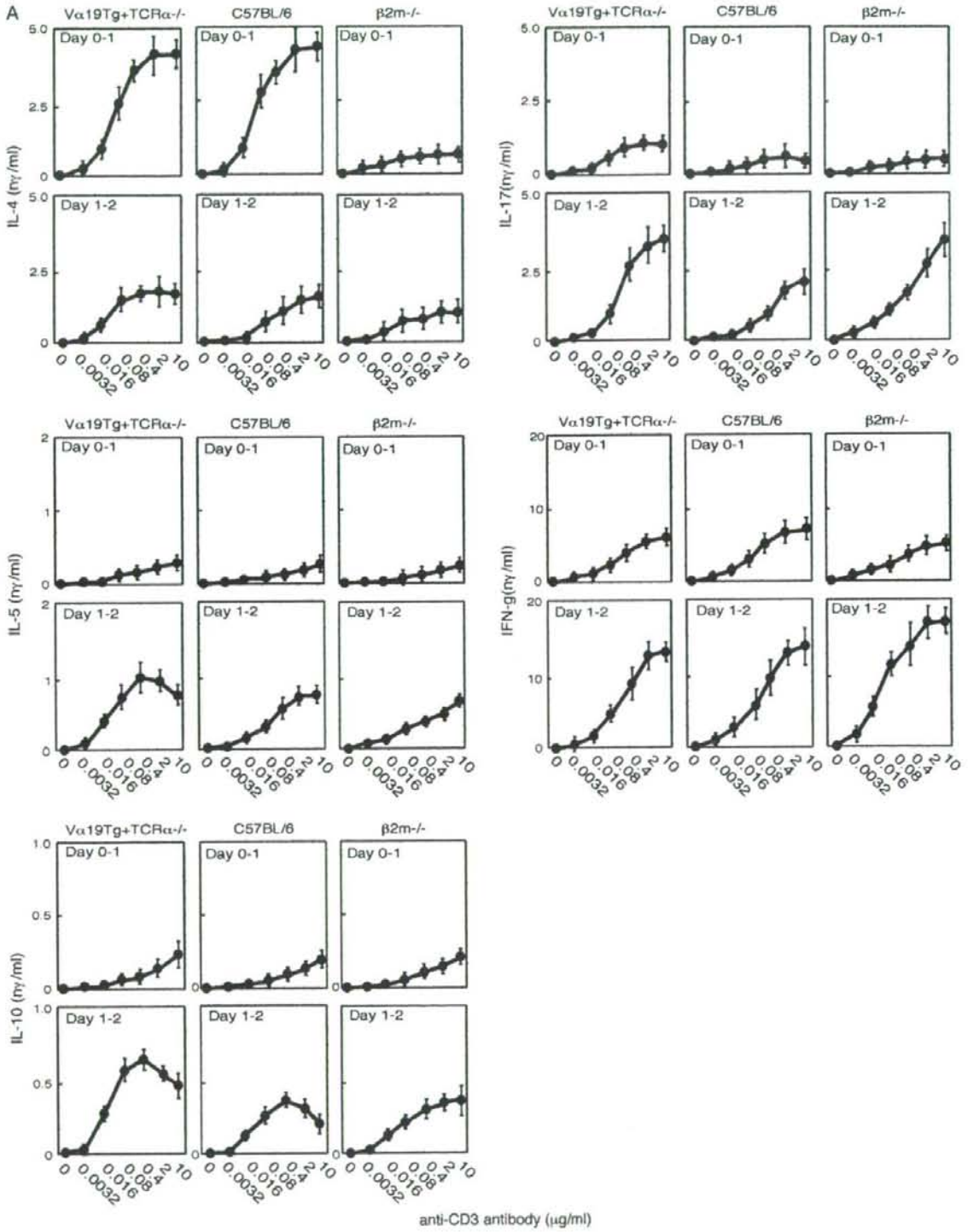

Fig. 4. *Continued.*

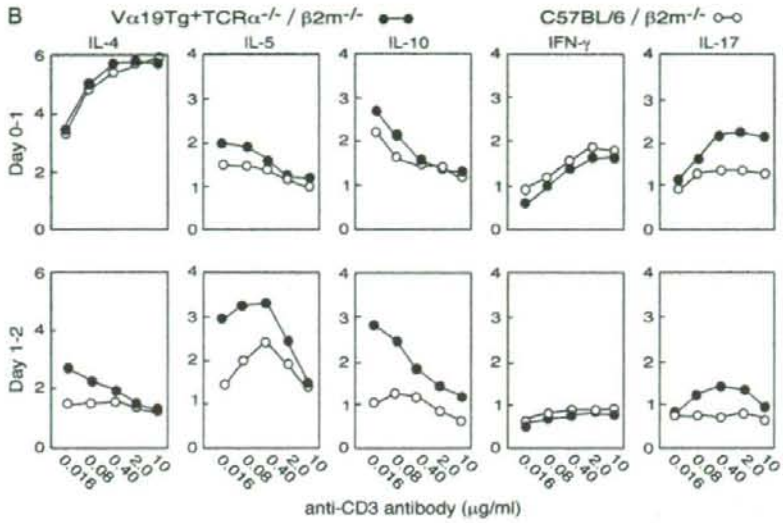

Fig. 4. Cytokine production by invariant Vα19 TCR Tg and non-Tg cells in culture stimulated with various concentrations of immobilized anti-CD3 antibody. (A) Liver MNCs (1×10^6 ml^{-1}) prepared from Vα19 Tg$^+$ TCR α$^{-/-}$, C57BL/6 and β2m$^{-/-}$ mice were cultured on a plate previously coated with various concentrations of anti-CD3 antibody. After 1 day, the culture medium was exchanged with fresh medium. The concentration of cytokines in the supernatants was determined by ELISA. The horizontal axis represents the concentration of anti-CD3 antibody used to coat the culture plates (micrograms per milliliter). The results indicate the average of a triplicate culture. The bars indicate standard deviations. Experiments were repeated four times, and essentially the same profiles were obtained. (B) The relative production of cytokine by Vα19 Tg$^+$ and B6 cells to β2m$^{-/-}$ cells following stimulation with different dose of anti-CD3 antibody is indicated.

Discussion

Cells in Vα19 Tg$^+$ mice promptly secrete T_h2-dominant and then pro-inflammatory cytokines following TCR stimulation *in vivo* (Fig. 1). The altered cytokine profiles of NK1.1$^+$ Vα19 Tg$^+$ cells along with the duration of TCR stimulation become clear when these cells are isolated and stimulated in culture with anti-TCR-CD3 antibody (Fig. 2). Similar kinetics of cytokine production by NK1.1$^+$ Vα19 Tg$^+$ cells was suggested by the intracellular cytokine staining experiments (Fig. 3) and the bulk culture of liver MNCs (Fig. 4). However, it remains possible that the delayed pro-inflammatory-dominant cytokine production found in the Tg liver cells in Fig. 4 is also ascribable to NK1.1$^-$ Tg$^+$ following their own TCR engagement and/or the bystander cells other than NK1.1$^+$ Tg$^+$ cells since pro-inflammatory-dominant cytokines from β2m$^{-/-}$ cells were observed after prolonged TCR stimulation (Fig. 2).

Recently, Sakuishi *et al.* (21) reported that the ratio of IL-5 to IFN-γ production by cells of human Vα24 invariant TCR-bearing lines was increased with a decrease in concentration of the anti-CD3 antibody used for stimulation. In accord with this report, we found T_h2-dominant cytokine production by invariant Vα19 TCR Tg$^+$ cells with the prolonged, attenuated stimulation to the invariant TCR (Fig. 4B).

The intensity of TCR engagement in invariant Vα19 TCR$^+$ cells may be controlled by antigen-presenting cells under the physiological conditions; these cells regulate the density of MR1 expression and select the antigens that they present. In Vα19 Tg mice, the disease progress of experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis, was suppressed (15). On the other hand, accumulating evidence suggests that T_h17 cells mediate certain inflammatory autoimmune diseases (22). In fact, invariant Vα19 TCR Tg$^+$ cells, especially those of the NK1.1$^+$ subset, preferably produced IL-10, but not IL-17, after priming of mice with a partial peptide of myelin oligodendrocyte glycoprotein (15). Presumably, the intensity of TCR engagement in the Vα19 TCR$^+$ cells of these mice may be in the range suitable for the induction of regulatory rather than pro-inflammatory-dominant immune responses. In the present study, enhanced production of IL-17 was observed in cells from Vα19 Tg mice in response to polyclonal stimulation of TCR-bearing cells using anti-CD3 antibody as a TCR stimulator. Further studies were thus required to elucidate how Vα19 TCR$^+$ cells contribute to preventing the progress of inflammatory autoimmune diseases.

We have found that certain α-mannosylated glycolipids have the potential to specifically activate invariant Vα19 TCR$^+$ cells in an MR1-dependent manner (23, 24) and that some of these induce T_h2-biased immune responses (23). Taking into account these findings, it is possible to speculate that certain natural ligands have potential to induce T_h2-dominant immune responses from invariant Vα19 TCR$^+$ cells.

The production of immunoregulatory cytokines by a novel NK1.1$^+$ T cell subset, Vα19 NKT cells, was characterized in the current study. Vα19 and Vα14 NKT cells partially share similar properties despite being subjected to the independent control of different antigen-presenting molecules. In addition, these two subsets are possibly involved differently in some immune responses. Thus, these NKT cell subsets are

possible targets for immunotherapies using activators specific to each subset.

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Conflict of Interest: The authors have no financial conflicts of interest in this study.

Abbreviations

MAIT	mucosal-associated invariant T
MNC	mononuclear cell
MR1	MHC-related protein 1
Tg	transgene or transgenic
Vα14 NKT	NK1.1$^+$ Vα14-Jα18 invariant TCR α$^+$
Vα19 NKT	NK1.1$^+$ Vα19-Jα33 invariant TCR α$^+$

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特集I　NKT細胞による免疫応答の制御

Invariant Vα19 TCR発現細胞のサイトカイン分泌能と免疫系制御*

島村道夫**

Key Words：NKT cell, invariant TCR, cytokine, immune regulation

はじめに

Vα19-Jα33（慣用的にはJα26）間で遺伝子再構成したinvariantなT細胞レセプター（TCR）α鎖を発現した細胞はTCRへの抗原刺激に応答して即時的にIL-4, IFN-γ, IL-17などの免疫系制御に重要なサイトカインを分泌する．しかも，自己免疫病患部への集積が観察されたことからこの細胞は免疫系調節に重要な機能をもつことが示唆され，実際，自己免疫モデル動物において病状の抑制に寄与することがわかってきた．この細胞の抗原刺激に応答した調節因子分泌の特徴を明確にすることは，免疫系調節機序を明らかにしてこの細胞の機能を生かした自己免疫病の治療法を開発するのに重要である．

Invariant Vα19 TCRα鎖発現細胞

Invariant Vα19-Jα33 TCR（ヒトではinvariant Vα7.2-Jα33 TCRα鎖，本稿では均一Vα19 TCRα鎖と省略する）はVα14-Jα18間で遺伝子再構成して形成される均一Vα14 TCRα鎖に続き第二の均一TCRα鎖として当初末梢血細胞についてのPCR法による分析からその存在が示された[1)2)]．均一Vα19 TCRα鎖は肝臓，骨髄，脾臓，リンパ節などの免疫系組織に主として第二のNKT細胞として発現していた（Vα19 NKT細胞）[3)]．その後，均一Vα19 TCRα鎖発現細胞の腸管粘膜固有層への局在が報告され[4)]，それゆえこの細胞はmucosal associated invariant T（MAIT）細胞とも称されるようになった．均一Vα19-Jα33 TCRα鎖遺伝子をTCRα鎖のプロモーター，エンハンサーの制御で発現させたトランスジェニック（Tg）マウスではこのTCR発現に依存してVα19 NKT細胞が過剰発生した[5)~7)]．正常マウスでもたとえば肝臓では既知のCD1拘束性均一Vα14 NKT細胞の1/30～1/20，単核球の1％の存在数が推測され，リンパ球クローンとして主要な存在である．均一Vα19 TCRα鎖発現細胞はMHCクラスIb分子MR1により正の選択を受けて発生する[4)7)]．この細胞はMR1に提示された末端にα-マンノシル基をもつある種の糖脂質を認識して活性化し[6)8)9)]，CD1に提示された末端にα-ガラクトシル基をもつある種の糖脂質を認識して活性化するVα14 NKT細胞とは作用機序が異なる．多発性硬化症の患者にみられる末梢血中のVα14 NKT細胞の減少が均一Vα19 TCR発現細胞ではみられず，代わりにこの細胞の患部への集積が観察された[10)]．しかも，多発性硬化症の動物モデルとなる実験的自己免疫性脳脊髄炎（EAE）が均一Vα19 TCR発現細胞により抑制されることが判明した[11)]．これらの事実から，均一Vα19 TCR発現細胞はVα14 NKT細胞とは異なる独自の機序により自己免疫病を個々の抗原特異性を超越して上流から制御する担い手として重

* Cytokine production and immune regulation by invariant Vα19-Jα33 TCR-bearing cells.

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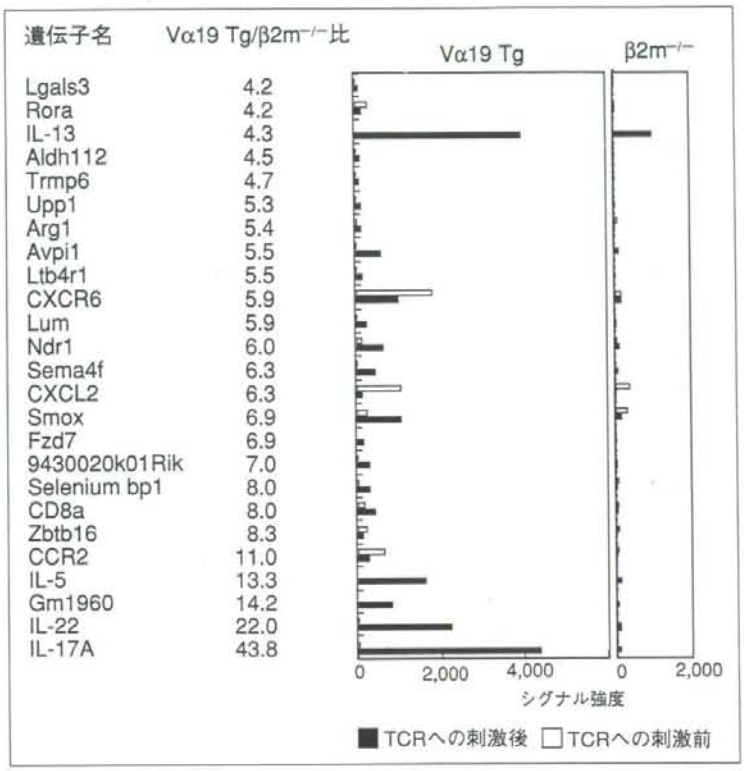

図1　均一Vα19-Jα33 TCR発現細胞のTCRへの刺激に応答した遺伝子発現
Vα19 TCR Tgマウス(TCRα$^{-/-}$バックグランド)肝臓単核球細胞，対照としてβ2m$^{-/-}$マウス細胞を固相化抗CD3抗体で刺激前後の細胞について，DNAマイクロアレイにより遺伝子発現を分析した．補正後のシグナルの蛍光強度を示す．活性化前の細胞を白抜きで，活性化後の細胞を黒のカラムで表し，Vα19 Tg，β2m$^{-/-}$細胞それぞれについて結果を示した．使用した39,000種のプローブで検出した転写物のうちVα19 Tg/β2m$^{-/-}$細胞の比率の上位のものにしぼって表示した．

要な存在と考えられる．Vα19 NKT細胞はTCRへの刺激に応答して即時的に免疫系調節機能をもつIL-4, IL-10, IFN-γ, IL-17などのサイトカインを分泌する．また，これが引き金となり二次応答として周囲の細胞のサイトカイン分泌を誘導する．このサイトカイン産生能が実際どのような機構で自己免疫抑制をもたらすかを知るために，この細胞の抗原刺激に伴うサイトカインの分泌能と免疫系ホメオスタシス効果について詳細な検討を加えた．

Vα19 NKT細胞のサイトカイン分泌能

はじめにVα19-Jα33 TCR発現細胞が抗原刺激を受けたときに特異的に発現するサイトカインやケモカインなどの細胞因子，それらのレセプター遺伝子を網羅的に調べた．Vα19 NKT細胞が過剰発生するTgマウスの肝臓から単核球細胞を調製し，これを抗CD3抗体でコートしたウェル上で培養したときの前後での遺伝子発現をDNAマイクロアレイ法により分析した．対照としてβ2-ミクログロブリン遺伝子欠損(β2m$^{-/-}$)マウス肝臓単核球細胞を用いた(図1)．重複を含めて39,000種の転写物の分析の結果，Vα19 Tg細胞の活性化に伴いIL-17A, IL-22遺伝子発現の顕著な亢進が観察された．これはVα19 NKT細胞の活性化に伴うTh17細胞の分化誘導を暗示している．一方でIL-5, IL-13の遺伝子高発現も観察された．これは後述の蛋白レベルでの分析で示すIL-4などの高発現と合わせ，均一Vα19 TCR発現細胞の存在によりEAEなどの臓器特異的自己免疫病動物モデル

における病状進行抑制を支持する．

次に，Vα19 Tg細胞および対照としてB6およびβ2m$^{-/-}$マウスから調製した肝臓単核球細胞の培養後の上清のサイトカイン濃度を実際にELISA法で分析した(図2)．その結果，Vα19 Tg細胞のうち，NK1.1^{+}細胞除去操作を加えない細胞で即時的なIL-4, IFN-γ, IL-17の産生が観察された．これに対しNK1.1^{+}細胞除去後の細胞ではIL-4の分泌が弱くなり，IFN-γ, IL-17の分泌は培養2日目以降になってから顕著にみられた．一方，対照のB6細胞では即時的なIL-4, IFN-γの分泌はTgマウス細胞同様にNK1.1^{+}細胞除去操作前の細胞で顕著であるが，IL-17の産生はVα19 TCR Tgマウス細胞より小さかった．β2m$^{-/-}$細胞では培養1日目のサイトカイン分泌は小さかった．以上から，即時的サイトカイン分泌はVα19 TCR Tg中のVα19 NKT細胞あるいはB6マウス細胞に約30%の細胞数で含まれるVα14 NKT細胞によりもたらされ，このとき高いIL-17の分泌はVα19 TCR Tgマウス細胞，おそらくVα19 NKT細胞によりもたらされることが強く示唆された．これは実際，Vα19 TCR TgマウスからVα19 NKT細胞を分取して同様の実験を行った結果確認された．NK関連遺伝子群の一つNKR-P1遺伝子産物であるNK1.1は，ある種の活性化T細胞でも発現し，また逆に活性化Vα14 NKT細胞での発現の減少が観察される[12]．したがって，コンベンショナルT細胞と区別されるいわゆるNKT細胞系譜の厳密なマーカーとしては不適切であることは事実であるが，それでもNK1.1の発現をもとに均一Vα19 TCRα鎖発現細胞のサイトカイン分泌パターンは区別され，事実上特定の細胞系譜のマーカーとして機能している．最近の報告ではNK1.1^{+}, Vα19 TCR^{+}細胞はMR1遺伝子欠損を背景としたマウスでは大きく減少することから，Vα19 NKT細胞の表現型をもつ細胞の多数がMR1拘束性であることが示唆された[7]．

NK1.1^{+} Tg^{+} (Vα19 NKT)細胞の高いIL-17分泌誘導能は脾臓細胞を使った細胞内サイトカイン染色実験からも示唆された(図3)．Vα19 Tg脾臓細胞，および対照としてB6脾臓細胞を抗CD3抗体，PMA/ionomycinにより刺激したときの細胞内サイトカイン産生を調べると，IL-4, IFN-γ同

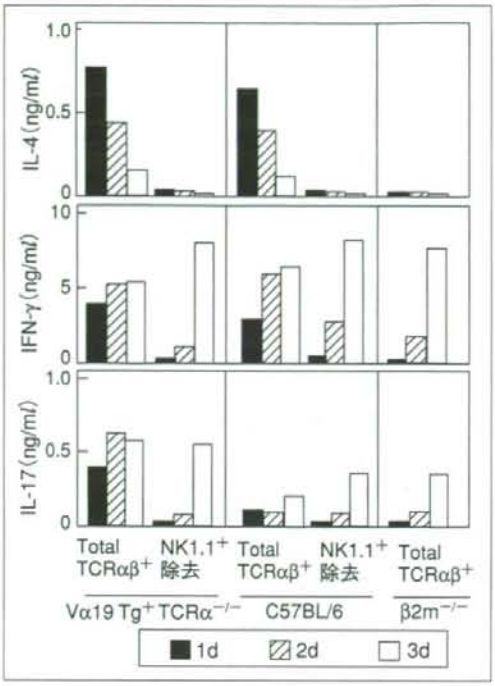

図2　均一Vα19-Jα33 TCR発現細胞のTCRへの刺激に応答したサイトカインの分泌

Vα19 TCR Tgマウス(TCRα$^{-/-}$バックグランド)肝臓単核球細胞，対照としてC57BL/6およびβ2m$^{-/-}$マウス細胞を固相化抗CD3抗体で刺激し，培養上清中のサイトカイン濃度をELISA法で分析した．Vα19 Tg, およびC57BL/6細胞についてはあらかじめNK1.1陽性細胞を除去した細胞についても同様に実験を行った．

様IL-17も当初NKT細胞による産生が主体で，次いでコンベンショナルT細胞による産生が主体となった．このときVα19 Tg脾臓細胞ではB6脾臓細胞に比べてIL-17産生細胞の割合が増大していた．

均一Vα19 TCRα鎖発現細胞活性化が分泌するIL-17発現誘導因子

均一Vα19 TCRα Tgマウス細胞の高いIL-17分泌能はどのような機構でもたらされたのか，それにVα19 NKT細胞はどのように関与しているのかについて次に検討した．Vα19 Tg脾臓細胞，および対照としてB6脾臓細胞をリスポンダーとして培養中でのIL-17産生を調べた．このときNKT細胞の存在比が大きい肝臓の単核球をVα19 TgおよびB6マウスから調製しあらかじめ抗CD3抗体

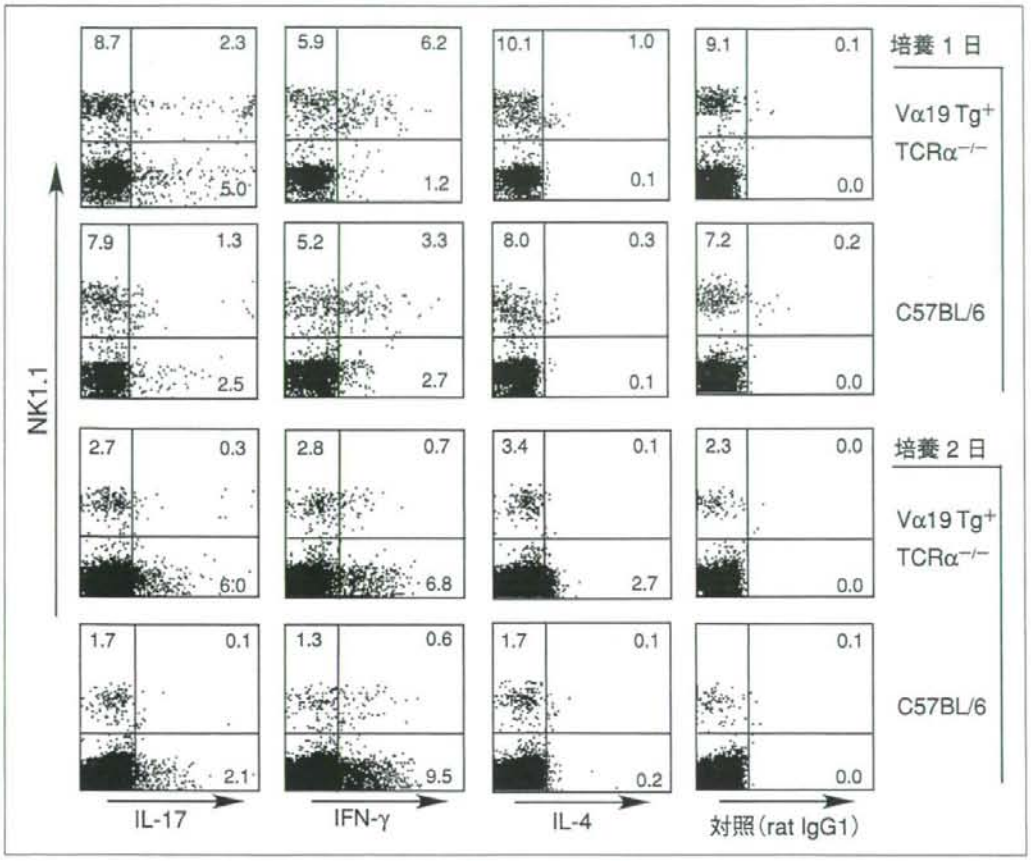

図3　抗体染色による均一Vα19-Jα33 TCR発現細胞のTCRへの刺激に応答した細胞内サイトカイン産生の検出
Vα19 TCR Tgマウス(TCRα$^{-/-}$バックグランド)脾臓単核球細胞，対照としてC57BL/6マウス細胞を固相化抗CD3抗体で1～2日刺激し，次いでPMA/ionomycinで5時間刺激し，ただちに細胞表面抗原およびサイトカインを免疫染色し，フローサイトメトリーで分析した．TCRαβ発現細胞にゲートをかけてデータを示した．

により短時間刺激し，その細胞あるいは培養上清をリスポンダー細胞の培養系に加え，両者の刺激能を検討した(図4)．その結果，TCRへの刺激を行った肝臓のTCR発現細胞の培養上清の添加のみによりリスポンダー細胞のIL-17産生が誘導されることが判明した．Vα19 Tg肝臓細胞培養上清の効果はB6細胞培養上清より大きく，かつVα19 Tgマウス由来のリスポンダーのときにB6のものより大きなIL-17分泌が起こった．また，IL-23の添加はVα19 Tgリスポンダー細胞の効果的なIL-17分泌を誘導した．このとき同時に測定したIFN-γの産生誘導は観察されなかった．

以上の結果から，Vα19 NKT細胞のTCR刺激に応答した自身のIL-17分泌に加え，液性因子のカスケードによるシグナル伝達がもたらすエフェクターT細胞によるIL-17分泌が起こり，これにVα19 TCR発現細胞が関与していることが示唆された．

Vα19 NKT細胞の抗原刺激の強さに応答したサイトカイン分泌

Vα19 NKT細胞の抗原刺激に応答してIL-17が高発現することが示唆された．しかしながら，IL-17は多発性硬化症などの炎症性自己免疫病の進行を推進する[13][14]．Vα19 NKT細胞の自己免疫病モデルでの病状抑制効果を考慮すると生理的環境下ではなんらかのサイトカイン分泌の調節機構が存在することが予測される．最近，ヒトVα24 invariant TCR発現細胞株(マウスVα14 NKT細胞に相当)を濃度の異なる固相化抗TCR抗体で

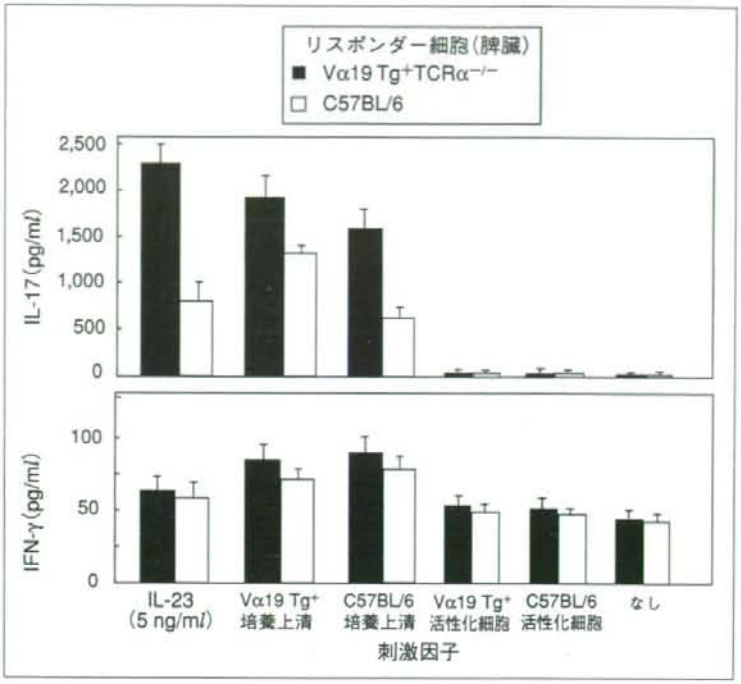

図4　TCR刺激を受けた均一Vα19 TCR発現細胞分泌因子によるIL-17産生誘導
Vα19 TCR Tgマウス(TCRα$^{-/-}$バックグランド)肝臓単核球細胞，対照としてC57BL/6マウス細胞を固相化抗CD3抗体で18時間培養し，上清と細胞に分離した．おのおのを別に調製したVα19 TCR TgおよびC57BL/6マウス脾臓単核球細胞に加え1日培養し，上清のIL-17, IFN-γをELISA法で分析した．ここで刺激因子として加えた培養上清にもともと含まれていたIL-17, IFN-γのリスポンダー細胞培養上清中の濃度への寄与はそれぞれ150, 50pg/ml以下である．

培養中刺激したときに，コートした抗体の濃度に依存して発現するサイトカインの種類が変化することが報告された[15]．そこで，実際TCR刺激に用いる抗CD3抗体の濃度をこれまでに用いた抗体濃度から段階的に低くして均一TCRへの刺激の強さを調節したときのVα19 NKT細胞の免疫応答を分析した．その結果，IL-4, IL-10などのTh2サイトカインの産生がIL-17やIFN-γに優先して起こる一定の濃度範囲が存在することがわかった(Shimamura, et al., 論文投稿中)．

おわりに

Vα19 NKT細胞の抗CD3抗体を使用したTCRへの刺激に応答して見かけ上，昂炎症性のIL-17の高発現がみられたが，これはVα19 NKT細胞の炎症性自己免疫病抑制効果を考える上でなんらかの説明づけが必要であった．均一Vα19 TCRα鎖発現細胞の抗原刺激に応答したサイトカイン分泌にはおそらく抗原提示細胞上でのMR1の発現濃度や抗原の種類に依存して多様性があり，Th2免疫応答を誘導して炎症抑制機能が発揮される抗原刺激が生理的条件下において存在することが示唆された．

IL-17産生Th17細胞はIL-23の作用を受けて炎症亢進性を示すが，TGF-β, IL-6, あるいはIL-27の作用を受けるとtype I regulatory T 細胞様に機能分化し，IL-10を産生して炎症抑制的に機能することが最近報告された[16)~18)]．均一Vα19 TCR発現細胞の適度なTCRへの刺激によりIL-6やTGF-β, IL-27が産生されたことから，IL-27産生細胞と共同してtype I regulatory T 細胞様分化にこの細胞が関与している可能性も考えられる．Vα19 TCR発現細胞のTh2サイトカインの分泌を優先的に誘導する特異的活性化物質を見出すことが炎症性