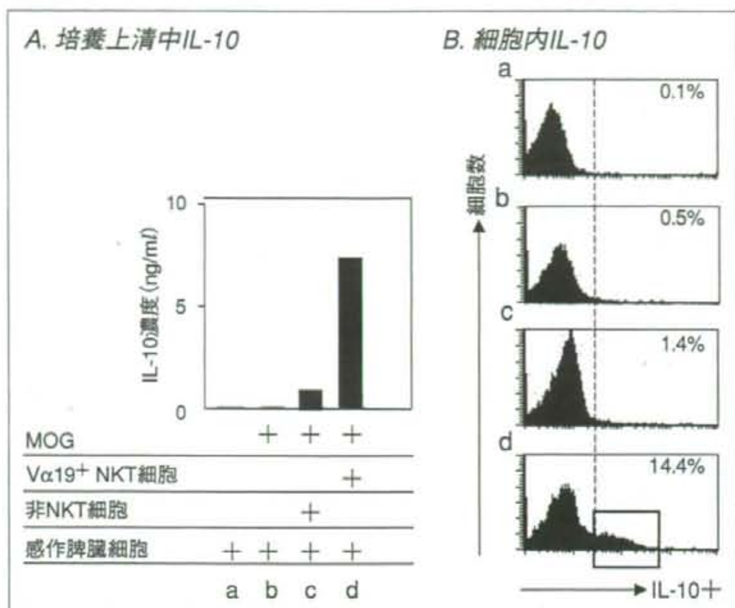

図3　Vα19-Jα33 T 細胞と B 細胞は協調的にIL-10を産生する

A：パネルの下に示したように，感作脾臓細胞単独(a)，感作脾臓細胞＋MOGペプチド(b)の培養条件では，上清中にIL-10は検出されない(ELISA法による)．また，感作脾臓細胞＋MOGペプチドにVα19 Tg x CD1d KOマウス肝臓から分離したNK1.1$^-$CD3$^+$ T 細胞を4：1の割合で加えたが，IL-10の産生は軽度であった(c)．しかし，NK1.1$^+$ T 細胞(Vα19-Jα33 T 細胞)を同じ割合で加えると(d)，著しいIL-10産生の亢進を認めた．

B：図4のa～dのそれぞれについて，細胞内IL-10を特異抗体で染色して解析した．

$MOG_{35\text{-}55}$を完全フロイントアジュバントに混和してから免疫することによって行った．EAEを発症したマウスでは，体重減少，尾の麻痺，後肢麻痺，四肢麻痺などの明確な臨床症状を呈する．EAEは自己免疫における特定の分子や細胞の関与を評価するのに優れたモデルであり，世界中の研究室で広く使われている．

実験の結果，野生型マウスに比較して，Vα19 TgではEAEの症状が軽くなった．また，Vα19 Tg x CD1d KOでは，CD1d KOに比べてEAEが軽症化した(図2A)．この結果から，Vα19-Jα33 T 細胞はCD1d拘束性NKT細胞を必要とせず，単独でEAEを抑制することが推測された．さらに，Vα19 Tg x CD1d KOの肝臓からVα19-Jα33 T 細胞を分離し，EAEを誘導した野生型B6マウスに移入したところ，Vα19-Jα33 T 細胞を移入されたマウスではEAEが軽症化した(図2B)．さらに，Vα19-Jα33 T 細胞を欠損するMR1 KOマウスにEAEを誘導したところEAEの臨床症状は増悪した(図2C)．以上の結果から，Vα19-Jα33 T 細胞にはEAEの炎症病態を制御する機能があることが示された．

$MOG_{35\text{-}55}$に対するrecall responseを評価したが，細胞増殖反応でみるかぎり，Vα19 Tgマウスと野生型マウスでは差を認めなかった．しかし，Vα19 TgマウスではIFN-γ，TNF-α，IL-17などの炎症性サイトカイン産生が低下していた．これらの結果から，Vα19 TgにおけるEAEの抑制は，$MOG_{35\text{-}55}$に反応する T 細胞レパトアが減少した結果ではなく，MOG反応性 T 細胞が機能的に偏倚したためと理解できる．

Vα19-Jα33 T 細胞は B 細胞と協調してIL-10を産生する

Vα19 TgマウスのEAEでは，野生型マウスに比べてIL-10の産生が亢進していることがわかったので，その産生細胞や発現誘導機序を明らかにすることを試みた．解析を容易にするために，Vα19 Tg x CD1d KOの肝臓から分離したVα19-

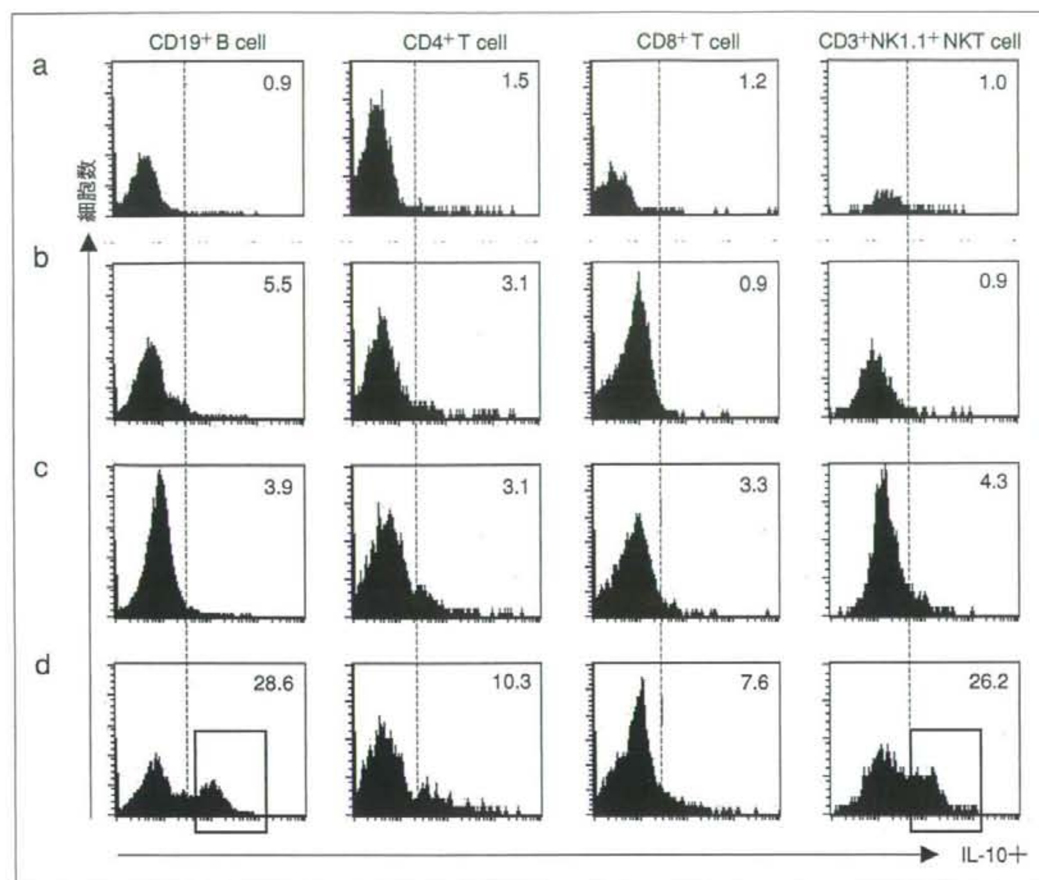

図4　IL-10産生細胞の同定
IL-10産生細胞を同定するため，リンパ球分画(B細胞，T細胞，NKT細胞など)特異的蛍光標識抗体による染色を追加した．細胞内IL-10が検出されたのは(d)の条件(感作脾臓細胞＋MOG＋Vα19-Jα33 T細胞)で，B細胞とNKT細胞のそれぞれ4分の1以上がIL-10を発現することがわかった．

Jα33 T細胞を，$MOG_{35\text{-}55}$で感作されたB6マウスの脾臓細胞(SpC)に，1：4の割合で添加したところ，IL-10の有意な産生が認められた(図3)．次に，細胞内サイトカイン染色で，IL-10産生細胞を調べたところ，B細胞とVα19-Jα33 T細胞がIL-10の主な産生細胞であることがわかった(図4)．脾臓B細胞を欠損する免疫グロブリンμ鎖欠損マウスを$MOG_{35\text{-}55}$で免疫し，そのSpCを用いたところ，IL-10の産生は顕著に低下した．このことから，IL-10産生はVα19-Jα33 T細胞とB細胞の協調的な相互作用の結果誘導されることが示唆された．

IL-10産生にはVα19-Jα33 T細胞がB細胞の発現するMR1抗原を認識する必要があるのではないかと考えた．この仮説を検証するために，MR1 KOマウスを$MOG_{35\text{-}55}$で免疫し，そのSpCを混合培養に供した．しかし，IL-10は培養上清中に検出され，その値はむしろ若干高い傾向にあった．したがって，IL-10産生にMR1分子認識は必要でないことがわかった(図5)．

Vα19-Jα33 T細胞は，細胞表面にICOS(inducible costimulatory；CD278)，B7.2(CD86)，CD40L(CD154)を発現している(図6A)．ICOSはCD28のファミリー分子で，ICOS補助刺激はIL-10産生を誘導することが報告されている．これらの補助刺激分子が関与する可能性を考え，ICOS-ICOSL，B7-CD28，あるいはCD40-CD40L相互作用を阻害する抗体を添加してその影響をみた．その結果，

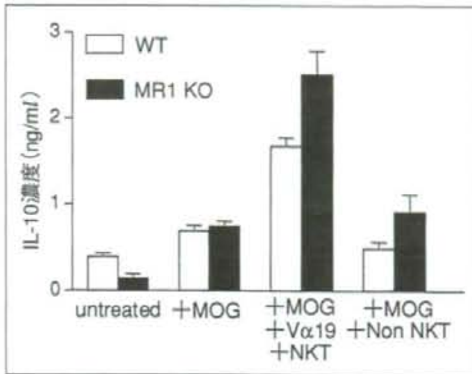

図5　IL-10産生はB細胞のMR1発現に依存しない
野生型B6マウスまたはMR1 KOマウスを$MOG_{35\text{-}55}$で感作して感作脾細胞を分離した．そのまま培養したグループ(untreated)，$MOG_{35\text{-}55}$のみを添加したグループ(＋MOG)，およびMOGとVα19-Jα33 T細胞を添加したグループ(＋MOG＋Vα19＋NKT)，MOGと非NKT細胞を添加したグループについて，培養上清中のIL-10濃度を測定した．Vα19-Jα33 T細胞添加によるIL-10の誘導は，B細胞の発現するMR1には依存しないことがわかった．

ICOSL特異的抗体の存在下では，IL-10産生が強く抑制されることがわかった(図6B)．さらに，精製B細胞とVα19-Jα33 T細胞のみの混合培養によってもIL-10が誘導され，抗ICOSL抗体の添加によって，IL-10産生は抑制された．これらの結果から，B細胞とVα19-Jα33 T細胞は，それぞれの発現するICOSLとICOSを介して相互作用し，協調的にIL-10を産生するものと考えられる(図7)．

おわりに

近年，先進諸国におけるアレルギー疾患と自己免疫疾患の増加が顕著になっている[15)16)]．長年にわたって信奉されてきた「Th1/Th2バランス(Th1/Th2 balance)」学説によれば，Th1細胞とTh2細胞は互いに拮抗する．しかし，Th1細胞の誘導する自己免疫とTh2細胞の介在するアレルギーの両者が平行して増加しているという事実は，Th1とTh2が互いに拮抗してバランスをとるという考え方では説明できない．むしろ，Th1とTh2

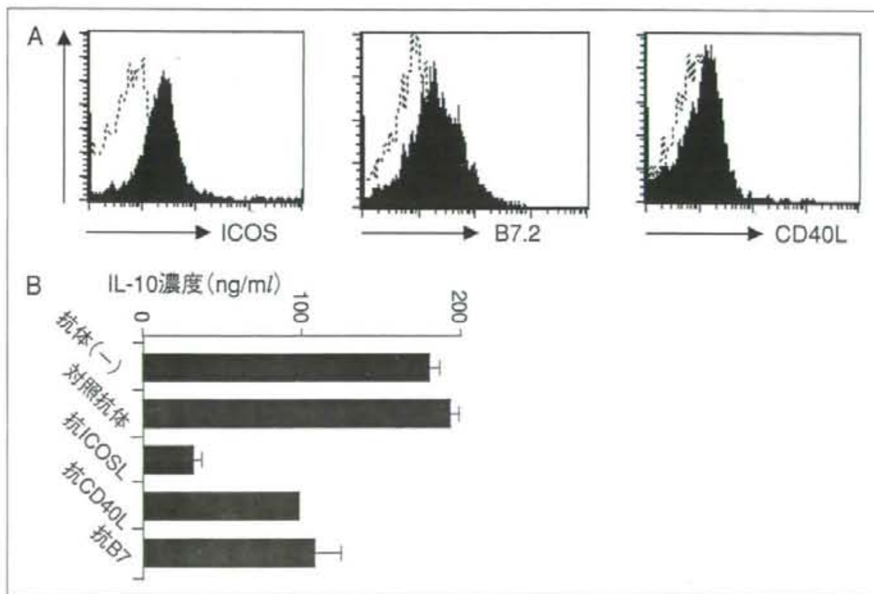

図6　ICOS-ICOSL補助刺激の役割

A：Vα19-Jα33 T細胞の補助刺激分子発現．Vα19 Tg x CD1d KOの肝臓$NK1.1^{+}$ T細胞(Vα19-Jα33 T細胞)，および野生型B6マウスのT細胞を，抗ICOS, 抗B7.2, 抗CD40Lで染色し，それぞれの発現レベルを比フローサイトメーターで比較した．破線は対照抗体による染色後の蛍光強度を，黒ぬり波は抗ICOS, 抗B7.2, または抗CD40L抗体による染色後の蛍光強度を示す．

B：IL-10産生におけるICOS-ICOSL補助刺激の関与．感作脾臓細胞＋MOG＋Vα19-Jα33 T細胞の培養に，抗体を添加して阻害効果を検討した．抗ICOSL抗体の添加によって，IL-10産生は著明に抑制された．

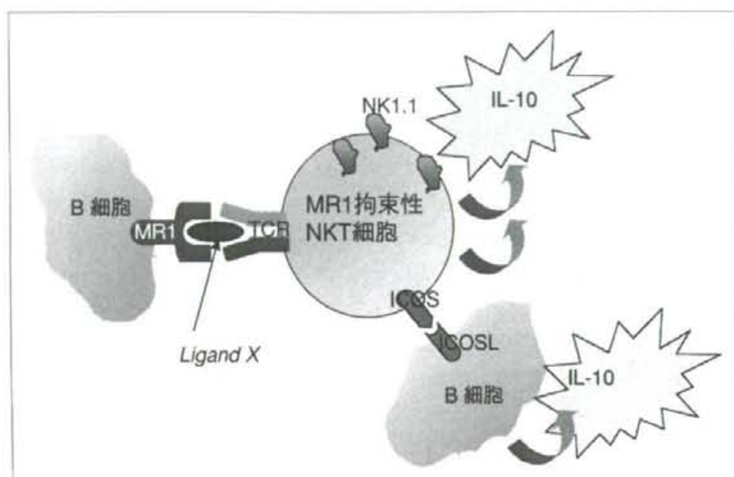

図７ MR1拘束性NKT細胞とＢ細胞の相互作用
MR1拘束性NKT細胞とＢ細胞のクロストークの結果，IL-10産生が誘導される．その際に重要なのはICOS-ICOSLを介した補助刺激シグナルである．一方，TCRを介したMR1認識はIL-10産生には必要でないが，他の目的で機能している可能性がある．

を一括して制御する免疫制御システム(immunoregulatory system)の機能が，現代人では徐々に低下している可能性を示唆する．Mucosal associated invariant T(MAIT)細胞でとくに興味深いのは，腸管粘膜に集積し腸内細菌叢(gut flora)の強い影響を受ける点である[1]．免疫制御システムが遺伝因子と環境要因(後天因子)の影響を受けることは明白であるが，このリンパ球は環境要因の影響を受けることが明確にされた最初の制御性細胞と言ってもよい．現代における免疫疾患の増加という大きな問題を解明する鍵を握る細胞かもしれない．

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Localization of NK1.1$^+$ invariant Vα19 TCR$^+$ cells in the liver with potential to promptly respond to TCR stimulation

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ABSTRACT

Previously, we found that more than a half of the NK1.1$^+$ T cell lines prepared from CD1$^{-/-}$ livers expressed invariant Vα19-Jα33 TCR α chains. Over-expression of the invariant Vα19-Jα33 TCR α transgene (Tg) with a natural TCR α promoter and an enhancer in mice induced the development of NK1.1$^+$ T cells (Vα19 NKT cells) in the lymphoid organs, especially in the liver. Preferential usage of the Vα19 Tg by NKT cells in the transgenic mouse livers was indirectly indicated by the observation that few NK1.1$^+$ TCR$\alpha\beta^+$ cells of the Vα19 Tg livers were stained with a cocktail of anti-TCR Vα antibodies in the FACS analysis. Upon invariant TCR engagement *in vivo* following injection of mice with anti-CD3 antibody, NKT cells of the Tg mouse livers as well as spleens promptly produced immunoregulatory cytokines such as IL-4 and IFN-γ and altered surface receptor expression. Collectively, localization of Vα19 NKT cells in the liver is suggested that are ready to immediately response against antigen stimulation.

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1. Introduction

Natural killer T (NKT) cells are defined as lymphocytes bearing both the common NK marker NK1.1, a product of a member of the NKR-P1 gene family, and TCR–CD3 complex [1,2]. The major component of NKT cells (Vα14 NKT cell) express the invariant TCR α chain (mouse Vα14-Jα18, human Vα24-Jα18) [1,2].

The 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 [3].

Recently, the presence of another invariant TCR α chain (Vα19-Jα33 (conventionally Jα26), AV19-AJ33) was shown by quantitative PCR analyses in mouse, human and bovine lymphoid cells [4,5]. We demonstrated that this invariant TCR α chain was preferentially expressed in NKT but not conventional T cells of the lymphoid organs including the liver and that more than a half of the hybrid cell lines produced from NKT cells of CD1-deficient livers expressed this invariant TCR α chain [6]. The localization of the invariant Vα19 TCR$^+$ in gut lamina propria was then demonstrated in the recent reports [7,8]. Positive selection of these cells (designated as mucosal-associated invariant T (MAIT) cells) by one of the evolutionarily conserved MHC-class Ib molecules MR1 [9] was also indicated in these studies.

In the current study, we characterized the mice over-expressing invariant Vα19-Jα33 TCRα transgene with a natural TCRα promoter and an enhancer to analyze the development of invariant Vα19 TCR$^+$ NK1.1$^+$ (Vα19 NKT) cells. We found that the invariant TCR transgene was always more frequently used by NKT cells than conventional T cells in the lymphoid organs, especially in the liver, and that the proportion of Vα19 NKT cells was the largest in the liver among the lymphoid organs examined. Thus these findings indicate the preferential differentiation of Tg$^+$ lymphoid precursors into NKT cell lineage in the liver.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Sankyo Service Co. (Tokyo, Japan). Beta2m-deficient mice with C57BL/6 genetic background

Abbreviations: Vα19 NKT cell, NK1.1+ Vα19-Jα33 invariant TCR α+ cell; Vα14 NKT cell, NK1.1+ Vα19-Jα18 invariant TCR α+ cell; Tg, transgene or transgenic; MNC, mononuclear cell; MAIT, mucosal-associated T lymphocyte.

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were obtained from Jackson Laboratory (Bar Harbor, ME, USA). CD1-deficient mice were provided by Dr. M.J. Grusby (Harvard University) [10]. They were backcrossed with C57BL/6 mice for six generations, and mice with phenotypes of H-2^b, NK1.1$^+$ and CD1$^{-/-}$ were selected. TCR Cα-deficient mice, backcrossed with C57BL/6 mice for more than 10 generations [11], were given by Drs. H. Ishikawa (Keio University) and M. Nanno (Yakult Co.).

2.2. Establishment of Vα19 Tg mice

A Vα19-Jα33 transgene with the endogenous TCR α promoter and the enhancer was injected into C57BL/6 or TCR Cα-deficient fertilized eggs and transgenic mouse lines were established. Details are shown in Supplemental Figure S1 online. A Vα19Tg$^+$CD1$^{-/-}$ mouse line was established from one of the three transgenic lines with the C57BL/6 background by cross with CD1-deficient mice. Vα19 Tg mice were compared with non-Tg mice in the same litter or with those with an appropriate genetic background (C57BL/6, 129/Sv or BALB/c) possibly included in each Tg line.

2.3. Cell preparations

MNCs were prepared from single cell suspension of mouse organs by density gradient centrifugation using Lymphosepar II (IBL, Gunma, Japan, $d = 1.090$) for spleen and bone marrow and Percoll (Pharmacia, Uppsala, Sweden) for liver as described previously [12]. Lamina propria lymphocytes were prepared as described by Treiner et al. [7].

2.4. Flow cytometry and antibodies

Mouse cells were pre-treated with anti-FcγRII, III monoclonal antibody, 2.4G2 (Pharmingen, San Diego, CA, USA), to saturate Fc receptors. Specific staining was performed with a combination of the following conjugated antibodies purchased from Pharmingen; H57-597 (anti-TCR Cβ), PK136 (anti-NK1.1), RM4.5 (anti-CD4), 53-6.7 (anti-CD8α), 53-5.8 (anti-CD8β), RR4-7 (anti-Vβ6), F23.1 (anti-Vβ8), B20.1 (anti-Vα2), RR3-16 (anti-Vα3.2), B21.14 (anti-Vα8.3), RR8-1 (anti-Vα11.1, 11.2). Tetramer of CD1-α-Gal-Cer was prepared from a DNA construct (provided by Dr. M. Kronenberg, La Jolla Institute for Allergy and Immunology) as described by Matsuda et al. [13]. The stained cells were analyzed on a FACS can flow cytometer equipped with the Cell Quest Software (Becton Dickinson, San Jose, CA, USA).

2.5. In vivo stimulation of Vα19Tg lymphocytes by TCR engagement

Mice of Vα19Tg$^+$ CD1$^{-/-}$ and CD1$^{-/-}$ in the same litter, and C57BL/6 (8 weeks of age) were intravenously injected with anti-CD3 antibody (2C11, Pharmingen, 1.5 or 3.0 μg/mouse) in 200 μl PBS. Livers were removed from mice 90 min after antibody injection, and MNCs were immediately prepared from them as described above. They were cultured at the concentration of 5×10^6 per ml in the DMEM (10% FCS, 50 μg/ml streptomycin, 50 U/ml penicillin) without further supplements. Cytokines in the supernatants were determined by ELISA using antibodies that were purchased from Becton Dickinson. In some experiments, livers were removed from mice 1 day after antibody injection, and MNCs were immunostained and analyzed by flow cytometry.

3. Results

3.1. Preferential development of Vα19 NKT cells in the liver of invariant Vα19-Jα33 TCR Tg mice

An invariant Vα19-Jα33 TCR gene segment was cloned into a TCR α vector containing the TCR α endogenous promoter and enhancer, and Tg mice (Vα19 Tg mice) were produced to examine the role of the invariant TCR in Vα19 NKT cell development (Supplementary Figure S1 online).

The development of lymphocytes expressing the invariant TCR was demonstrated by the presence of TCRαβ$^+$ cells in the lymphoid organs of the Vα19 Tg mice with the TCRα-deficient (TCRα$^{-/-}$) background (Fig. 1A). A remarkable proportion of the Tg$^+$ cells was differentiated into NK1.1$^+$ TCRαβ$^+$ NKT cells in the Tg organs, especially in the liver (liver, 29.5%; bone marrow, 7.5%; spleen, 3.6%). The proportion was comparable to that in the non-Tg mice with the same genetic background (C57BL/6) (26.4%, 5.1%, 3.2%, respectively, Fig. 1B [14]). The cellularity of these organs in the transgenic mice (liver, 4.8×10^6; bone marrow, 4.5×10^7; spleen, 1.3×10^8 at 8 weeks of age) was not significantly altered from that in non-Tg mice (liver, 4.4×10^6; spleen, 1.2×10^8; bone marrow, 4.3×10^7 at a similar age). Thus, these findings suggest that the expression of the invariant TCR α transgene induced preferential development of Vα19 NKT cells in these organs compared with the development of NKT cells in normal mice.

The preferential development of NKT cells in the Tg livers was also observed in the livers of the Tg mice with the genetic background of C57BL/6 (28.7%, Fig. 1B)) and CD1$^{-/-}$ (31.0%, Supplementary Figure S2) where TCR αβ$^+$ cells are allowed to use endogenous TCR α chains due to the incomplete allelic exclusion of the TCR α locus. The preferential use of the transgene by the cells of liver rather than spleen or thymus of the Tg mice with C57BL/6 background was supported by the expression of the transgene analyzed by RT-PCR (Supplemental Figure S1D). Development of Vα14 NKT cells was suppressed not only in the Tg livers with CD1$^{-/-}$ background but also in the Tg livers with C57BL/6 background, because only a limited fraction of the Tg liver cells were stained with CD1-α-Gal-Cer tetramers (1.6%) compared with the non-Tg mouse liver cells with C57BL/6 background (17.1%) (Fig. 2). Taken together, most NKT cells in the Tg livers with these genetic backgrounds were probably Vα19 but not Vα14 NKT cells in spite of the non-stringent pressure of TCR α usage. A similar increase in NKT cell development was observed in the Vα14-Jα18 TCRα Tg mice [3]. In contrast, few NKT cells were generated in Vα11-Jα2B4 or Vα8-Jα37 TCRα Tg mice [3,15]. Thus, NKT cell development is possibly dependent on the use of invariant TCR α chains expressed by lymphoid precursors.

The facilitated development of Vα19 NKT cells in the Tg mice was supported by a comparison of the TCR structure between Tg and non-Tg mouse cells. Liver mononuclear cells (MNCs) isolated from Vα19 Tg$^+$ CD1$^{-/-}$ and C57BL/6 mice were triply stained with fluorescence-conjugated anti-TCRαβ, anti-NK1.1 and a cocktail of anti-Vα2, 3, 8 and 11 antibodies. Staining profiles in the NKT cell fraction (NK1.1$^+$, TCRαβ$^+$) and the conventional T cell fraction (NK1.1$^-$, TCRαβ$^+$) with the anti-Vα antibody cocktail are shown in Fig. 3 and Table 1. Since Vα14 NKT cells were a main component of the NKT cell population in the non-Tg normal liver, few NKT cells were stained with the anti-Vα antibody cocktail; whereas, a substantial fraction of conventional T cells was positive for the Vα staining. Here in the Tg livers, almost all the NKT cells were negative for the Vα staining despite the lack of Vα14 NKT cells; whereas, a substantial fraction of the conventional T cells was positive for the Vα expression. These observations indicate that the invariant Vα19 TCRα-bearing cells were directed to develop preferentially into NKT

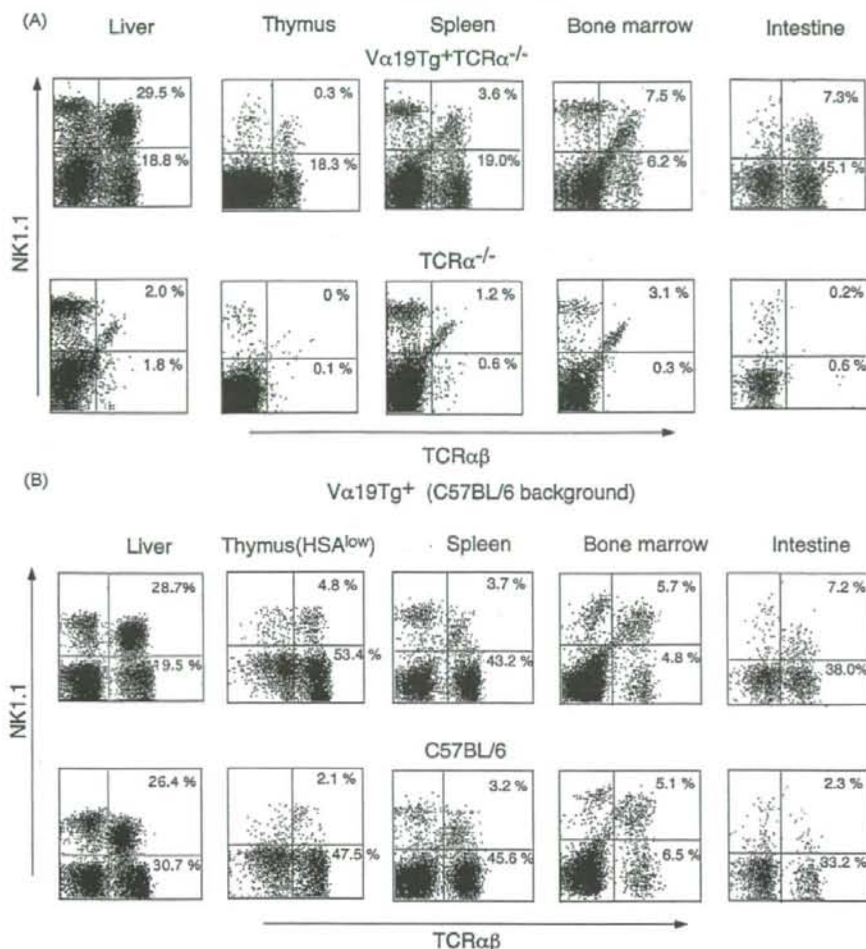

Fig. 1. Development of Vα19 NKT cells in Vα19 Tg mice. MNCs were isolated from liver, thymus, spleen, bone marrow and intestinal lamina propria of mice (at 8–12 weeks of age, female). They were stained with fluorescence-labeled antibodies and analyzed by FACS. (A) Staining profiles of the Vα19 Tg+ TCRα$^{-/-}$ and TCRα$^{-/-}$ cells with anti-NK1.1 and anti-TCR αβ antibodies. (B) Staining profiles of the Vα19 Tg+ and non-Tg cells with the same genetic background (C57BL/6). Note that plots of thymocytes are gated on the HSA^{low} cells in (B). In (A) and (B), one of the three representative experiments, each using pools of cells from 2 to 3 mice is shown.

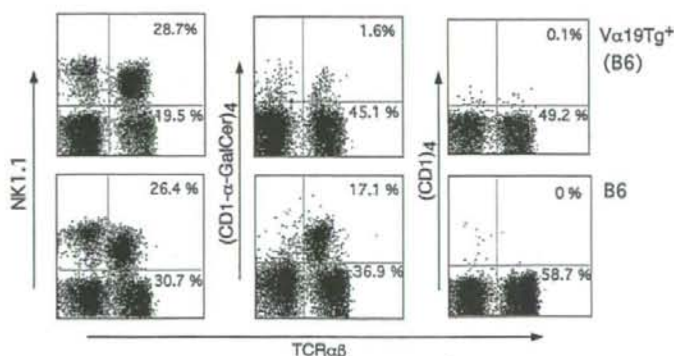

Fig. 2. Staining profiles of Tg- and non-Tg liver MNCs with CD1-α-Gal-Cer tetramers. Liver MNCs isolated from Tg and non-Tg mice on the C57BL/6 background (at 8–10 weeks of age, female, both in the range of 3–7 × 10^6 per animal) were stained with anti-TCR αβ antibody and either anti-NK1.1 antibody, CD1-α-Gal-Cer tetramers, or empty CD1 tetramers. The stained cells were analyzed by FACS.

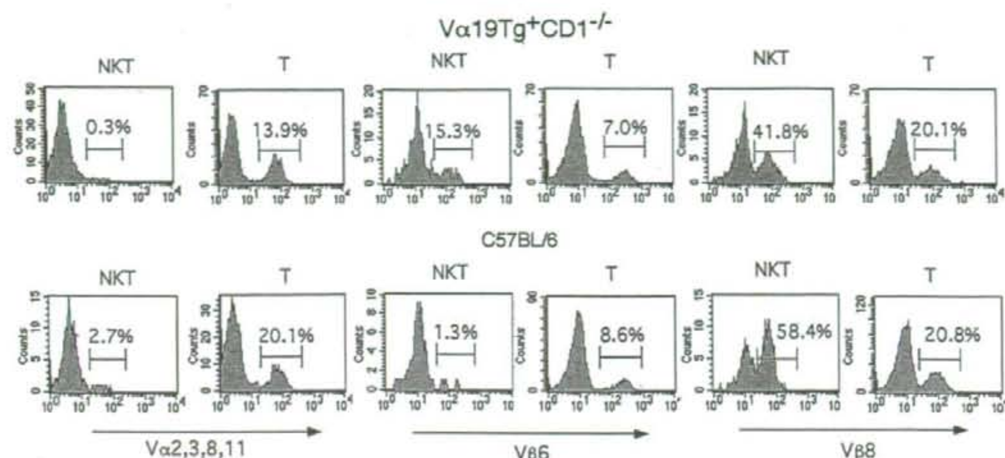

Fig. 3. Preferential expression of the invariant Vα19 TCR transgene by the NK1.1$^+$, TCRα($^+$ cells in the Tg livers. Liver MNCs from Vα19 TCR Tg (CD1$^{-/-}$ background) and non-Tg mice (at 8 weeks of age, female) were triply stained with an anti-TCRαβ, anti-NK1.1 and anti-TCR Vα mixture (Vα2, 3.2, 8, 11) or anti-Vβ antibodies. The histograms of the NK1.1$^+$, TCR αβ$^+$ (NKT) and the NK1.1$^-$, TCR αβ$^+$ (T) cells stained with anti-TCR Vα cocktail, anti-Vβ6 and anti-Vβ8 antibody are indicated.

cells in accord with our previous observation that invariant Vα19 TCR α chains are predominantly expressed as NKT cells in the liver [6]. The preferential development of Vα19 NKT cells was also found in the lymphoid organs other than the liver in the Tg mice judging from the Vα usage (Table 1).

Similar to invariant Vα19 TCR α$^+$ hybridomas [4,5], NKT cells used Vβ6 and Vβ8 relatively frequently in Vα19 Tg$^+$ TCR α$^{-/-}$ mice, where the TCR α chain is fixed to the Vα19 transgene (Table 1). This characteristic Vβ usage was also found in Vα19 Tg$^+$ CD1$^{-/-}$ mice. These findings suggest that lymphoid precursors bearing invariant Vα19$^+$/confined Vβ$^+$ semi-invariant TCR are preferentially differentiated into NKT cells.

A substantial number of NKT cells was observed in the α19 Tg$^+$ TCR α$^{-/-}$ gut lamina propria (Fig. 1) in accordance with the

Table 1
Vα and Vβ usage of the NKT and conventional T cells in the transgenic and non-transgenic lymphoid organs

	Liver	Spleen	Bone marrow	Intestine	Thymus (lamina propria)
Vα cocktail* (%)					
Vα19Tg$^+$CD1$^{-/-}$					
NKT cell	0.6	8.6	10.6	6.8	6.1
T cell	14.3	13.6	16.3	12.1	11.5
C57BL/6					
NKT cell	2.6	17.4	15.5	14.3	5.9
T cell	20.3	21.3	21.6	16.9	17.8
Vβ6$^+$ (%)					
Vα19Tg$^+$CD1$^{-/-}$					
NKT cell	17.1	13.3		17.4	14.0
T cell	7.8	8.6		10.9	7.7
Vα19Tg$^+$TCRα$^{-/-}$					
NKT cell	18.4	11.7		14.8	14.0
T cell	11.9	10.1		12.9	8.0
C57BL/6					
NKT cell	1.3	8.5		10.9	5.6
T cell	8.3	7.9		6.8	7.5
Vβ8$^+$(%)					
Vα19Tg$^+$CD1$^{-/-}$					
NKT cell	43.4	34.1		30.9	30.0
T cell	24.0	24.3		29.9	25.4
Vα19Tg$^+$TCRα$^{-/-}$					
NKT cell	53.4	33.9		35.0	31.1
T cell	33.2	28.3		30.1	20.0
C57BL/6					
NKT cell	62.0	27.3		27.2	28.8
T cell	21.4	21.1		20.2	20.7

Vα cocktail*, positive for staining with anti-Vα2, 3, 8, and 11 antibody cocktail. The average of 2–4 experiments each using pools of cells from 2 to 3 mice (8–12 weeks old) is shown.

reports on the localization of invariant Vα19 TCR⁺ cells there [7,8]. The proportions of NK1.1⁺ T cells were increased in the lamina propria of the Tg mice, irrespective of their genetic background ($6.0 \pm 1.5\%$ in Vα19 Tg⁺ TCRα$^{-/-}$ intestine, $2.8 \pm 1.0\%$ in C57BL/6 intestine, Supplementary Table S1). Besides, the proportion of the cells stained with the anti-Vα cocktail in lamina propria NKT cells was less than that in spleen or bone marrow NKT cells in Vα19 Tg⁺ CD1$^{-/-}$ mice (Table 1). Taken together, it is suggested that Vα19 Tg⁺ NKT cells are preferentially localized in the lamina propria as well as in the liver.

Vα19 NKT cells were rarely found in the Vα19 Tg⁺ TCR α$^{-/-}$ thymus (0.3%, Fig. 1A). The proportion of CD4⁺ or CD8⁺ single positive cells was reduced in the Tg thymus (4.4% in the Vα19 Tg⁺ TCR α$^{-/-}$ thymus, 9.2% in the C57BL/6 thymus), suggesting the impaired maturation of T-lineage cells in general with the TCR α$^{-/-}$ background. Above all, the development of NKT cells in the Tg thymus seemed to be especially limited (cf. 0.6% in the Vα19 Tg⁺ thymus with the C57BL/6 background, 0.7% in the C57BL/6 thymus). However, comparison between the proportion of NKT cells among HSA^{low} thymocytes in the Tg and that in non-Tg mice with the same background (C57BL/6) (4.8% and 2.1%, Fig. 1B) suggests that the thymus is also the organ where Vα19 Tg⁺ NKT cells are distributed.

A quite unique CD4, CD8 co-receptor expression was observed in Vα19 NKT cells (Fig. 4). Different from Vα14 NKT cells, numbers of CD4⁺ and CD8⁺ Vα19 NKT cells are comparable. Rather, the CD8⁺ NKT cell subset predominates in the Vα19 Tg⁺TCR α$^{-/-}$ liver. The CD8 molecules expressed by the NKT cells in the Tg liver and spleen consisted of the αβ hetero-dimer, whereas those expressed by the Tg bone marrow NKT cells were mostly the αα homo-dimer, thus suggesting the presence of heterogeneity in the CD8⁺ NKT cell subset depending on the tissue distribution in the Tg mice. However, the CD4⁻, CD8⁻ double negative NKT cell population was present as the major component in all the Tg organs analyzed here including the lamina propria (CD4⁺:CD8⁺:CD4⁻CD8⁻ = 3:1:6, data not shown). The preferential generation of CD4⁻, CD8⁻ T cells has also been reported in Vα14-Jα18 invariant TCRα Tg mice [3]. Therefore, it is not clear whether the double negative population is generated as the major subset during normal development.

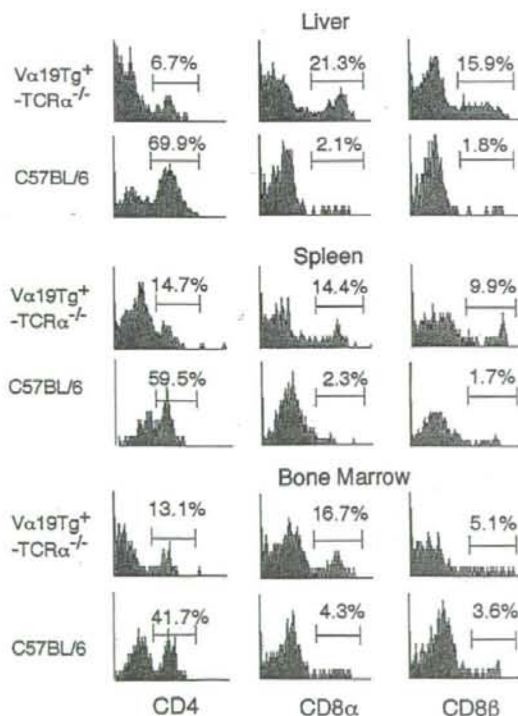

Fig. 4. CD4/CD8 co-receptor expression by NK1.1⁺, TCR αβ⁺ cells of Vα19 Tg⁺ and non-Tg mice. MNCs were isolated from liver, thymus, spleen, and bone marrow of Vα19 Tg⁺ TCRα$^{-/-}$ and C57BL/6 mice (at 8–12 weeks of age). They were immunostained and analyzed by flow cytometry. Profiles of CD4/CD8 co-receptor expression in the NK1.1⁺, TCR αβ⁺ cells of Vα19 Tg⁺ TCRα$^{-/-}$ and C57BL/6 organs are indicated.

3.2. Prompt activation of Vα19 NKT cells in the liver upon TCR engagement in vivo

Cytokine production by liver Vα19 NKT cells was examined to assess their immunoregulatory potential. It has been reported that Vα14 NKT cells in the spleen promptly produce immunoregulatory cytokines in response to *in vivo* challenge with anti-CD3 antibody [15]. Thus, production of cytokines by Vα19 Tg⁺ CD1$^{-/-}$ liver cells was compared with the production by CD1-deficient or normal liver cells that were prepared from mice previously injected with anti-CD3 antibody (Fig. 5). Production of IL-4 and IFN-γ was reduced in CD1$^{-/-}$ liver cells compared with C57BL/6 cells. This reduction was restored by the introduction of invariant Vα19 TCR transgene into CD1$^{-/-}$ mice. The prompt production of cytokines by liver lymphocytes was similarly observed in Vα19 Tg⁺ TCR α$^{-/-}$ mice (data not shown), thus indicating that invariant Vα19 TCR⁺ cells are the producer of the cytokines. In addition, NK1.1⁺ but not NK1.1⁻ Vα19 Tg⁺ liver cells were responsive to the stimulation to TCR–CD3 complex in culture and primarily produced immunoregulatory cytokines (Shimamura et al., submitted for publication). Thus it is suggested that certain subsets of the Tg TCR⁺ cells in Vα19 Tg⁺ mice, probably Vα19 NKT cells take the place of Vα14 NKT cells in the normal mice and are responsible for the prompt cytokine production responding to TCR stimulation. The prompt production of IL-4 and IFN-γ by hepatic NKT cells almost reached maximum with administration of 1.5 μg of anti-CD3 antibody. This dose of antibody was in accord with the dose required for the maximum induction of the IL-4 mRNA expression from the spleen cells isolated from mice with the antibody injection [15].

IL-5 and IL-10 production by Vα19 Tg⁺ and C57BL/6 liver cells increased with anti-CD3 antibody administration to some extent. The increase was more significant than the change in the production of these cytokines by CD1$^{-/-}$ liver cells. Nevertheless, liver cells showed relatively high IL-5 and IL-10 production in the culture without sensitization by injection of anti-CD3 antibody in contrast to the IL-4 and IFN-γ production. The background levels of IL-5 and IL-10 in the culture supernatants of spleen cells of the same mice were kept to be very low (data not shown).

3.3. Surface receptor down-regulation of hepatic Vα19 NKT cells upon TCR engagement in vivo

It has been reported that Vα14 NKT cells respond to the stimulation in vivo with α-Gal-Cer and down-regulate the expression of NK1.1 marker [16,17]. The proportion of NK1.1⁺ T cells in the liver of Vα19 Tg⁺ TCR α$^{-/-}$ mice reduced when the mice were previously injected with anti-CD3 antibody (Fig. 6). Conversely, the proportion of NK1.1⁻ T cells, especially TCRαβ^{low} cells, increased. Thus, Vα19 NKT cells, as well as Vα14 NKT cells, are suggested to down-modulate surface expression of NK1.1 receptors promptly responding to stimulation to TCR.

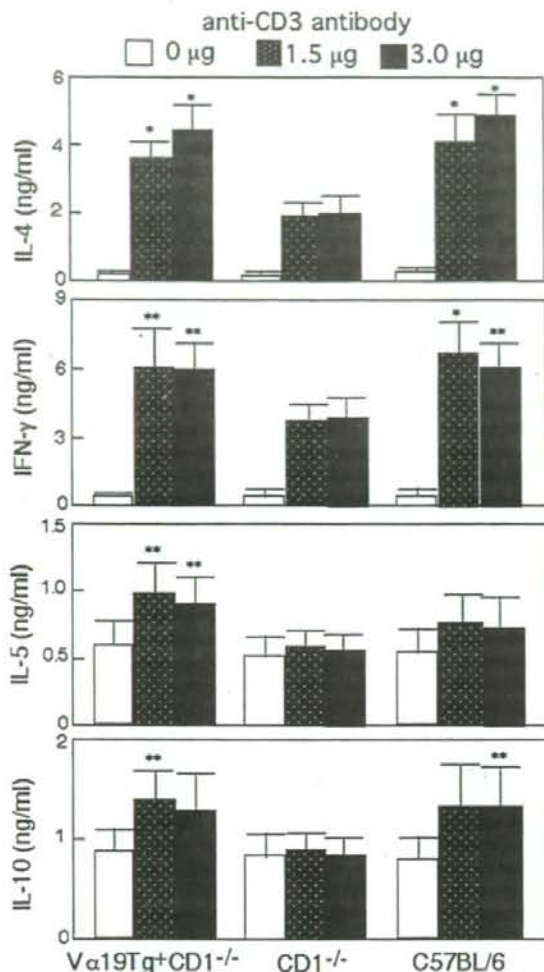

Fig. 5. Cytokine production by Tg and non-Tg mice in response to *in vivo* treatment with anti-CD3 antibody. Liver MNCs prepared from Vα19 Tg^+ $CD1^{-/-}$, $CD^{-/-}$, and C57BL/6 mice injected 90 min previously with a different dose of anti-CD3 antibody (2C11) were cultured in DMEM (10% FCS) without additional stimulation for 2 h. Culture supernatants were harvested and tested for production of cytokines by ELISA. The mean ± S.D. for three mice in each strain is shown. Experiments were repeated twice, and essentially the same profiles were obtained. Statistical significance in the Vα19Tg^+ and C57BL/6 cell responses compared with the $CD1^{-/-}$ cell responses was assessed by Student's *t*-test (*$p < 0.01$ and **$p < 0.05$).

4. Discussion

In the present study, invariant Vα19-Jα33 TCR α Tg mice were generated to examine the roles of invariant TCR α expression in Vα19 NKT cell development. Over-expression of the invariant Vα19 transgene induced the development of NKT cells in the lymphoid organs of Tg mice especially in the liver (Fig. 1, Table 1). In Vα19 Tg^+ $CD1^{-/-}$ organs, the proportion of cells stained with the anti-Vα antibody cocktail in the NKT cell population was less than that in the conventional T cell population (Fig. 3, Table 1), suggesting that lymphoid precursors bearing invariant Vα19 TCR preferentially differentiate into an NKT lineage under the non-stringent pressure of TCR α usage. Invariant Vα19 TCR α chains prefer to pair with $V\beta6^+$ and $V\beta8^+$ TCR β chains. Such a characteristic Vβ usage was found not only in the NKT cells but also in the conventional T cells to a degree of the Vα19 Tg^+ TCR $\alpha^{-/-}$ mice where the TCR α chain is fixed to the Vα19 transgene (Table 1). These findings raise the possibility that NKT and T cells with expression of the semi-invariant $V\alpha19^+/V\beta6^+$ or $V\beta8^+$ TCR frequently present in Vα19 Tg^+ TCR $\alpha^{-/-}$ mice are at least partially of an identical or similar lineage despite their difference in phenotypes. In fact, NKT cells in the livers of Vα19 Tg^+ TCR $\alpha^{-/-}$ mice reduced the expression of NK1.1 marker upon TCR engagement (Fig. 6). The tissue distribution of invariant Vα19 TCR^+ cells in normal mice has not been definitively determined because of a lack of reagents specifically identifying this population, but is probably reflected in the invariant Vα19 TCR Tg mice to some extent because the expression of the invariant TCR transgene is controlled by the natural TCR α promoter and enhancer. The predicted tissue distribution of Vα19 NKT cells is similar to that of Vα14 NKT cells in normal mice [18].

Our previous study on $CD1^{-/-}$ liver lymphocytes indicated that invariant Vα19 TCR-bearing cells preferentially developed in the liver [6]. Vα19 NKT cells were estimated to comprise about 50% of the NKT cell population in the $CD1^{-/-}$ liver. Provided that Vα19 NKT cells develop similarly in normal and $CD1^{-/-}$ livers, they account for about 0.5–1% of normal liver MNCs, corresponding to 1/40–1/20 the number of Vα14 NKT cells. The estimated population of Vα19 NKT cells is so large as a clone that they may have their own roles in the immune system.

The MHC restriction molecules for the preferentially generated Vα19 NKT cells in the Tg livers were not determined. However, the expression of invariant Vα19 TCR was reduced in the $\beta2m^{-/-}$ livers [6]. In addition, Kawachi et al. reported that development of NKT cells in the spleen or lymph node of another invariant Vα19 TCR Tg line was reduced under the MR1-deficient background [8]. Thus, it is possible to speculate that a large proportion of the Vα19 NKT cells in the present Tg livers are restricted by MR1.

Localization of the invariant Vα19 TCR^+ cells in the gut lamina propria (MAIT cells) compared with mesenteric lymph nodes, skin and gut intraepithelium has been reported [7,8]. Consistent with these reports, increased percentages of $V\alpha19^+$ NKT cells in the Tg lamina propria were observed (Supplementary Table S1). The stain-

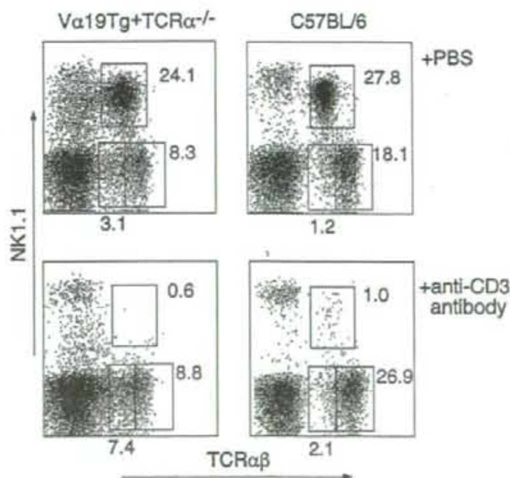

Fig. 6. Surface receptor down-regulation of hepatic Vα19 NKT cells upon TCR engagement in vivo. Liver MNCs were prepared from Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice injected 24 h previously with anti-CD3 antibody (2C11, 2 μg/mouse). Cells were stained with anti-TCRαβ and anti-NK1.1 antibodies and analyzed by flow cytometry.

ing of lamia propria lymphocytes in Vα19 Tg⁺ $CD1^{-/-}$ mice with anti-Vα antibodies (6.8% of NKT cells, 12.1% of T cells), suggests that invariant Vα19 TCR-bearing precursors preferentially differentiate into NKT-lineage even in the gut lamina propria. Invariant Vα19 TCR⁺ MAIT cells are suggested to have roles in the control of IgA production [7,19]. We found that the serum IgA level of Vα19 Tg mice ($132 \pm 56\,\mu g/ml$) was comparable to that of non-Tg mice ($115 \pm 59\,\mu g/ml$) with the same genetic background (C57BL/6). Thus, MAIT cells may participate in the control of IgA production for mucosal immunity.

Vα19 NKT cells in the liver were induced to produce immunoregulatory cytokines such as IL-4, IFN-γ, and to some extent IL-5 and IL-10 following administration of anti-TCR antibody (Fig. 5), and altered surface receptor expression (Fig. 6). Thus it is possible that Vα19 NKT cells are ready to respond to antigen stimulation and initiate the following immune responses not only in the intestine but also in the other lymphoid organs including the liver. These cells may contribute to the control of the immune responses and suppress the autoimmunity in certain cases. However, it is also possible in other situations that either Th1- or Th2-biased cytokine production by them is potentially pathogenic and deteriorates diseases such as inflammatory autoimmune diseases, allergy or fibrosis [20,21]. Vα19 and Vα14 NKT cells share similar properties regarding the potential to produce cytokines, tissue distribution and morphology (revealed by electron microscopy (Supplemental Figure S3)). However, these two subsets are subjected to independent MHC controls and are possibly involved in certain immune responses in an individual manner as previously suggested by their behavior in the autoimmune diseases [22,23].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.imlet.2008.08.002.

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Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines

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Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) mediated by Th17 and Th1 cells. DNA microarray analysis previously showed that NR4A2, an orphan nuclear receptor, is strongly up-regulated in the peripheral blood T cells of MS. Here, we report that NR4A2 plays a pivotal role for mediating cytokine production from pathogenic T cells. In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, NR4A2, was selectively up-regulated in the T cells isolated from the CNS. Strikingly, a forced expression of NR4A2 augmented promoter activities of IL-17 and IFN-γ genes, leading to an excessive production of these cytokines. Conversely, treatment with siRNA for NR4A2, resulted in a significant reduction in the production of IL-17 and IFN-γ. Furthermore, treatment with NR4A2 siRNA reduced the ability of encephalitogenic T cells to transfer EAE in recipient mice. Thus, NR4A2 is an essential transcription factor for triggering the inflammatory cascade of MS/EAE and may serve as a therapeutic target.

IL-17 | interferon-γ | EAE | Th17 | siRNA

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS), accompanying multiple foci of inflammatory lesions. MS is thought to have an autoimmune pathogenesis, involving autoimmune T cells reactive to myelin antigens (1). Development of the CNS inflammation is triggered by proinflammatory cytokines produced by the autoimmune T cells, which penetrate into the CNS parenchyma after being activated in the periphery (2, 3). Although the precise mechanism for the peripheral T cell activation remains obscure, studies indicated possible roles for cross-reactive peptides, cytokines, or superantigen (4).

Experimental autoimmune encephalomyelitis (EAE) is a prototype autoimmune disease model (5) that can be induced in laboratory animals by active immunization with myelin antigens (mAg) or by passive transfer of mAg-specific T cells. Because Th1 cell clones reactive to mAg are capable of inducing clinical and pathological manifestations of EAE in naive mice, it has long been believed that Th1 cells producing IFN-γ play a central role in the pathogenesis of EAE and MS. This postulate is also supported by the past experience that clinical application of IFN-γ treatment for MS turned out to worsen the disease (6). Furthermore, treatment with a peptide analogue of myelin basic protein (MBP) resulted in disease exacerbation along with an expansion of MBP-reactive Th1 cells (7). These results have been repeatedly mentioned to support the Th1-mediated pathogenesis of MS. However, this dogma has recently been challenged. Namely, despite an obvious reduction of Th1 cells, mice deficient for IFN-γ or IFN-γ receptor (8) or for IL-12 signaling were susceptible to EAE (9, 10). Subsequent studies have clarified that IL-23 rather than IL-12 is essential for EAE induction. Lately, the IL-23-dependent pathogenic T cells were identified as Th17 cells, a novel helper T cells producing IL-17 (11, 12). Currently, it is widely appreciated that Th17 cells are crucial in the development of autoimmune diseases either independently or collaboratively with Th1 cells (13).

DNA microarray analysis revealed an up-regulation of IL-17 in the brain lesions of MS (14). More recently, a pathological study has demonstrated that IL-17 secreting T cells are present in active lesions of MS (15). Gene expression profiling provided a number of potential candidate molecules that might be appropriate as a therapeutic target (14, 16). We recently characterized gene signature of peripheral blood T cells from Japanese MS patients and found that a nuclear orphan receptor NR4A2 is most significantly overexpressed in MS (17). NR4A2 mutations are reported to cause familial Parkinson's disease, reflecting its essential role in the development and survival of substantia nigra neurons (18). In contrast, much less attention has been paid onto its role in T cells. NR4A family members (NR4A1 and -3) were shown to mediate apoptotic processes of mature (19, 20) and immature T cells (21, 22). However, these studies do not give insights into an overexpressed NR4A2 in MS. Here, we report that NR4A2 is a transcription factor regulating the expression of key cytokines in the pathogenesis of MS, including IL-17. Furthermore, we revealed that silencing NR4A2 expression by specific siRNA effectively prevents the production of the cytokines, thereby inhibiting their pathogenic potentials to mediate EAE.

Results

Up-Regulation of NR4A2 in Peripheral Blood T Cells of MS. We analyzed gene expression profiles of peripheral blood T cells from MS and control subjects (17, 23). Comparison of the patients and healthy donors has revealed that 286 of 1,263 genes are differentially expressed between MS and controls. Among genes up-regulated in MS, NR4A2 was most significantly overexpressed in MS in statistical *P* values and an increase ratio (3.6-fold). To consolidate the overexpression of NR4A2 in MS, we performed quantitative RT-PCR for NR4A2 expression, using the same samples previously analyzed. Expression of NR4A2 in T cells from MS increased ≈5-fold on average compared with healthy donors (Fig. 1; $P < 0.01$).

T Cell Expression of NR4A2 in EAE. NR4A2 is a transcription factor of steroid/thyroid receptor family implicated in various cellular responses such as steroidogenesis, neuronal development, atherogenesis, and cell cycle regulation (24). However, its role in

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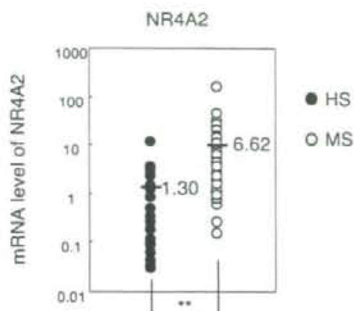

Fig. 1. Quantitative analysis of NR4A2 transcription between MS and controls. CD3+ T cells were isolated from PBMC of 57 MS patients and of 19 healthy donors, and total RNA was extracted. cDNA was synthesized and the expression levels of NR4A2 transcript were analyzed by quantitative RT-PCR. Each sample was normalized to GAPDH to adjust for variations. Open circles, MS patients; filled circles, healthy controls. Bars indicate mean values of each group. The statistical difference was determined by two-sided Student t test (**, $P < 0.01$).

T cell-mediated autoimmune diseases is unknown. Therefore, we explored the functional involvement of NR4A2 in EAE induced in C57BL/6 (B6) mice by immunization with MOG_{35-55}. $CD3^+$ T cells were isolated from SPL, dLN, and PBMC after EAE induction and the expression levels of NR4A2 gene were measured by quantitative RT-PCR (Fig. 2*a Right*). NR4A2 expression was detectable in PBMC-T cells on days 14, 21, and 28, showing a maximum value on day 21, which was well correlated with the clinical severity of EAE (Fig. 2*a Left*). NR4A2 expression in SPL-T cells and dLN-T cells was also correlated with the severity of EAE, but only marginally.

In the course of EAE, mAg-primed T cells would accumulate into the CNS and produce inflammatory cytokines, leading to the formation of inflammatory lesions (25). We next examined a kinetic change of NR4A2 in the T cells infiltrating into the CNS. As assessed by quantitative RT-PCR, remarkable expression of NR4A2 was observed in the CNS-T cells on day 9, when an early EAE sign became evident (Fig. 2*b*). The expression level decreased gradually thereafter, but was still significant until day 21. These results suggest that the CNS-T cells also express NR4A2, but the expression kinetics significantly differed from that of PBMC-T cells.

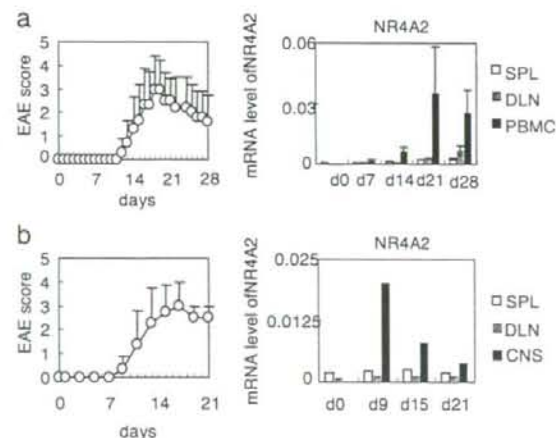

Fig. 2. Kinetic analysis of NR4A2 expression in the disease course of EAE. (*a*) (*Left*) EAE was induced in B6 mice by immunization with MOG_{35-55} in CFA. Mice were killed on days 7, 14, 21 and 28 after immunization, and T cells were isolated from dLN, SPL, or PBMC, using anti-CD3 magnetic beads. (*Right*) Total RNAs were isolated from the T cell populations, and the expression levels of NR4A2 were determined by quantitative RT-PCR. One representative data from three independent experiments is shown, and data are expressed as mean ± SEM (n = 5 for each). (*b*) EAE induced in B6 mice with MOG_{35-55}. Clinical scores were expressed as mean ± SEM (n = 4). Here, we determined NR4A2 expression in CD3+ T cells isolated by using EPICS ALTRA cell sorter. The lymphoid cells (SPL, dLN, and CNS) were pooled from four mice on days 0, 9, 15, and 21 and used for cell sorting and RT-PCR analysis. The purity of the CNS-derived CD3+ T cells was >93%.

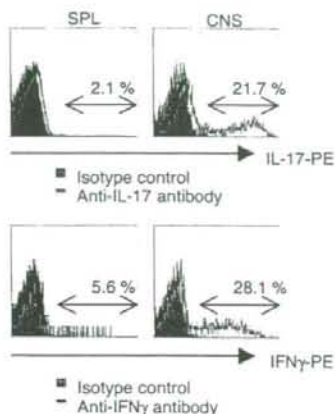

Fig. 3. Accumulation of IL-17 or IFN-γ-producing inflammatory T cells in the CNS. Mononuclear cells were isolated from spleen or CNS on day 17 after immunization and stimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) in the presence of 2 mM monensin for 4 h. Production of IL-17 and IFN-γ was analyzed for the gated CD4+ T cell population by intracellular cytokine staining. Black line represents samples stained with either anti-IL-17 or anti-IFN-γ Ab, and the filled histogram represents samples stained with isotype control. Given values show the percentage of cytokine producing-T cells present in each panel.

Accumulation of IL-17- and IFN-γ-Producing T Cells in the CNS of EAE. Th1 cells specific for mAg have long been thought to induce EAE through their production of IFN-γ. However, recent studies indicate that Th17 rather than Th1 cells may play a central role (13). To make this point clear in our experimental setting, we examined the ability of the CNS-T cells to produce IFN-γ and IL-17. Mononuclear cells were recovered from the CNS and SPL on day 17, and stimulated with PMA and ionomycin (P/I). After immunostaining, expression of IL-17 or IFN-γ in the $CD4^+$ T cells was analyzed by flow cytometry. Major proportions of the CNS-T cells were found to produce IL-17 (21.7% of the cells) or IFN-γ (28.1%) after stimulation (Fig. 3). In contrast, spleen cells contained a lower number of cells producing these cytokines.

Transcriptional Up-Regulation of IL-17 and IFN-γ After Introduction of NR4A2. The concomitant expression of inflammatory cytokines and NR4A2 has guided us to investigate whether NR4A2 directly affects cytokine gene expression as a transcription factor, using luciferase reporter plasmids containing the promoter fragment of IL-17, IFN-γ, or IL-2. NR4A2 gene transduction would result in a twofold augmentation of IL-17 promoter activity and, for IFN-γ, an even higher (5-fold) induction (Fig. 4*a*). A significant induction of IL-2 promoter activity was also noted. Intriguingly, an introduction of NR4A2 plasmid

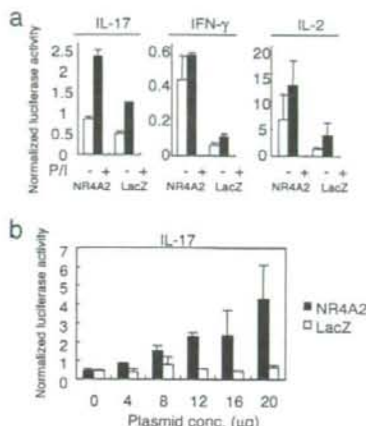

Fig. 4. Promoter activities of cytokine genes in the presence of NR4A2. (*a*) The effect of NR4A2 expression on IL-17, IFN-γ, and IL-2 promoter activity. A reporter plasmid containing promoter of cytokine gene (10 μg) and *Renilla* luciferase plasmid (100 ng) were introduced into EL4 cells by electroporation, together with pcDNA4-NR4A2 or pcDNA4-LacZ (10 μg). Cells were stimulated for 18 h with P/I. Luciferase activity was determined for each cell lysate after normalization to the *Renilla* luciferase activity. One representative data from three independent experiments is shown. Data are expressed as mean ± SD. (*b*) The effect of NR4A2 expression on basal promoter activity of IL-17 gene. EL4 cells transfected with pcDNA4-NR4A2 or pcDNA4-LacZ together with IL-17 reporter plasmid and *Renilla* luciferase plasmid as desribed in a were cultured for 18 h without stimulation. One representative data from three independent experiments is shown. Data are expressed as mean ± SD.

without P/I stimulation also augmented basal promoter activity of IL-17 genes in a dose dependent manner (Fig. 4*b*). Similarly, basal promoter activity of IFN-γ was promoted (data not shown).

Retroviral Transduction of NR4A2 Gene Enhances Expression of Inflammatory Cytokine in Primary T Cells. The results obtained in EL4 lymphoma cells need to be verified in more physiological settings. Next, we examined whether forced expression of NR4A2 may affect the expression of cytokines in primary rodent T cells. Bicistronic retroviral vector containing NR4A2 gene fragment (pMIG-NR4A2) or empty vector (pMIG) were used for production of retroviruses (Fig. 5*a*). We infected the B6 T cells with either of the retroviruses as described in ref. 26 and compared the cytokine production between GFP-positive (infected) and GFP-negative (uninfected) CD4⁺ T cells by intracellular cytokine staining (Fig. 5*b Top*). CD4⁺ T cells infected with pMIG-NR4A2-introduced retrovirus showed a twofold enhancement of IL-17 expression (8.4%) compared with those infected with control retrovirus (4.1%) after stimulation with P/I. In contrast, IL-17 production by uninfected T cells in either panel was almost equivalent (Fig. 5*b Middle*). Furthermore, one-third of the CD4⁺ T cells infected with pMIG-NR4A2-introduced retrovirus showed a massive IFN-γ expression (35.1%) compared with control retrovirus (14.1%) (Fig. 5*b Bottom*).

Silencing of NR4A2 Gene Expression Results in a Reduced Production of IL-17 and IFN-γ. Reporter gene analysis and retroviral transduction experiments demonstrated that T cell production of IL-17 and IFN-γ is controlled by NR4A2 (Figs. 4 and 5). We further explored whether silencing of NR4A2 gene may affect the production of inflammatory cytokines by CD4⁺ T cells. An NR4A2-specific siRNA was selected from three siRNAs based on the inhibitory efficacy. The targeting sequence of the NR4A2

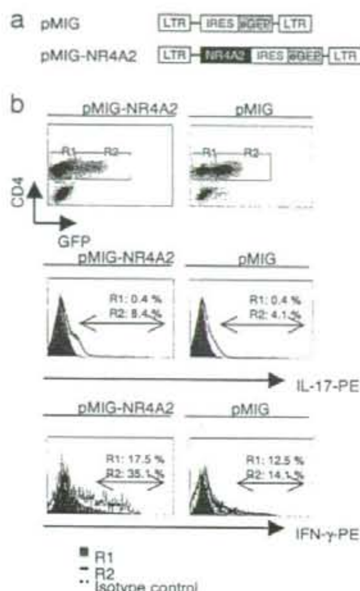

Fig. 5. The effect of retrovirally transduced NR4A2 on cytokine production by primary murine CD4⁺ T cells. (*a*) DNA fragments encoding wild-type NR4A2 were cloned into the pMIG(W) bicistronic retroviral vector. LTR, long terminal repeat; IRES, internal ribosome entry site; eGFP, enhanced green fluorescence protein b. (*b*) Splenic CD4⁺ T cells were infected with retrovirus encoding NR4A2 or control retrovirus, and CD4⁺ GFP⁻ T cells and CD4⁺ GFP⁺ T cells were gated as R1 and R2, respectively. Forced expression of NR4A2 increased the number of CD4⁺ T cells producing IL-17 or IFN-γ. The histogram shows intracellular cytokine staining on the gated cells (R1 or R2). Black line represents cells in R2 gate (GFP+) stained with either anti-IL-17 or anti-IFN-γ Ab, and the filled histogram represents cells in R1 gate (GFP−) stained with isotype control. Given values show the percentage of cytokine producing-T cells present.

siRNA is completely conserved between mice and human. Therefore, we could apply it to human T cells and study whether NR4A2 could be a therapeutic target in human MS. In a preparatory experiment, using FITC-labeled siRNA, the transfection efficiency was found to be 95%. We purified CD4⁺ T cells from human PBMC and transfected them with the NR4A2 siRNA or control RNA, using nucleofector II. The cells were stimulated with immobilized anti-CD3 Ab. As shown in Fig. 6*a*, silencing NR4A2 gene expression resulted in a 50% reduction of IL-17 and IFN-γ production. However, production of TNF-α, IL-4, or IL-5 was not changed significantly after siRNA treatment (Fig. 6*b*). Intriguingly, the siRNA treatment also induced a modest reduction of IL-10 production. The molecular mechanism of this inhibition is not clarified yet. Because silencing of NR4A2 expression rather selectively inhibited the expression of inflammatory cytokines, it is arguable that NR4A2 may be a good target for therapeutic intervention of MS. In this line, we next examined whether the NR4A2 siRNA is effective for inhibiting a production of inflammatory cytokines in MS. For this aim, CD4⁺ T cells were isolated from pairs of an MS patient and an age- and sex-matched healthy donor and were stimulated with anti-CD3 Ab after being transfected with the NR4A2 siRNA or control RNA. We found that the siRNA treatment significantly reduced the production of IL-17 and IFN-γ by T cells from MS or healthy donors [supporting information (SI) Fig. S1]. Again we observed some reduction of IL-10 after siRNA treatment. However, the siRNA showed little effect on

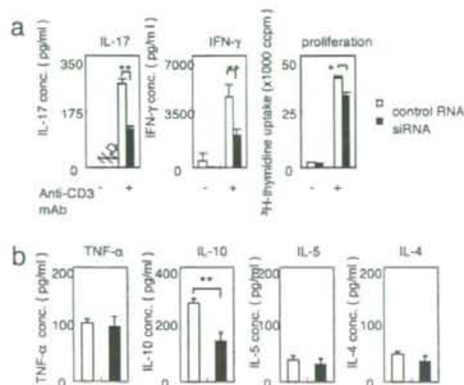

Fig. 6. The effect of NR4A2 gene silencing on T cell cytokine production. (*a*) Specific inhibition of T cell production of IL-17 and IFN-γ by siRNA treatment. Human CD4⁺ T cells derived from PBMC were transfected with siRNA or control RNA and stimulated by immobilized anti-CD3 Ab for 48 h. Cytokine levels in the culture supernatant were determined by ELISA or a CBA human Th1/2 cytokine kit. Proliferation rate was measured by ³H-TdR uptake. (*b*) Effect of siRNA treatment for T cell production of TNF-α, IL-10, IL-5, and IL-4 after stimulation with immobilized anti-CD3 Ab. The data are expressed as mean ± SD (*, $P < 0.05$; **, $P < 0.01$; Mann–Whitney *U* test).

production of TNF-α, IL-5, and IL-4 from T cells used for assays (Table S1).

Amelioration of EAE by Silencing of NR4A2. Finally, we investigated the therapeutic implication of the siRNA experiments in a model of passively induced EAE, induced by adoptive transfer of mAg-activated LN cells. We prepared lymphoid cells from dLN of SJL/J mice 10 days after immunization with $PLP_{139-151}$. The dLN cells were transfected with the NR4A2 siRNA or control RNA and stimulated with $PLP_{139-151}$ *in vitro*. Three days later, the cultured cells enriched in lymphoblasts were transferred to irradiated naive SJL/J mice. In addition to evaluating clinical manifestations, histology was assessed by hematoxylin-eosin (HE) and luxol fast blue (LFB) staining of paraffin-embedded spinal cord sections. Notably, severity of clinical (Fig. 7*a*) and histological EAE on day 31 (Fig. 7*b*) was significantly prevented in siRNA-treated group compared with control RNA-treated group (Fig. 7*b*). These results suggest that modulation of NR4A2 expression by specific siRNAs or other chemical compounds might be a promising treatment for active MS that are harboring potent encephalitogenic T cells.

Discussion

Although mAg-specific T cell clones isolated from the peripheral blood has been widely used to gain insights into the pathogenesis of MS (27), analysis of polyclonal T cells has been undervalued for a long time. However, it was recently demonstrated that peripheral T cells from MS and healthy subjects significantly differ in surface phenotype or gene expression profiling (17, 23, 28). Using cDNA microarray, we have identified NR4A2 as a gene most significantly up-regulated in the peripheral T cells of MS (17). We conducted the present study to clarify the implication of this interesting observation. Inspired by the recent discovery that retinoid-related orphan receptor γt (RORγt) is essential for Th17 cell differentiation (29) and that retinoic acids play a regulatory role in Th17 cell differentiation (30), we have focused our efforts to explore the possible role of NR4A2 in cytokine regulation. Reporter gene analysis and retroviral transduction of NR4A2 clearly demonstrated that T cell production

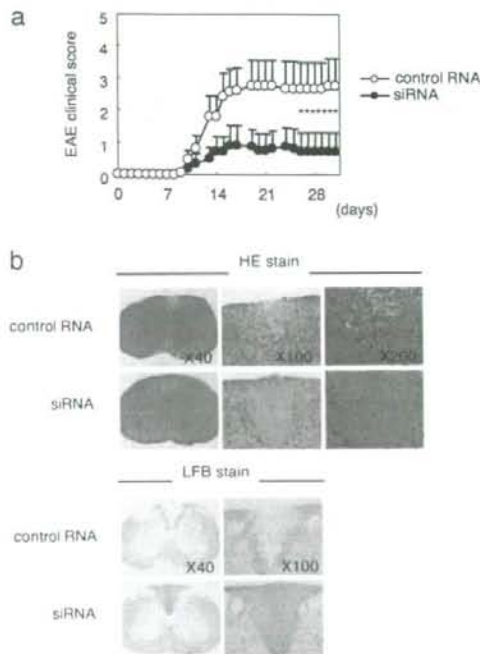

Fig. 7. The effect of T cell silencing of NR4A2 expression on passive EAE. (*a*) Inguinal and popliteal LNs cells were collected from female SJL/J mice 10 days after immunization with $PLP_{139-151}$, and were transfected with siRNA for NR4A2 or control RNA, using HVJ-E vector kit. The cells were cultured in complete media for 8 h. Then the media were replaced with fresh complete media containing 35 μg/ml $PLP_{139-151}$, and the cells were stimulated for another 3 days. After expansion, cells were harvested and transferred i.p. (5×10^6 cells per mouse) into 3Gy-irradiated naive SJL/J mice ($n = 10$) followed by i.p. injection of PT. Mean ± SEM clinical scores were indicated. (*, $P < 0.05$ by Mann–Whitney *U* test.) (*b*) Histological analysis of spinal cords removed on day 31 after adoptive transfer of $PLP_{139-151}$-reactive T cells. Sections obtained from cervical cord regions were stained with HE or LFB. Infiltration of mononuclear cells and demyelination of the cervical cord regions were analyzed for mice injected with $PLP_{139-151}$-reactive T cells pretreated with control RNA or siRNA for NR4A2.

of inflammatory cytokines, including IL-17 and IFN-γ, is regulated by NR4A2, whereas silencing of NR4A2 by a specific siRNA prevents expression of these cytokines. Furthermore, treatment with the siRNA reduced the ability of pathogenic T cells to adoptively transfer EAE. These results have identified a previously uncharacterized role for NR4A2 in the regulation of T cell production of inflammatory cytokines.

NR4A2 is a member of the orphan nuclear NR4A subfamily that consists of NR4A1 (also referred to as Nur77), NR4A2 (Nurr1), and NR4A3 (NOR-1) (24). The NR4A members share a highly conserved zing finger DNA binding domain and a less conserved putative ligand-binding domain. All these members bind to the DNA sequence NBRE (AAAGGTCA) or NurRE to activate target gene expression. NR4A1 and NR4A2 can also heterodimerize with retinoic X receptor (RXR) and activate gene expression through DR5 (24). They exert pleiotropic functions and are classified as immediate early genes induced by physiological and physical stimuli. Studies of gene-targeted mice have shown that NR4A1 and NR4A3 play a critical role in T cell apoptosis during the thymocyte development (20–22, 31). In contrast, developing thymocytes in NR4A2 deficient mice ap-

pear to be normal (21, 32), which distinguishes NR4A2 from other NR4A members.

Involvement of orphan nuclear receptor in T cell differentiation has recently attracted broad attention, because RORγt, a splice variant of RORγ, was found to play an essential role in the development of Th17 cells (29). RORγ/RORγt were reported to play an essential function in survival of $CD4^+CD8^+$ thymocytes (33, 34) and in the generation of fetal lymphoid tissue inducer (LTi) cells (35). It is particularly intriguing that the consensus binding sequence for RORγ [$(A/T)_5$AGGTCA] overlaps with that for NR4A (NBRE; AAAGGTCA), which has encouraged us to explore the functional role of NR4A2 in the production of IL-17 and IFN-γ. Although the molecular mechanism of cytokine production through the induced expression of NR4A2 is not clear yet, NR4A2 and RORγt may have an overlapping role in regulating the development and effector functions of Th17 cells.

NR4A2 expression in the CNS-infiltrating T cells showed a peak value at a very early phase of EAE (day 9–12) (Fig. 2*b*). We speculate that this probably coincides with the entry of encephalitogenic cells into the CNS (2, 3). Consistently, a similar kinetic change was found in expression of T-bet and RORγt in the CNS-T cells (data not shown). In contrast, up-regulation of NR4A2 in peripheral blood T cells was significantly delayed. This is likely to result from a late activation of peripheral T cells after peripheral recruitment of antigen presenting cells engulfing myelin and/or peripheral dispersion of myelin protein or its fragments.

By applying a specific siRNA, we showed that blocking NR4A2 expression is effective for inhibiting production of IL-17 and IFN-γ from T cells from healthy donors and MS patients. Therapeutic implication was further demonstrated by using an adoptive transfer EAE model. Because Th17 cells were identified as a major player in autoimmunity (12, 15), it is sometimes argued that Th17 cells would be a sole potent inducer of autoimmune inflammation. However, T-bet-deficient mice and Stat4-deficient mice that obviously lack Th1 cells would resist against induction of EAE, although they maintain a large number of Th17 cells (36, 37). This suggests that both Th1 and Th17 cells are required for induction of full-blown EAE (38). In this context, the ability of the NR4A2 siRNA to inhibit production of both IL-17 and IFN-γ suggests the advantage of NR4A2 targeting therapy in controlling autoimmune inflammation.

Materials and Methods

EAE Induction. Active EAE was induced with myelin oligodendrocyte glycoprotein (MOG) amino acids 35–55 (MOG_{35-55}; MEVGWYRSPFSRVVHLYRNGK) as described in ref. 39. Female B6 mice were immunized s.c. with 100 μg of MOG_{35-55} mixed with 1 mg of heat-killed *Mycobacterium tuberculosis* H37RA emulsified in Freund's adjuvant (CFA). Pertussis toxin (PT) (200 ng) was injected i.p. on days 0 and 2 after immunization. Clinical signs were scored daily as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, flaccid tail; 3, partial hind limb paralysis; 4, total hind limb paralysis; and 5, fore and hind limb paralysis.

Quantitative RT-PCR. DNase-treated total RNAs were processed for cDNA synthesis, using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen). cDNAs were amplified by PCR on Light Cycler ST300 (Roche Diagnostics) by using a Light Cycler-FastStart DNA Master SYBR Green I kit (Roche). Values for each gene were normalized to those of a housekeeping gene GAPDH to adjust for variations between different samples. Forward primer for amplifying human NR4A2 gene was 5′-CGACATTTCTGCCTTCTCC-3′ and reverse primer 5′-GGTAAAGTGTCCAGGAAAAG-3′. Mouse NR4A2 forward primer was designed as 5′-GCATACAGGTCCAACCCAGT-3′ and reverse primer 5′-AATGCAGGAGAAGGCAGAAA-3′. To evaluate silencing efficacy of *NR4A2*-specific siRNAs, expression of NR4A2 gene was quantified by RT-PCR, using the primers to flank the siRNA target sequence (forward, 5′-TGCCACCACTTCTCTCCCCA-3′; reverse, 5′-GCGGCATCATCTCCTCAGAC-3′).

Luciferase Assays. Ten million of EL4 thymoma cells suspended in 500 μl of cold PBS and transfected with 4–20 μg of pcDNA4-NR4A2 or pcDNA4-LacZ in the presence of 10 μg of reporter plasmid, 100 ng of Renilla luciferase plasmid, and 5 μg of DEAE-DEXTRAN (Sigma) by electroporation (250 V, 975 μF, time constant = 30–34 ms) with a GenePulser electroporator II (Bio-Rad). Six hours later, cells were stimulated with 20 ng/ml PMA and 1 μg/ml ionomycin for 24 h, followed by analysis for luciferase activity. The data were normalized for internal controls of Renilla luciferase activity.

Retroviral Infection. Mouse $CD4^+$ T cells purified by AutoMACS using mouse CD4 T isolation kit (Miltenyi Biotec) were stimulated with immobilized anti-CD3 Ab and soluble anti-CD28 Ab in complete medium supplemented with IL-2 (100 units/ml) for 24–48 h before infection. The primed $CD4^+$ T cells were infected twice with retroviruses produced by 293T cells cotransfected with pMIG retroviral vector and pCL-Eco packaging vector. The T cells were cultured in the presence of 30 units/ml of IL-2 for 3 days and were then subjected to intracellular cytokine staining.

Silencing Effects of NR4A2 siRNA on Passive EAE. To evaluate an effect of NR4A2 siRNA, an adoptive transfer EAE model in SJL/J mice was applied, because consistent disease could be induced relatively easily. Female SJL/J mice (8–12 weeks old) (Charles River Laboratories) were immunized s.c. with 100 μg of proteolipid protein (PLP) amino acids 139–151 ($PLP_{139-151}$; HSLGKWLGHPDKF) and 1 mg of heat-killed *M. tuberculosis* H37RA in CFA. Inguinal and popliteal LNs harvested 10 days after immunization were transfected with siRNAs, using hemaggultinating Virus of Japan envelope (HVJ-E) vector kit (GENOMEONE; Ishihara Sangyo). Eight hours later, the cells were stimulated with $PLP_{139-151}$ peptide (35 μg/ml). After 3 days, collected cells were injected i.p. (5×10^6 cells per body) into irradiated mice (3 Gy/body) with intrapelitoneal injection of PT. For conventional histological analysis of EAE, paraffin-embedded spinal cords were stained with either HE or LFB.

Statistics. For statistical analysis, a nonparametric Mann–Whitney *U* test or Student *t* test was used. $P < 0.05$ was considered statistically significant.

Supporting Information. For further details, see *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Mayumi Fujita for EAE induction, Miho Mizuno, Chiharu Tomi, and Yuki Kikai for excellent technical assistance. This work was supported by grants from the Ministry of Health, Labour and Welfare of Japan.

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Differential Expression of CD11c by Peripheral Blood NK Cells Reflects Temporal Activity of Multiple Sclerosis[1]

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Multiple sclerosis (MS) is an autoimmune disease, showing a great degree of variance in temporal disease activity. We have recently demonstrated that peripheral blood NK cells biased for secreting IL-5 (NK2 bias) are associated with the remission state of MS. In this study, we report that MS patients in remission differentially express CD11c on NK cell surface (operationally defined as CD11c^{high} or CD11c^{low}). When we compared CD11c^{high} or CD11c^{low} patients, the expression of IL-5 and GATA-3 in NK cells supposed to endow a disease-protective NK2 phenotype was observed in CD11c^{low} but not in CD11c^{high} patients. In contrast, the CD11c^{high} group showed a higher expression of HLA-DR on NK cells. In vitro studies demonstrated that NK cell stimulatory cytokines such as IL-15 would up-regulate CD11c expression on NK cells. Given previous evidence showing an association between an increased level of proinflammatory cytokines and temporal disease activity in MS, we postulate that inflammatory signals may play a role in inducing the CD11c^{high} NK cell phenotype. Follow-up of a new cohort of patients showed that 6 of 10 CD11c^{high} MS patients developed a clinical relapse within 120 days after evaluation, whereas only 2 of 13 CD11c^{low} developed exacerbated disease ($p = 0.003$). As such, a higher expression of CD11c on NK cells may reflect the temporal activity of MS as well as a loss of regulatory NK2 phenotype, which may allow us to use it as a potential biomarker to monitor the immunological status of MS patients. ***The Journal of Immunology*, 2006, 177: 5659–5667.**

Multiple sclerosis (MS)[3] is a chronic inflammatory disease of the CNS, in which autoreactive T cells targeting CNS Ags are presumed to play a pathogenic role (1). A large majority of the patients with MS (~70%), known as relapsing-remitting MS, would develop acute exacerbations of disease between intervals of remission. It is currently believed that relapses are caused by T cell- and Ab-mediated inflammatory reactions to the self-CNS components, and could be controlled at least to some degree by anti-inflammatory therapeutics, immunosuppressants, or plasma exchange.

The clinical course of MS varies greatly among individuals, implicating difficulties to predict the future of each patient. For example, patients who had been clinically inactive in the early stage of illness could abruptly change into active MS accompanying frequent relapses and progressive worsening of neurological conditions. There are a number of unpredictable matters in MS, including an interval between relapses, responsiveness to remedy and the prognosis in terms of neurological disability. To provide better quality of management of the patients, searches of appropriate biomarkers are currently being warranted (2).

We have recently shown that surface phenotype and cytokine secretion pattern of peripheral blood NK cells may reflect the disease activity of MS (3, 4). A combination of quantitative PCR and flow cytometry analysis has revealed that NK cells in clinical remission of MS are characterized by a higher frequency of CD95$^+$ cells as well as a higher expression level of IL-5 than those of healthy subjects (HS) (3). As IL-5-producing NK cells, referred to as NK2 cells (5), could prohibit Th1 cell activation in vitro (3), we interpreted that the NK2 bias in MS may contribute to maintaining the remission state of MS. More recently, we have found that MS patients in remission can be further divided into CD95^{high} and CD95^{low}, according to the frequency of CD95$^+$ cells among NK cells (4). Notably, memory T cells reactive to myelin basic protein, a major target Ag in MS, were increased in CD95^{high} patients, compared with CD95^{low}. Of note, CD95^{high} NK cells exhibited an ability to actively suppress the autoimmune T cells, whereas those from CD95^{low} patients did not. These results suggest that NK cells may accommodate their function and phenotype to properly counterregulate autoimmune T cells in the remission state of MS.

Recently, a distinct population of NK cells that express CD11c, a prototypical dendritic cell (DC) marker, was identified in mice (6, 7). As the CD11c$^+$ NK cells exhibited both NK and DC functions, they are called as "bitypic NK/DC cells." CD11c associates with integrin CD18 to form CD11c/CD18 complex and is expressed on monocytes, granulocytes, DCs, and a subset of NK cells. Although precise functions are unclear, it has been reported that CD11c is involved in binding of iC3b (8), adhesion to stimulated endothelium (9) or phagocytosis of apoptotic cells (10). The initial purpose of this study was to evaluate CD11c expression and function of CD11c$^+$ NK cells in MS in the line of our research to characterize NK cells in MS. On initiating study, we noticed that there was no significant difference between MS and HS in the frequency of CD11c$^+$ NK cells. However, expression levels of CD11c were significantly higher in MS. We further noticed that up-regulation of CD11c is seen in some, but not all, patients with MS. So we have operationally classified MS into CD11c^{low} and CD11c^{high}.

In this study, we demonstrate that IL-5, characteristic of NK2 cells (5), were significantly down-regulated in CD11c^{high} than

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[3] Abbreviations used in this paper: MS, multiple sclerosis; HS, healthy subject; DC, dendritic cell; MFI, mean fluorescence intensity; ECD, energy-coupled dye.

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CD11c^{low} NK cells. In contrast, expression of HLA-DR class II molecule was up-regulated in CD11c^{high} NK cells. Notably, both CD11c and HLA-DR on NK cells were reproducibly induced in vitro in the presence of IL-15 (11) or combination of inflammatory cytokines, known to be increased in the blood of MS (12–14). Furthermore, we found that the remission state of CD11c^{high} is unstable in comparison to CD11c^{low}, as judged by an increased number of the patients who exacerbated during the 120 days after examining NK cell phenotypes. These results suggest that the CD11c^{high} group of patients may be in more unstable condition than CD11c^{low}, presenting with reduced regulatory functions of NK cells.

Materials and Methods

Subjects

Twenty-five patients with relapsing-remitting MS (15) (male (M)/female (F) = 8/17; age = 37.7 ± 11.1 (year old)) and 10 sex- and age-matched HS (M/F = 3/7; age = 39.9 ± 12.2 (year old)) were enrolled for studying NK cell phenotypes. All the patients were in the state of remission at examination as judged by magnetic resonance imaging scanning and clinical assessment. They had not been given immunosuppressive medications, or corticosteroid for at least 1 mo before examination. They had relatively mild neurological disability (expanded disability status scale <4) and could walk to the hospital without any assistance during remission. The same neurologist followed up the patients regularly (every 3–4 wk) and judged the occurrence of relapse by using magnetic resonance imaging and clinical examinations. Information on NK cell phenotype or other immunological parameters was never given to either the neurologist or the patients at the time of evaluation. To precisely determine the onset of relapse, patients were allowed to take examination within a few days after a new symptom appeared. Written informed consent was obtained from all the patients and the Ethics Committee of the National Center of Neuroscience (NCNP) approved the study.

Reagents

Mouse IgG1 isotype control-PE, anti-CD3-energy-coupled dye (ECD), anti-CD4-PE, anti-CD8-PC5, anti-CD56-PC5, anti-CD69-PE, and anti-HLA-DR-FITC mAbs were purchased from Immunotech. Anti-CD11c-PE and anti-CD95-FITC were purchased from BD Pharmingen. Recombinant human cytokines were purchased from PeproTech. AIM-V (Invitrogen Life Technologies) was used for cell culture after supplementing 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen Life Technologies).

Cell preparation and NK cell purification

PBMC were separated by density gradient centrifugation with Ficoll-Hypaque PLUS (Amersham Biosciences). To purify NK cells, PBMC were treated with NK isolation kit II (Miltenyi Biotec) twice, according to the manufacturer's protocol. Briefly, PBMC were labeled with a mixture of biotin-conjugated mAbs reactive to non-NK cells and magnetic microbead-conjugated anti-biotin mAbs. The magnetically labeled non-NK cells were depleted with auto-MACS (Miltenyi Biotec) and this procedure always yielded >95% purity of NK cells when assessed by the proportions of CD3^{-}CD56^{+} cells with flow cytometry.

Flow cytometry

To evaluate the expression of CD11c, CD95, or other surface molecules on NK cells, PBMC were stained with anti-CD3-ECD, anti-CD56-PC5, and FITC- or PE-conjugated mAbs against molecules of our interest and were analyzed with EPICS flow cytometry (Beckman Coulter). Mean fluorescence intensity (MFI) of CD11c was measured on gated CD11c^{+} fraction or whole NK cells.

Stimulation of purified NK cells with proinflammatory cytokines

Purified NK cells (1×10^5/well) were stimulated in the presence or absence of IL-4, IL-8, IL-12, IL-15, IL-18, IL-23, TNF-α, and GM-CSF or combination of IL-12, IL-15, and IL-18 for 3 days. We analyzed CD11c expression after staining the cells with anti-CD11c-PE, anti-CD3-ECD, and anti-CD56-PC5. The concentration of IL-12 was at 10 ng/ml, and those of the other cytokines were at 100 ng/ml.

RT-PCR

Total RNA were extracted with a RNeasy Mini kit (Qiagen) from purified NK cells, and the cDNA were synthesized with Super Script III first strand systems (Invitrogen Life Technologies) according to the manufacturer's protocol. For quantitative analysis of IL-5, IFN-γ, GATA-3, and T-bet, the LightCycler quantitative PCR system (Roche Diagnostics) was used. Relative quantities of mRNA were evaluated after normalizing each expression levels with β-actin expression. PCR primers used were as follows: β-actin-sense, AGAGATGGCCACGGCTGCTT, and -antisense, ATTTGCGGTGGACGATGGAG; IFN-γ-sense, CAGGTCATTCAGATGTAGCG, and -antisense, GCTTTTCGAAGTCATCTCG; IL-5-sense, GCACACTGGAGAGTCAAACT, and -antisense, CACTCGGTGTTCATTACACC; GATA-3-sense, CTACGGAAACTCGGTCAGG, and -antisense, CTGGTACTTGAGGCACTCTT; T-bet-sense, GGAGGACACCGACTAATTTGGGA, and -antisense, AAGCAAGACGCAGCACCAGGTAA.

Statistical analysis of remission rate

We set the first episode of relapse after blood sampling as an end point, although we followed clinical course of each patient for up to 120 days, regardless of whether they developed relapses. No patients developed second relapse during the 120 days. When the neurologist prescribed corticosteroids without knowing any information on the NK cell phenotype, the patient was considered as the dropout at that time point. Remission rate was calculated as Kaplan-Meier survival rate, and statistical difference between CD11c^{low} and CD11c^{high} MS was evaluated with the log-rank test.

Results

CD11c on NK cells is up-regulated in MS remission

First, we confirmed that PBMC from healthy individuals and MS contain CD11c^{+} NK cells (Fig. 1), which constitute a major population of whole NK cells. We then noticed that proportion of CD11c^{+} NK cells as well as its levels of expression greatly varied among individuals, particularly in MS. To examine this issue further, we systemically examined 25 MS patients in remission and 10 HS for NK cell expression of CD11c. Whereas 20–80% of NK cells are CD11c^{+} in HS (Fig. 1*c*), almost all NK cells were CD11c^{+} in some MS patients (Fig. 1, *c* and *e*). However, reflecting a great degree of variance, comparison between HS and MS did not reveal a significant difference (Fig. 1*c*). In contrast, when we measured the MFI of CD11c expression on CD11c^{+} NK cells, it was significantly higher in MS as compared with HS (Fig. 1*a*). This difference was also noticed when MFI of CD11c was measured for all the NK cell populations (Fig. 1*b*). It was interesting to know whether the levels of CD11c expression may correlate with NK cell functions. Therefore, we operationally divided the MS patients into CD11c^{low} and CD11c^{high} subgroups (Fig. 1*a*), by setting the border as (the average + 2 × SD) of the values for HS.

CD11c^{high} NK cells express HLA-DR more brightly than CD11c^{low} NK cells

It was previously reported that infection with certain viruses would accompany up-regulation of CD11c on NK cells (16). This raises a possibility that the increased expression of CD11c in CD11c^{high} MS may reflect an activation state of NK cells caused by some sort of stimuli. To verify this hypothesis, we examined surface expression of cell activation markers (CD69 and HLA-DR). Although CD69, an early activation marker, was not detectable on NK cells (Fig. 2*a*), NK cells from MS, particularly CD11c^{high} MS, significantly overexpressed HLA-DR on surface (Fig. 2). Interestingly, HLA-DR expression was also up-regulated on CD4^{+} T cells from CD11c^{high} MS compared with those from HS (data not shown). These results indicate that NK cells and T cells are differentially activated in CD11c^{high} MS, CD11c^{low} MS, and HS.

Absence of NK2 bias in CD11c^{high} MS

We have previously reported that a higher level of IL-5 expression (NK2 bias) is one of the characteristics of NK cells of MS in