with the expansion of allergen-specific Foxp3$^+$ Treg cells, suggesting that the suppressive effect was mediated by Foxp3$^+$ Treg cells. Moreover the protective effect of H3N1 infection could be replicated by treating suckling mice with NKT cell–activating glycolipids from *H. pylori* or with α-C-GalCer. These studies are particularly important not only because they characterize an NKT cell population that suppresses AHR, but also because they provide a plausible mechanism for the hygiene hypothesis and for epidemiological studies indicating that infection with respiratory viruses (9) and *H. pylori* (2, 3) protect against the development of asthma.

NKT cells comprise a small subset of T lymphocytes that share characteristics with NK cells and conventional T cells, with potent functions in modulating immunity that have only recently become appreciated (33). NKT cells express a relatively unique transcription factor, PLZF, specific for NKT cells (34) and other innate or activated T cells (35), and an invariant TCR, Vα14Jα18 in mice and Vα24 in humans, and are restricted by the MHC class I-like molecule, CD1d. The conservation of this invariant TCR across many mammalian species suggests that it is a pattern recognition receptor, and that NKT cells play an important role in innate immunity. Activation of NKT cells through this invariant TCR results in the rapid production of large amounts of cytokines, including IL-4 and IFN-γ, particularly from mature NKT cells found in adult mice and humans. In contrast, NKT cells in neonates or in cord blood are immature, and produce only small amounts of cytokines (36, 37). Nevertheless, the ability of mature NKT cells to rapidly produce very large quantities of cytokines endows that NKT cell with the capacity to play very important regulatory roles in autoimmunity, cancer, asthma, and infectious diseases (38).

NKT cells participate in immune responses to a growing list of infectious microorganisms, driven either by direct TCR recognition of specific glycolipids expressed by microorganisms, as in the case of *Borrelia burgdorferI* (39) and *Sphingomonas paucimobilis* (32, 40), or by indirect responses to cytokines released by activated DCs, as in the case of *Salmonella typhimurium* (41), *E. coli*, *Staphylococcus aureus*, *Listeria monocytogenes* (42), and *Mycobacteria tuberculosis* (43, 44). During influenza A infection in adult mice, NKT cells abolished the suppressive activity of influenza A–induced myeloid-derived suppressor cells, thereby enhancing survival (18). Our current studies also suggest that NKT cells may respond during infection with influenza A, and to glycolipids (PI57) produced by *H. pylori*, resulting in inhibitory effects on immunity, though primarily in young mice. The capacity of *H. pylori* glycolipids to activate a regulatory NKT cell subset (but only in young mice) may also explain the protective effects of *H. pylori* infection in neonatal but not older mice against gastritis and malignant metaplasia (45) as well as the observation that only WT, and not cholesterol-α-glucosyltransferase–deficient, *H. pylori* can infect the gastric mucosa of mice (28), given that cholesterol-α-glucosyltransferase is required for synthesis of PI57 (46). Finally, we would like to point out that the structure and function of PI57 is unique, since it includes a cholesterol-containing tail distinct from previously described NKT cell ligands, and since it represents the first demonstration of cholesterol as a target for TCR recognition.

NKT cells thus react to a diverse group of pathogens by functioning as an innate immune cell that can sense and rapidly respond to the presence of infectious agents. The capacity to respond to such pathogens, however, may be limited in neonates and young children due to limited numbers and to the immaturity of NKT cells (36, 37). On the other hand, the immaturity of NKT cells in young children may provide an opportunity for infection and therapeutic intervention to influence the subset composition of NKT cells, thereby preventing the development of asthma and allergy.

In asthma, NKT cells have been suggested to play a very important pathogenic role (20, 47). This idea has become controversial, since some patients, particularly those with mild or well-controlled asthma, have few detectable pulmonary NKT cells, although patients with severe or poorly controlled asthma have a significant increase in pulmonary NKT cells (19, 48, 49). Nevertheless, in many distinct mouse models of asthma, the presence of specific NKT cell subsets was required for the development of AHR. For example, CD4$^+$IL-17RB$^+$ NKT cells are required in allergen-induced AHR (19, 20, 50, 51); in ozone-induced AHR, an NK1.1$^-$ IL-17–producing subset is required (21); and in Sendai virus–induced AHR, a CD4$^+$ NKT cell population that interacts with alternatively activated alveolar macrophages is required (22). While previous studies have suggested that some (DN) NKT cells could not induce AHR (50), we now show for the first time that a population of NKT cells, enriched for a DN, T-bet–dependent, and IFN-γ–producing subset, has a potent regulatory role, suppressing the development of AHR. Although previous studies have suggested an inhibitory role for NKT cells in asthma, since adoptive transfer of NKT cells acutely activated with α-GalCer (1 hour prior to transfer) inhibit the development of experimental asthma in a C57BL/6 mouse model (52), we believe that our current studies are quite distinct. We showed that H3N1 infection in suckling mice expanded a population of NKT cells that, when examined 42 days after infection, specifically suppressed allergen-induced AHR without the need for acute activation with exogenous glycolipids.

While H3N1 infection affects many different cell types, the fact that the protective effect of H3N1 infection could be transferred with purified NKT cells, and the fact that the protective effect could be replicated by treatment of suckling mice with α-C-GalCer or a glycolipid from *H. pylori* (PI57) that specifically activated NKT cells in a CD1d-restricted fashion, strongly suggests that the protective effect of H3N1 infection in young mice was primarily mediated by a subset of NKT cells. The NKT cell subset activated by PI57 in suckling mice appeared to be a subset of invariant NKT cells, since DN NKT cells in suckling mice expanded after treatment with PI57, and since CD1d tetramers loaded with PI57 could stain NKT cells. The precise mechanism by which the DN NKT cells suppressed AHR is not clear, but may involve the preferential production of IFN-γ but not IL-4, since DN NKT cells from H3N1-infected suckling *Tbet*$^{-/-}$ mice failed to inhibit AHR. A role for IFN-γ is also supported by our observation that treatment of suckling mice with α-C-GalCer, which preferentially induces IFN-γ (26), also prevented the development of OVA-induced AHR 42 days later, whereas treatment with α-GalCer or with *Sphingomonas* glycolipid (PBS30) did not.

The "regulatory" NKT cells mediating the inhibitory effect of H3N1 and of PI57 and α-C-GalCer may be similar to previously described DN NKT cells that protected against the development of type I diabetes in humans and in mice (53, 54), to IFN-γ–producing NKT cells that were required for allograft tolerance (55), or to IL-4–producing NKT cells that induced Treg cells in the prevention of graft versus host disease (56–58). In our experiments, increased numbers of both natural and adaptive OVA-specific Treg cells were associated with the regulatory NKT cells and were blocked by treatment with anti-CD25 mAb (Figure 4, F and G). Moreover, we believe that our studies are the first to demonstrate the existence of a

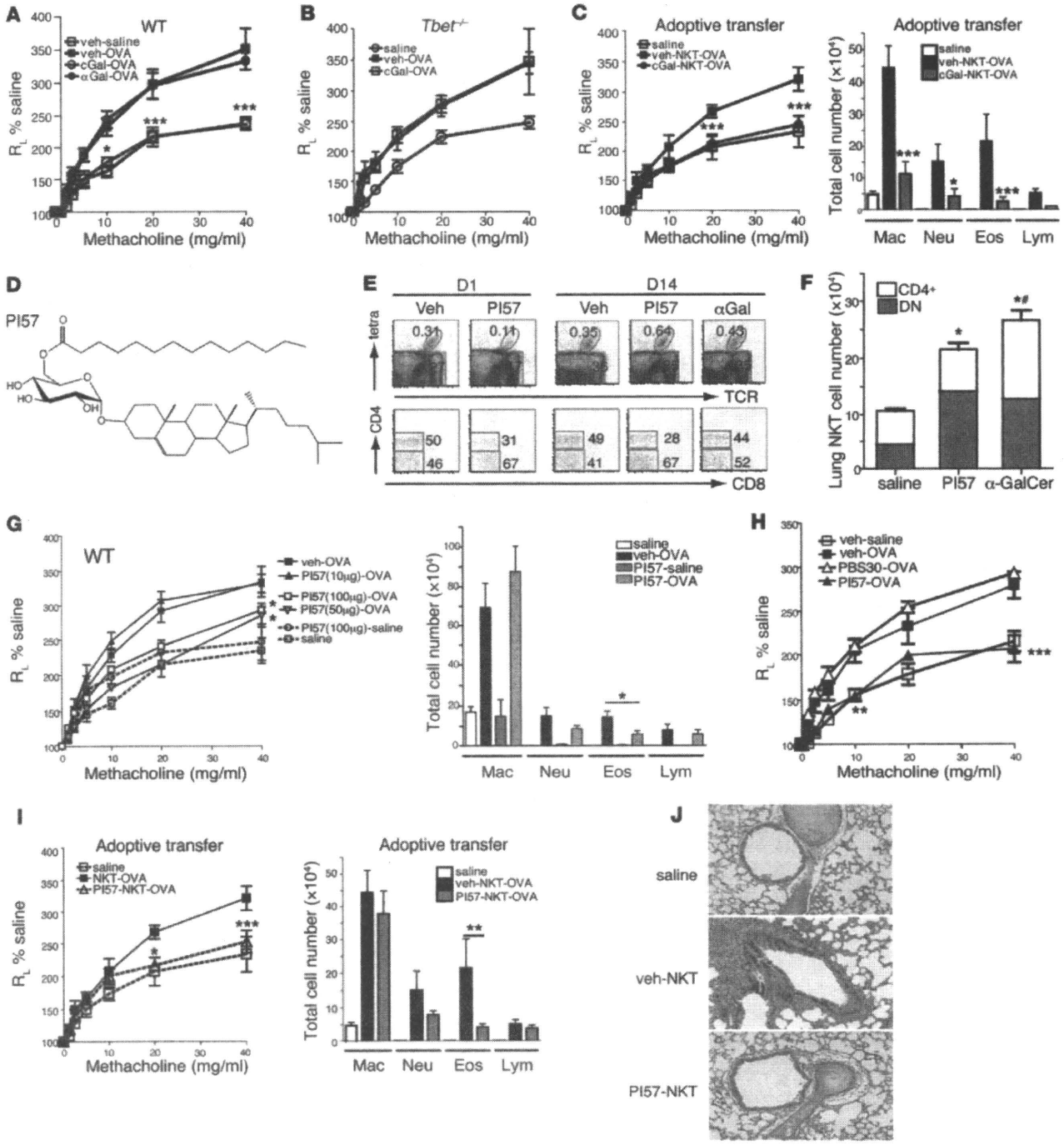

Figure 6

Induction of protection with α-C-GalCer and a glycolipid from *H. pylori*. (**A**) Two-week-old BALB/c mice ($n = 6–8$/group) or (**B**) *Tbet*$^{-/-}$ mice ($n = 4–6$ per group) received 5 μg α-GalCer (cGal), 2 μg α-GalCer, or vehicle. After OVA sensitization and challenge, AHR was measured on day 44. (**C**) Donor mice were treated with α-C-GalCer (5 μg) or vehicle i.p. NKT cells served as donors, as in Figure 4A ($n = 4$ per group). Lung resistance (left) and cell counts in BAL (right) were assessed. (**D**) Structure of PI57. (**E**) Mice received PI57 (50 μg), α-GalCer (2 μg), or vehicle i.p., and lungs were examined 1 or 14 days later for CD4 and CD8 expression. (**F**) Absolute numbers of CD4$^+$ NKT and DN NKT subsets from **E** were assessed. (**G**) BALB/c mice ($n = 5–8$/group) received PI57 or vehicle i.p. Lung resistance (left) and BAL cells (right) were assessed. (**H**) BALB/c mice treated with PI57 (50 μg), PBS30 (*Sphingomonas* glycolipid) (50 μg), or vehicle i.p. were assessed for AHR as in **G**. (**I**) Donor mice were treated with PI57 (50 μg) or vehicle i.p. NKT cells served as donors as in Figure 4A. Lung resistance (left) and BAL cells (right) were assessed ($n = 4$ per group). (**J**) Representative lung sections from **I** stained with H&E (original magnification, ×10). Data represent 2–3 independent experiments. $^*P < 0.05$, $^\#P < 0.05$, $^{***}P < 0.001$ versus vehicle-OVA (**C**, **G**, and **I**), DN NKT saline (**F**), and CD4$^+$ NKT saline (**F**).

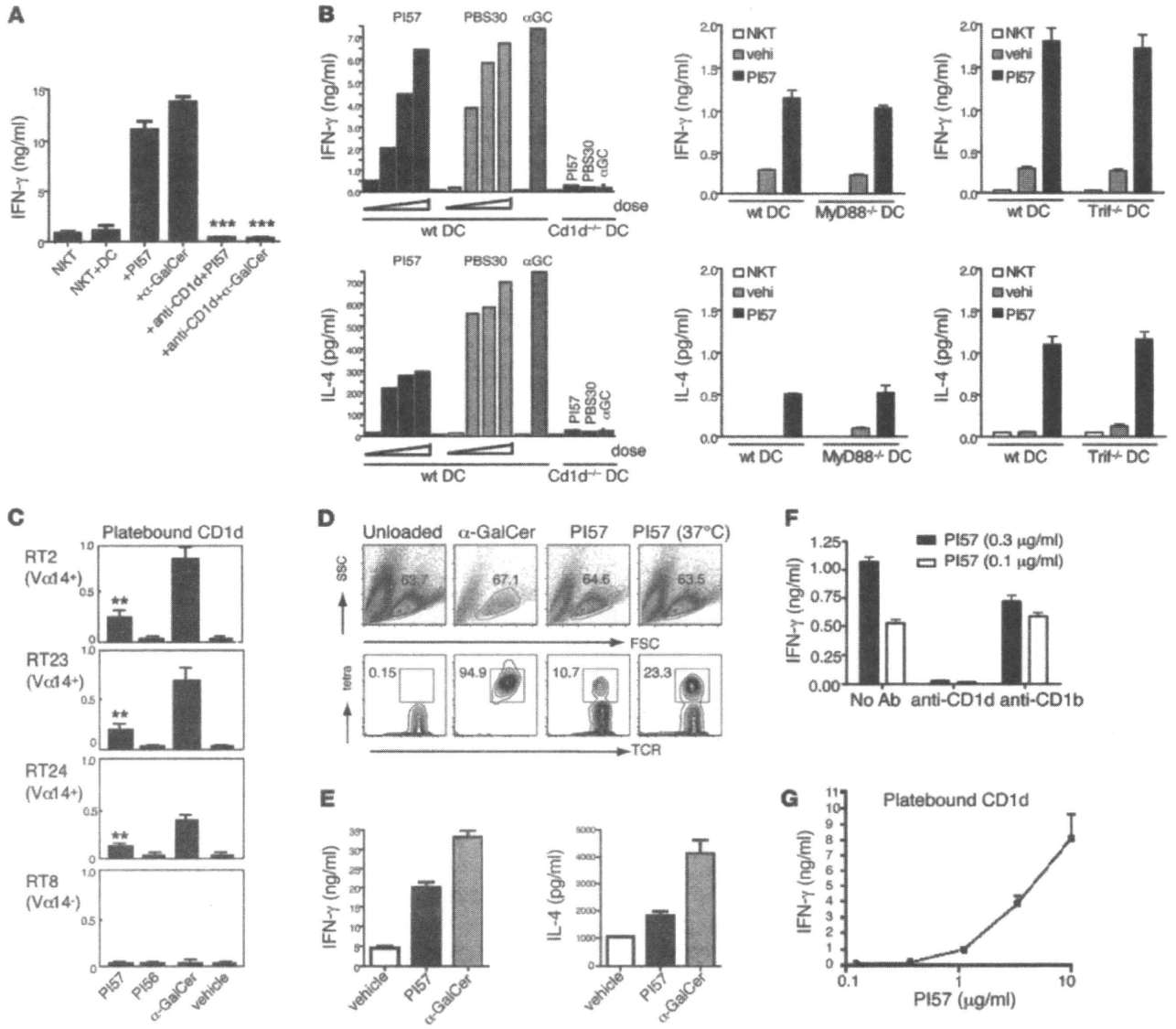

Figure 7

PI57 directly activates NKT cells. (**A**) NKT cell lines were cocultured with BM-derived DCs (BMDCs) and α-GalCer (100 ng/ml), PI57 (10 μg/ml), or vehicle for 48 hours, with or without pre-incubation with anti-CD1d (10 μg/ml). IFN-γ was measured by ELISA. (**B**) Murine NKT cell lines were cocultured as in **A** with BMDCs from WT, *Cd1d*$^{-/-}$, *Myd88*$^{-/-}$, or *Trif*$^{-/-}$ mice. Cells were treated with α-GalCer (100 ng/ml), PI57 (2.5, 5, or 10 μg/ml), PBS30 (1, 2.5, or 5 μg/ml), or vehicle for 48 hours. IFN-γ and IL-4 were measured by ELISA. (**C**) IL-2 production from hybridomas derived from invariant Vα14 NKT cells (RT2, RT23, and RT24) and an irrelevant Vβ8$^+$ T cell (RT8; control) (see Supplemental Methods). (**D**) Mouse NKT cell lines were stained with PE-labeled CD1d tetramers of PI57 or α-GalCer at 4°C for 45 minutes or 37°C for 25 minutes, and with anti-TCRβ–APC antibody. Top: Lymphocytes were gated in the FSC/SSC window. Bottom: Percentage of CD1d tetramer$^+$ cells. (**E**) IFN-γ and IL-4 production from human NKT cell lines by treatment with α-GalCer (100 ng/ml), PI57 (10 μg/ml), or vehicle for 48 hours in vitro (see Supplemental Methods). (**F**) IFN-γ production from CD1d-transfected NKT cell clone BM2a.3 in presence of PI57 and blocking mAb against human CD1d or CD1b (see Supplemental Methods). (**G**) CD1d Fc-coated Maxisorp plates were loaded with lipid and cultured with 5 × 10^4 NKT cells. IFN-γ was analyzed by ELISA after 24 hours. Data represent 3 or 5 independent experiments.

subpopulation of NKT cells that can suppress the effects of other subpopulations of NKT cells that enhance the development of experimental asthma. These results suggest that a balance exists between NKT cells that induce, and those that protect against, AHR, and that stimulation with H3N1, α-C-GalCer, or *H. pylori* glycolipids, but not a *Sphingomonas* glycolipid or α-GalCer, may selectively expand this regulatory NKT cell population in young mice. The inability of α-GalCer to protect may be due to the fact that it nonselectively stimulates all invariant NKT cells or because it may anergize NKT cells, including suppressive populations. Nevertheless, these data support the idea that under normal, pathogen-free conditions, CD4$^+$ NKT cells that induce AHR predominate, but that in very young mice, exposure to Th1-skewing reagents that can alter the composition of NKT cell subpopulations may change subsequent lung immunity.

Therefore, it appears that the balance between $CD4^+$ versus regulatory (presumably DN) NKT cells is determined or imprinted early in life but might be influenced by exposure to specific types of infections, particularly those that can affect NKT cells. In our studies, H3N1 infection in 2-week-old pups activated the immature NKT cells and preferentially expanded a DN NKT cell subset. In addition, our studies suggest that α-C-GalCer and glycolipids from *H. pylori* can profoundly affect this NKT cell subpopulation, which may explain epidemiological studies showing an association of *H. pylori* infection with protection against asthma (2, 3). Although these studies were performed in mice, which mature from neonates to adults in only 35 days versus many years in humans, taken together, our results suggest that infection with certain microorganisms can prevent the subsequent development of asthma and allergy by expanding the relative proportion of a specific subset of NKT cells, thus providing an immunological mechanism for the hygiene hypothesis. Finally, these results predict that treatment of children with compounds such as α-C-GalCer and others derived from microorganisms (e.g., *H. pylori*) might expand this regulatory NKT cell subset and be effective in preventing the development of asthma.

Methods

Mice. WT BALB/c ByJ and *Tbet*$^{-/-}$ (C.129S6-Tbx21tm1Glm/J) mice were purchased from The Jackson Laboratory. *Jα18*$^{-/-}$ mice were gifts from M. Taniguchi and T. Nakayama (Chiba University, Chiba, Japan). *Tlr7*$^{-/-}$ mice were generated by Shizuo Akira, and the Vα14 Tg mice were provided by Albert Bendelac (University of Chicago, Chicago, Illinois, USA). These strains were backcrossed to BALB/c for more than 10 generations. DO11.10 X *Rag*$^{-/-}$ mice were provided by Abul Abbas (UCSF, San Francisco, California, USA). For studies in suckling mice, BALB/c, *Tlr7*$^{-/-}$, and *Tbet*$^{-/-}$ mice were bred, and the offspring were infected at 2 weeks of age, then weaned at 3 weeks. The Animal Care and Use Committee at Children's Hospital Boston approved all animal protocols.

Influenza A infection. Two-week-old pups (suckling mice) or 8-week-old adult mice were anesthetized with 3% isoflurane and inoculated intranasally (i.n.) with influenza A virus (strain Mem/71 [H3N1]) in 20 μl PBS for suckling mice or 50 μl PBS for adult mice. The virus is a reassortant influenza virus strain carrying the hemagglutinin of A/Memphis/1/71 (H3) and the neuraminidase of A/Bellamy/42 (N1). The virus was grown and harvested from 10-day embryonated chicken eggs as previously described (59). The dose of virus used (1.2×10^4 PFU/mouse) causes nonlethal pneumonia of both suckling and adult mice, with complete virus clearance around day 7 after infection. Control (mock-infected) mice were treated with i.n. allantoic fluid (AF) diluted 1:500 in PBS.

Reagents. α-GalCer and PBS30 (31) were synthesized by P.B. Savage (Brigham Young University, Provo, Utah, USA). *H. pylori* glycolipids were extracted and purified as described in the Supplemental Methods. The *H. pylori* glycolipid PI57 (cholesteryl 6-O-tetradecanoyl-α-D-glucopyranoside) was synthesized based on ^1H, ^{13}C NMR spectrometry, TLC analysis, ES-mass spectrometry of lipids from *H. pylori* SS1 and human *H. pylori* S strains (Supplemental Figure 4 and Supplemental Methods), and data reported for purified *H. pylori* glycolipids (30). An analog of α-C-GalCer, called "GCK151", which has activity with mouse and human NKT cells (27), was synthesized by Richard W. Franck (Hunter College of CUNY).

PI57-loaded CD1d tetramers. To generate PI57-loaded mCD1d monomers, a 10-fold molar excess of PI57 in DMSO at 2 mg/ml was incubated with biotinylated-mCD1d (from the NIH Tetramer facility) in 2 mM CHAPS and 20 mM Tris pH 7.0 overnight at room temperature. The mCD1d monomers were tetramerized by adding SA-PE (S868; Invitrogen) to the lipid-loaded monomers as previously described (60).

Induction of AHR and measurement of airway responsiveness in the OVA model. To induce AHR, BALB/c mice were sensitized with 100 μg of OVA (Sigma-Aldrich) in alum administered i.p (on day 0). After sensitization, mice were exposed to i.n. antigen (50 μg OVA/day) or normal saline for 1 day (day 7; single-dose challenge protocol) or for 3 consecutive days (days 7–9). AHR was assessed on the day after last OVA challenge. Control mice received i.p. injection of PBS and i.n. administrations of normal saline.

Collection and analysis of bronchoalveolar lavage. Immediately after the AHR measurement, mice were euthanized and the lungs were lavaged twice with 0.5 ml of PBS, and the fluid was pooled. Cells in bronchoalveolar lavage (BAL) were counted and analyzed as previously described (20). The relative number of different types of leukocytes was determined from slide preparations of BAL stained with Diff-Quik solution (Dade Behring).

Adoptive transfer of NKT cells. NKT cells were purified from splenocytes of WT BALB/c, influenza virus–infected BALB/c, influenza virus–infected *Tlr7*$^{-/-}$, influenza virus–infected *Tbet*$^{-/-}$, Vα14 TCR transgenic mice, PI57-treated BALB/c, and α-GalCer–treated BALB/c mice using magnetic cell sorting (MACS), as previously described (20). Splenic NKT cells were labeled with PE-conjugated CD1d tetramer, followed by anti-PE microbeads (Miltenyi Biotec) and then sorted with AutoMACS according to the manufacturer's instruction. Purity of NKT cells was approximately 93% (Supplemental Figure 2A), and there was no detectable Treg cell contamination (Supplemental Figure 2B). Purified NKT cells were adoptively transferred into immunized recipient mice by intravenous injection (10^6 for *Jα18*$^{-/-}$; 5×10^5 for BALB/c) 1 hour before the first challenge of OVA (day 7). For the OVA-specific Treg cell experiment, 5×10^4 DO11.10 $CD4^+$ T cells (from DO11.10 × *Rag*$^{-/-}$ mice) were adoptively transferred into recipient mice 5 hours before sensitization with OVA/alum (day 0). The recipients later received NKT cells 1 hour before the first challenge of OVA (day 7).

ELISA. Mouse or human IL-4 and IFN-γ levels were measured by ELISA, as previously described (20). Mean values of triplicate cultures were shown. Data are representative of 2 or 3 independent experiments.

Statistics. Differences between groups with parametric distributions were analyzed using the Student's 2-tailed *t* test. Otherwise, the Mann-Whitney *U* test was used. Data represent mean ± SEM. *P* values of 0.05 or less were considered statistically significant.

Acknowledgments

These studies were supported by grants from the NIH RO1 AI68085, R01 HL62348, R01 AI026322, and RC1 HL069507, by an award from the Bunning Food Allergy Project, and by grant 20570146 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The authors thank Dirk Zajonc for help in loading CD1d molecules, and the NIH NIAID Tetramer Facility for providing CD1d monomers and PBS57-CD1d tetramers.

Received for publication August 20, 2010, and accepted in revised form October 20, 2010.

Address correspondence to: Dale T. Umetsu, Karp Labs, Rm 10127, One Blackfan Circle, Boston, Massachusetts 02115, USA. Phone: 617.919.2439; Fax: 617.730.0384; E-mail: dale.umetsu@childrens.harvard.edu. Or to: Michio Shimamura, Tsukuba Research Center for Interdisciplinary Materials Science, University of Tsukuba, 1-1-1 Tennodai, Ibaraki 305-8571, Japan. Phone: 81.29.853.4527; Fax: 81.29.853.4507; E-mail: michio@chem.tsukuba.ac.jp. Or to: Petr Illarionov, University of Birmingham School of Biosciences, Edgbaston B15 2TT, Birmingham, United Kingdom. Phone: 44.121.4158123; Fax: 44.121.4145925; E-mail: illar@yahoo.com.

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Immunology Letters 121 (2008) 38–44

Contents lists available at ScienceDirect

Immunology Letters

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

Michio Shimamura[a,*], Yi-Ying Huang[a], Rika Migishima[b], Minesuke Yokoyama[b], Takako Saitoh[c], Takashi Yamamura[d]

[a] Developmental Immunology Unit, Mitsubishi Kagaku Institute of Life Sciences, Japan
[b] Mouse Genome Technology Center, Mitsubishi Kagaku Institute of Life Sciences, Japan
[c] Fine Structure Analysis Section, Mitsubishi Kagaku Institute of Life Sciences, Japan
[d] National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan

ARTICLE INFO

Article history:
Received 15 April 2008
Received in revised form 13 August 2008
Accepted 18 August 2008
Available online 27 September 2008

Keywords:
NKT cell
Development
Invariant TCR
Cytokine production

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$^+$ TCRαβ$^+$ 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.

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.

* Corresponding author at: Developmental Immunology Unit, 11 Minamiooya, Machida, Tokyo 194-8511, Japan. Tel.: +81 42 724 6315; fax: +81 42 724 6317.
E-mail address: michio@libra.ls.m-kagaku.co.jp (M. Shimamura).

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doi:10.1016/j.imlet.2008.08.002

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

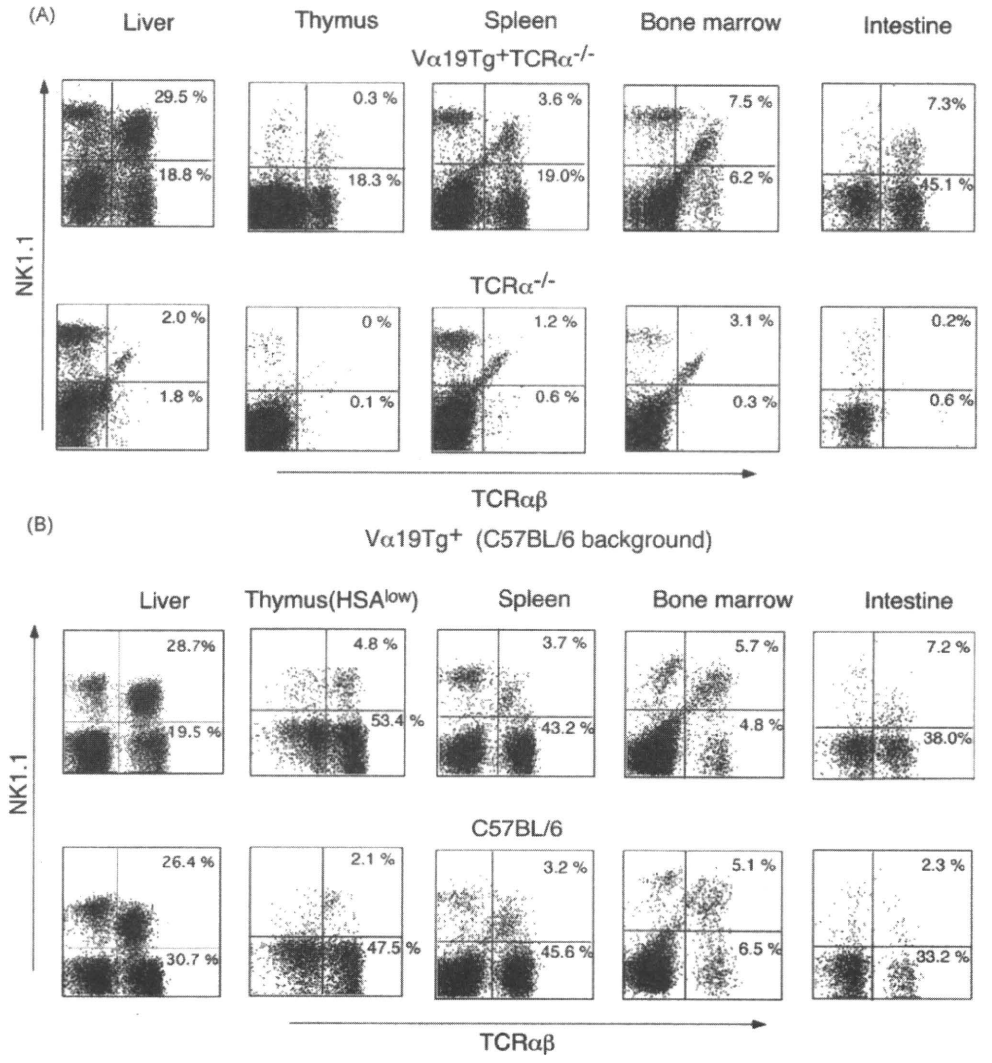

Fig. 1. Development of Vα19 NKT cells in Vα19 Tg mice. MNCs were isolated from liver, thymus, spleen, bone marrow and intestinal lamina propria of mice (at 8–12 weeks of age, female). They were stained with fluorescence-labeled antibodies and analyzed by FACS. (A) Staining profiles of the Vα19 Tg$^+$ 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.

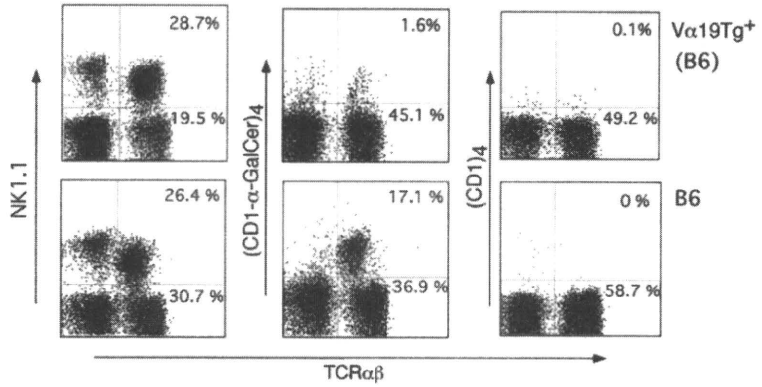

Fig. 2. Staining profiles of Tg- and non-Tg liver MNCs with CD1-α-Gal-Cer tetramers. Liver MNCs isolated from Tg and non-Tg mice on the C57BL/6 background (at 8–10 weeks of age, female, both in the range of 3–7 × 10^6 per animal) were stained with anti-TCR αβ antibody and either anti-NK1.1 antibody,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

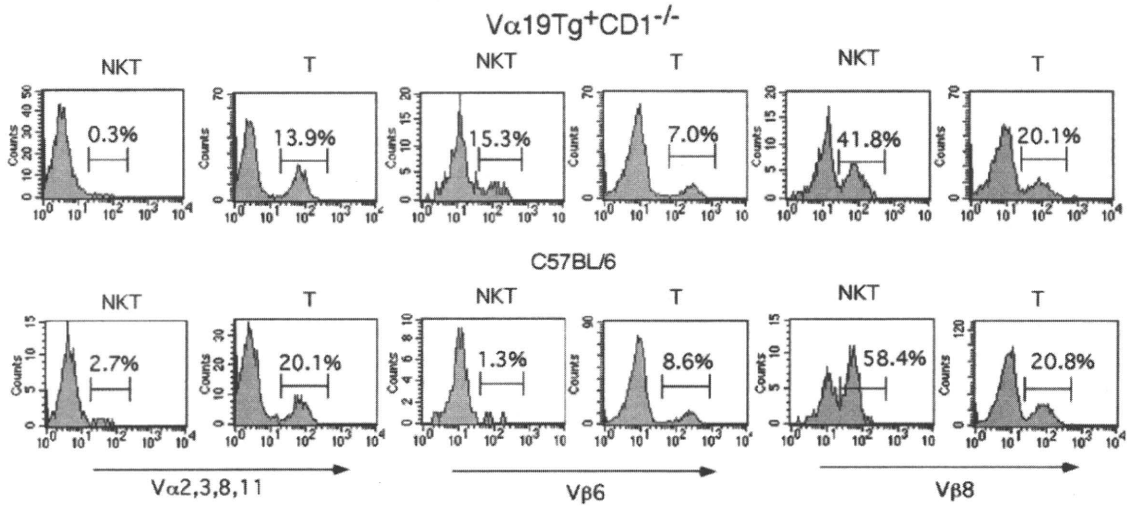

Fig. 3. Preferential expression of the invariant Vα19 TCR transgene by the NK1.1$^+$, TCRα($^+$ cells in the Tg livers. Liver MNCs from Vα19 TCR Tg (CD1$^{-/-}$ background) and non-Tg mice (at 8 weeks of age, female) were triply stained with an anti-TCRαβ, anti-NK1.1 and anti-TCR Vα mixture (Vα2, 3.2, 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 ± 1.5% in Vα19 Tg$^+$ TCRα$^{-/-}$ intestine, 2.8 ± 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.

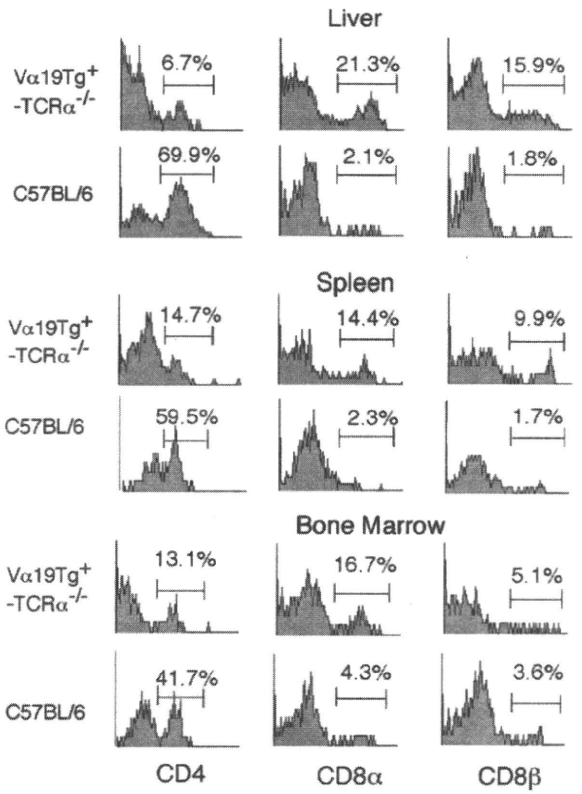

Fig. 4. CD4/CD8 co-receptor expression by NK1.1$^+$, TCR αβ$^+$ cells of Vα19 Tg$^+$ and non-Tg mice. MNCs were isolated from liver, thymus, spleen, and bone marrow of Vα19 Tg$^+$ TCRα$^{-/-}$ and C57BL/6 mice (at 8–12 weeks of age). They were immunostained and analyzed by flow cytometry. Profiles of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

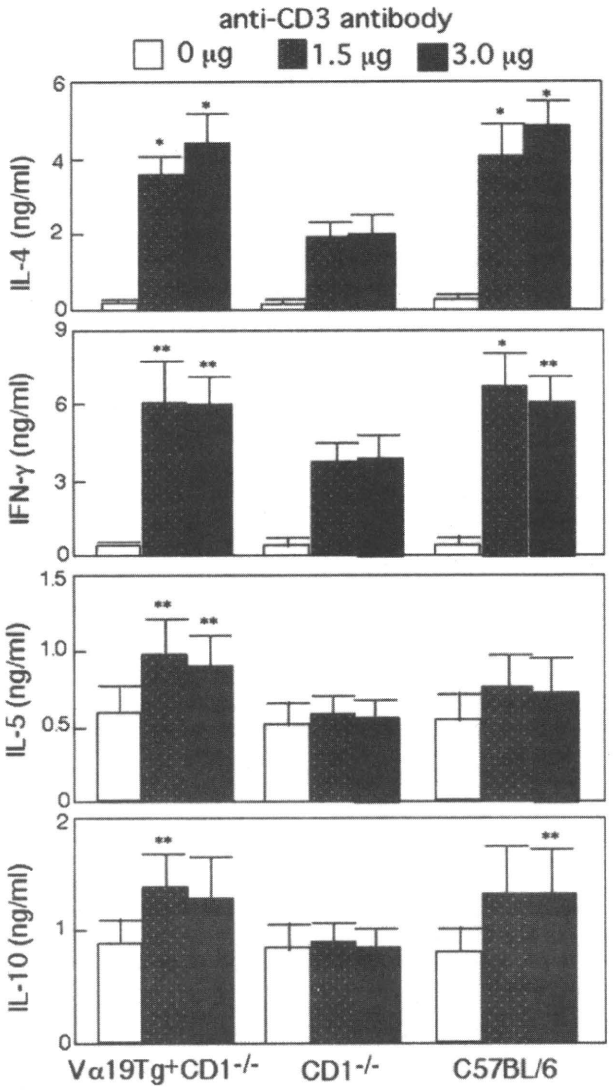

Fig. 5. Cytokine production by Tg and non-Tg mice in response to *in vivo* treatment with anti-CD3 antibody. Liver MNCs prepared from Vα19 Tg$^+$ CD1$^{-/-}$, CD$^{-/-}$, and C57BL/6 mice injected 90 min previously with a different dose of anti-CD3 antibody (2C11) were cultured in DMEM (10% FCS) without 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 Vβ6$^+$ and Vβ8$^+$ 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 α$^{-/-}$ 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 Vα19$^+$/Vβ6$^+$ or Vβ8$^+$ TCR frequently present in Vα19 Tg$^+$ TCR α$^{-/-}$ 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 α$^{-/-}$ 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 β2m$^{-/-}$ 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 Vα19$^+$ NKT cells in the Tg lamina propria were observed (Supplementary Table S1). The stain-

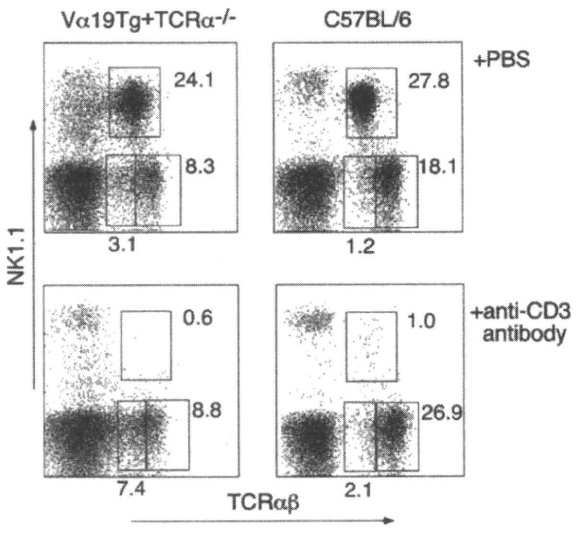

Fig. 6. Surface receptor down-regulation of hepatic Vα19 NKT cells upon TCR engagement in vivo. Liver MNCs were prepared from Vα19 Tg$^+$ TCRα$^{-/-}$ and C57BL/6 mice injected 24 h previously with anti-CD3 antibody (2C11, 2 μg/mouse). Cells were stained with anti-TCRαβ and anti-NK1.1 antibodies and 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 ± 56 μg/ml) was comparable to that of non-Tg mice (115 ± 59 μg/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].

Acknowledgements

The authors thank Drs. M. Kronenberg, M. Nanno, H. Ishikawa, M.J. Grusby, and M. Iwata for providing valuable materials. They also thank Dr. J. L. Croxford for the critical reading of the manuscript, and Mr. S. Kamijo, Ms. Y. Shirota, Ms. N. Suzuki and Ms. Y. Murakami for technical and secretarial assistance. This work was supported by a grant from the Ministry of Health, Welfare and Labor, Japan.

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

Yoshimitsu Doi*, Shinji Oki*†, Tomoko Ozawa*, Hirohiko Hohjoh‡, Sachiko Miyake*, and Takashi Yamamura*†

Departments of *Immunology and ‡Molecular Genetics, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan

Communicated by Tadamitsu Kishimoto, Osaka University, Osaka, Japan, April 11, 2008 (received for review March 11, 2008)

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

Author contributions: Y.D., S.O., H.H., S.M., and T.Y. designed research; Y.D., S.O., and T.O. performed research; Y.D., S.O., S.M., and T.Y. analyzed data; and S.O. and T.Y. wrote the paper.

The authors declare no conflict of interest.

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†To whom correspondence may be addressed. E-mail: soki@ncnp.go.jp or yamamura@ncnp.go.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0803454105/DCSupplemental.

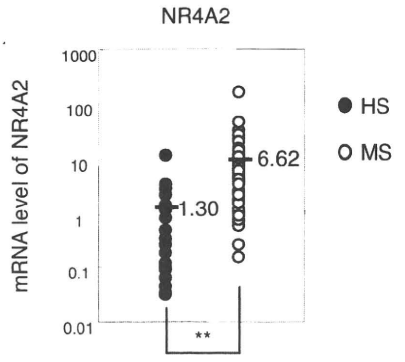

Fig. 1. Quantitative analysis of NR4A2 transcription between MS and controls. $CD3^+$ T cells were isolated from PBMC of 57 MS patients and of 19 healthy donors, and total RNA was extracted. cDNA was synthesized and the expression levels of NR4A2 transcript were analyzed by quantitative RT-PCR. Each 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.

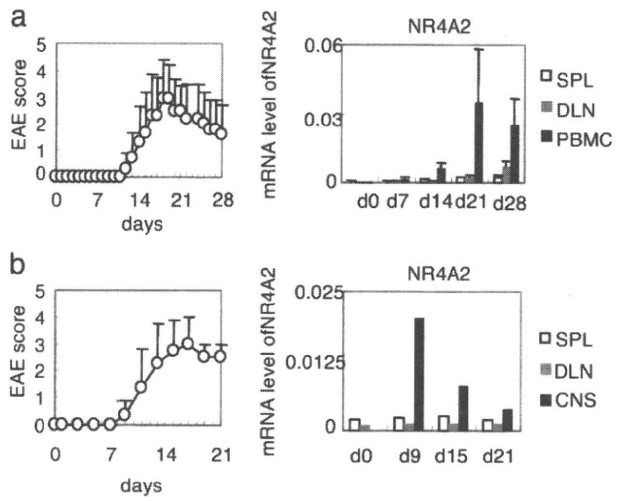

Fig. 2. Kinetic analysis of NR4A2 expression in the disease course of EAE. (*a*) (*Left*) EAE was induced in B6 mice by immunization with MOG_{35-55} in CFA. Mice were killed on days 7, 14, 21 and 28 after immunization, and T cells were isolated from dLN, SPL, or PBMC, using anti-CD3 magnetic beads. (*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%.

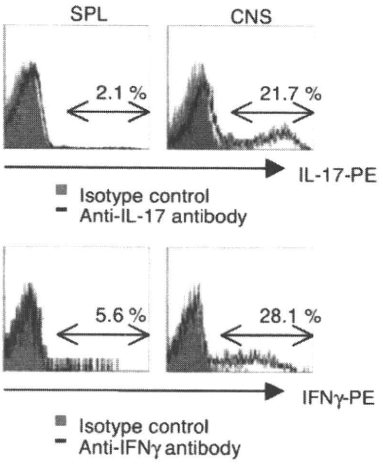

Fig. 3. Accumulation of IL-17 or IFN-γ-producing inflammatory T cells in the CNS. Mononuclear cells were isolated from spleen or CNS on day 17 after immunization and stimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) in the presence of 2 mM monensin for 4 h. Production of IL-17 and IFN-γ was analyzed 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.

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.

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

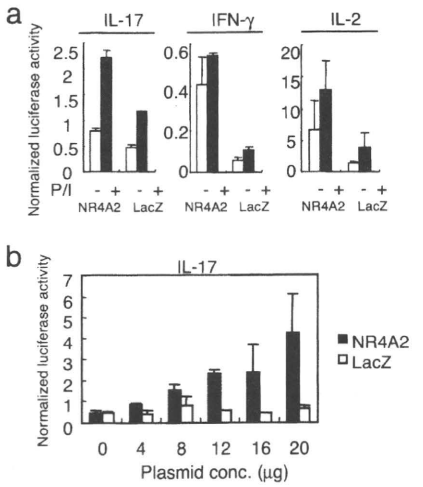

Fig. 4. Promoter activities of cytokine genes in the presence of NR4A2. (*a*) The effect of NR4A2 expression on IL-17, IFN-γ, and IL-2 promoter activity. A reporter plasmid containing promoter of cytokine gene (10 μg) and *Renilla* luciferase plasmid (100 ng) were introduced into EL4 cells by electroporation, 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.

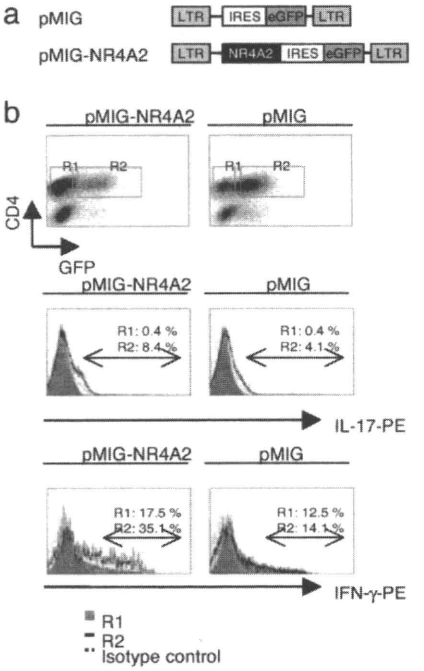

Fig. 5. The effect of retrovirally transduced NR4A2 on cytokine production by primary murine CD4$^+$ T cells. (*a*) DNA fragments encoding wild-type NR4A2 were cloned into the pMIG(W) bicistronic retroviral vector. LTR, long terminal repeat; IRES, internal ribosome entry site; eGFP, enhanced green fluorescence 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.

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 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

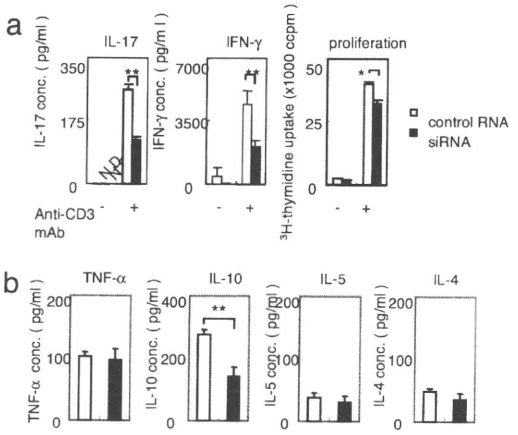

Fig. 6. The effect of NR4A2 gene silencing on T cell cytokine production. (*a*) Specific inhibition of T cell production of IL-17 and IFN-γ by siRNA treatment. Human CD4$^+$ T cells derived from PBMC were transfected with siRNA or control RNA and stimulated by immobilized anti-CD3 Ab for 48 h. Cytokine levels in the culture supernatant were determined by ELISA or a CBA human Th1/2 cytokine kit. Proliferation rate was measured by ^{3}H-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 naïve 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.

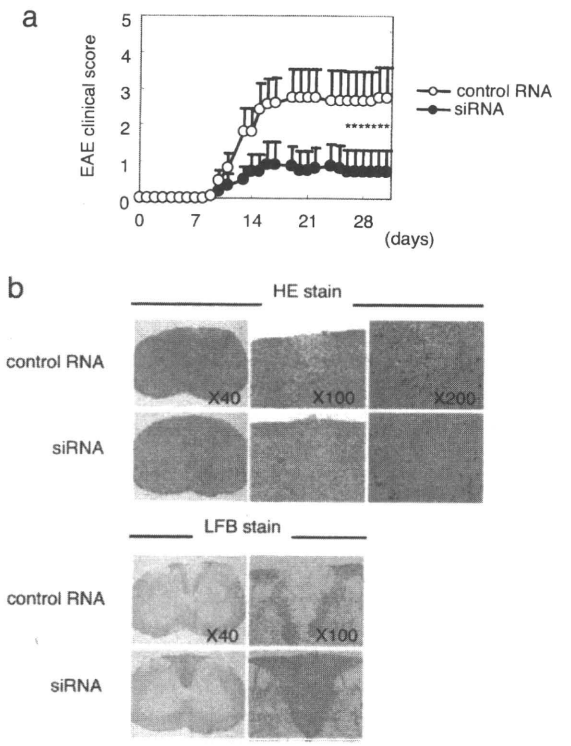

Fig. 7. The effect of T cell silencing of NR4A2 expression on passive EAE. (*a*) Inguinal and popliteal LNs cells were collected from female SJL/J mice 10 days after immunization with $PLP_{139-151}$, and were transfected with siRNA for NR4A2 or control RNA, using HVJ-E vector kit. The cells were cultured 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 naïve 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.

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 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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HMG Advance Access published March 18, 2010

Human Molecular Genetics, 2010 **1–8**
doi:10.1093/hmg/ddq092

Regulatory polymorphisms in *EGR2* are associated with susceptibility to systemic lupus erythematosus

Keiko Myouzen[1], Yuta Kochi[1,*], Kenichi Shimane[1,2], Keishi Fujio[2], Tomohisa Okamura[2], Yukinori Okada[3], Akari Suzuki[1], Tatsuya Atsumi[5], Satoshi Ito[6], Kazuki Takada[7], Akio Mimori[8], Shiro Ikegawa[4], Ryo Yamada[9], Yusuke Nakamura[10] and Kazuhiko Yamamoto[1,2]

[1]Laboratory for Autoimmune Diseases, Center for Genomic Medicine (CGM), RIKEN, Tokyo 113-0033, Japan, [2]Department of Allergy and Rheumatology, Graduation School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan, [3]Laboratory for Statistical Analysis and [4]Laboratory for Bone and Joint Diseases, CGM, RIKEN, Tokyo 106-8639, Japan, [5]Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan, [6]Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki 305-8575, Japan, [7]Department of Medicine and Rheumatology, Graduate School, Tokyo Medical and Dental University, Tokyo 113-8519, Japan, [8]Division of Rheumatic Diseases, International Medical Center of Japan, Tokyo 162-8655, Japan, [9]Statistical Genetics, Center for Genomic Medicine, Graduate School of Medicine Kyoto University, Kyoto 606-8501, Japan and [10]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Received October 31, 2009; Revised and Accepted February 24, 2010

Systemic lupus erythematosus (SLE) is an autoimmune disease induced by the combinations of environmental and genetic factors. Recently, mice in which the early growth response 2 (*EGR2*) gene, a zinc-finger transcription factor, is conditionally knocked out in CD2$^+$ T cells have been shown to develop a lupus-like autoimmune disease. Here, we evaluated if polymorphisms in the *EGR2* gene influence SLE susceptibility in humans. We first analyzed the effect of SNPs in the *EGR2* region on *EGR2* expression, and a significant positive correlation with expression was identified in an SNP located at the 5′ flanking region of *EGR2* (rs10761670, $R = 0.23$, $P = 0.00072$). We then performed a case–control association study using three sets of SLE cohorts by genotyping 14 tag SNPs in the *EGR2* gene region. A peak of association with SLE susceptibility was observed for rs10761670 [Pooled: OR = 1.23 (95% CI 1.10–1.37), $P = 0.00023$). This SNP was also associated with susceptibility to rheumatoid arthritis (RA) [OR = 1.15 (95% CI 1.05–1.26), $P = 0.0019$), suggesting that *EGR2* is a common risk factor for SLE and RA. Among the SNPs in complete linkage disequilibrium with rs10761670 ($r^2 = 1.0$), two SNPs (rs1412554 and rs1509957) affected the binding of transcription factors and transcriptional activity *in vitro*, suggesting that they may be candidates of causal regulatory variants in this region. Therefore, *EGR2* is a genetic risk factor for SLE, in which increased gene expression may contribute to SLE pathogenesis.

INTRODUCTION

Systemic lupus erythematosus (SLE, OMIM # 152700) is an autoimmune disease induced by the combinations of environmental and genetic factors. This disease is characterized by antinuclear autoantibodies, complement activation, hyperproduction of interferon and tissue destruction (1). Genetic studies using a candidate gene approach identified several SLE susceptibility genes, including *HLA-DRB1*, *FCGR2B/3A/3B*, *PTPN22*, *STAT4* and *IRF5* (2–7). In addition, recent genome-wide association studies (GWASs) have uncovered novel SLE susceptibility genes, including *TNFAIP3*, *BANK1*, *ITGAM*, *PXK*, *KIAA1542* and *C8orf13-BLK* (8–11). Among these genes, genes such as *PTPN22*, *STAT4*, *TNFAIP3* and

*To whom correspondence should be addressed at: Laboratory for Autoimmune Diseases, Center for Genomic Medicine, RIKEN, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel/Fax: +81 358008828; Email: ykochi@src.riken.jp

IRF5 are also associated with susceptibility to other autoimmune diseases, suggesting that some genetic dispositions are shared between SLE and other autoimmune diseases (7,12–14). These GWASs for SLE have enabled a comprehensive survey of the genome, and the statistical power of individual GWASs has been increasing as growing number of samples have been genotyped. However, a recent large-scale replication study that examined the suggested loci of GWAS demonstrated that the 26 confirmed risk loci only explain an estimated 8% of the total genetic susceptibility to SLE (15). This implicated many genes remained undiscovered probably due to their moderate risk. Therefore, the candidate gene approach is still a complementary tool for the identification of unknown genes with moderate risk that contribute to SLE susceptibility.

The early growth response 2 (*Egr2*) gene in mice is a member of the zinc-finger transcription factor *Egr* family (*Egr1*, *2*, *3* and *4*) that are expressed during thymic T-cell differentiation (16), and plays an essential role in hindbrain development and myelination of the peripheral nervous system (17). Whereas *Egr4* is constitutively expressed in T cells, *Egr1*, *2*, and *3* are up-regulated by T-cell receptor (TCR) engagement. Opposing functions of the *Egr*s have been described, where *Egr1* enhances T-cell function by up-regulating IL-2 and other molecules that stimulate T cells (18). On the other hand, *Egr2* and *Egr3* are considered to negatively regulate T cells, which are demonstrated in a study using *Egr2* and *Egr3*-deficient T cells (19). In that study, it was proposed that *Egr2* and *Egr3*, which are located in the downstream of transcription factor NF-AT signaling, inhibited T-cell function by both up-regulating negative regulators such as *Cbl-b* and inhibiting the expression of T-cell activators such as *Egr1* and *NAB2*. Interestingly, mice whose *Egr2* gene was conditionally knocked out in $CD2^+$ T cells were shown to develop a lupus-like autoimmune disease, characterized by accumulation of interferon-γ and interleukin-17-producing $CD4^+$ T cells, loss of tolerance to nuclear antigens, massive infiltration of T cells into multiple organs and glomerulonephritis (20). More recently, we described a new subset of IL-10-secreting regulatory T cells, termed $CD4^+CD25^-LAG3^+$ regulatory T cells, that characteristically express *Egr2* (21). Because *Egr2*-transduced naïve $CD4^+$ T cells differentiated into IL-10-secreting $CD4^+$ $CD25^-LAG3^+$ regulatory T cells, *Egr2* was considered to be a key transcription factor for the differentiation of these cells. A significant role in intestinal immunity was suggested for these cells by the observation that the frequency of $CD4^+$ $CD25^-LAG3^+$ regulatory T cells in the $CD4^+CD25^-$ T cell population was comparatively higher in Peyer's patch than in the spleen, and that experimentally induced colitis was effectively protected by these regulatory T cells.

In humans, chromosome 10q21, within which *EGR2* is located, has been identified as a candidate locus for Crohn's disease (CD) susceptibility in two independent GWASs (22,23). The landmark SNPs identified in these GWASs are located in the intergenic region between the *ZNF365* and the *EGR2* genes. T cells have been assumed to play a major role in the pathogenesis of CD, as an excess of T cells (both effector and regulatory T cells) were detected in the intestinal mucosa of patients (24,25). Indeed, genetic studies of CD identified several susceptibility genes that are involved in the differentiation of T cells, which further supports the significance of T cells for CD (25). Therefore, given the effects of *Egr2* on murine T cells and the role of $CD4^+CD25^-$ $LAG3^+Egr2^+$ regulatory T cells in the colitis model, *EGR2* is considered to be a strong candidate gene for CD susceptibility at this locus.

The described evidence derived from the murine disease models and from human genetic studies implies the existence of disease causal variations in the *EGR2* gene region, which might be shared between SLE and CD in humans. In the present study, we first searched for functional variants that may affect gene function or gene expression of *EGR2*, and then performed case–control association tests to examine the contribution of *EGR2* to SLE susceptibility.

RESULTS

Correlation between *EGR2* expression and SNP genotypes

To identify variants which potentially increase the risk of disease in the *EGR2* gene region, we first searched for functional variants that might alter the amino acid sequence, or affect the expression level, of *EGR2*. The coding region of *EGR2* was sequenced using DNA from 96 individuals affected with SLE (the power for detecting rare variants with frequency of 0.01 was estimated to be 0.86). In this sequencing analysis, no variation leading to amino acid substitution in *EGR2* was discovered.

We then examined possible correlations between *EGR2* expression and genotype. As *EGR2* has been reported to be expressed in B cells as well as in T cells (26,27), we analyzed a data set of gene expression in Epstein-Barr virus (EBV)-transformed lymphoblastoid B cell lines from HapMap individuals (28). Analysis of the genotype data of Japanese HapMap samples identified three LD blocks in a Japanese population in an 80-kb region that includes the *EGR2* gene on 10q21.3 (Fig. 1A). The correlation between SNP genotype and the expression of *EGR2* in this region was assessed for data sets of three individual HapMap ethnic groups (JPT + CHB, CEU and YRI). The observation of a common peak of correlation in the 5′ flanking region of *EGR2* among these three groups implied common regulatory factors for *EGR2* expression in this chromosomal region (Fig. 1B). We then selected 69 SNPs from this 80-kb region that are common (minor allele frequency; MAF > 0.10) to all three HapMap ethnic groups, and assessed the correlation between their genotypes and the expression of *EGR2* using the pooled data set of all populations ($n = 210$) (Fig. 1B). Rs10761670 (T/A) in the 5′ flanking region of *EGR2* showed the highest correlation ($R = 0.23$). When the expression level of *EGR2* was regressed with the number of alleles of rs10761670, the correlation was statistically significant ($P = 0.00072$, Fig. 1C). This correlation suggested that variations existed in the 5′ flanking region of *EGR2* that affect gene expression, which may potentially increase the risk of disease.

Case–control association study of the *EGR2* gene region

To evaluate the association between *EGR2* polymorphisms and SLE susceptibility, we performed a case–control