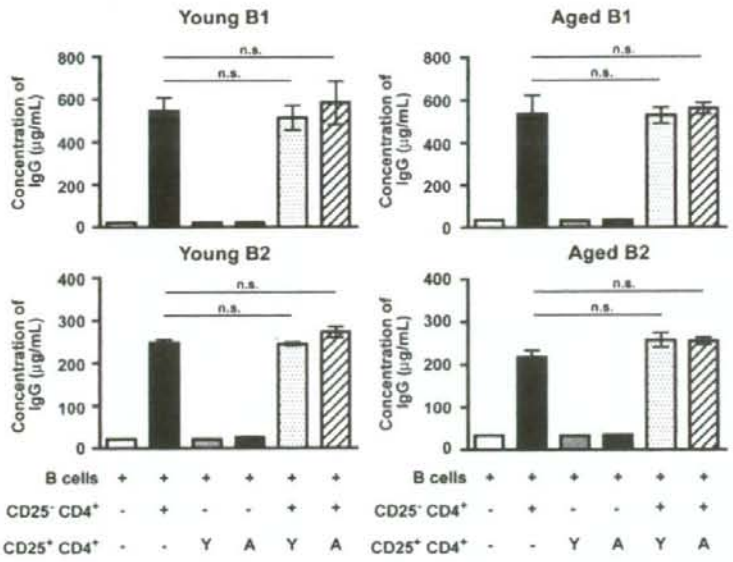

Figure 6. Inability of T_{reg} cells of BWF1 mice to suppress *in vitro* antibody production induced by $CD25^-$ $CD4^+$ T cells of aged BWF1 mice. Concentration of IgG antibody in the culture supernatant was measured by enzyme-linked immunosorbent assay after co-culture of T cells and B cells in the following combinations for 5 days. B cells alone, (white column); B cells + $CD25^-$ $CD4^+$ T cells (black column); B cells + $CD25^+$ $CD4^+$ T cells of young BWF1 (light gray column); B cells + $CD25^+$ $CD4^+$ T cells of aged BWF1 (dark gray column); B cells + $CD25^-$ $CD4^+$ T cells + $CD25^+$ $CD4^+$ T cells of young BWF1 (dotted column); B cells + $CD25^-$ $CD4^+$ T cells + $CD25^+$ $CD4^+$ T cells of aged BWF1 (striped column). $CD25^-$ $CD4^+$ T cells of aged BWF1 mice were used for all combinations. B-cell subsets used for each combination were indicated above each panel. Data are presented as mean ± SEM. n.s., not significant by one-way analysis of variance with Bonferroni's multiple comparison test. Representative of three independent experiments is shown.

compensate for that repertoire. It is therefore feasible that accumulation of T_{reg} cells is too late to control the pathogenic autoimmune response in aged BWF1 mice, or that antigen specificity of T_{reg} cells in aged BWF1 mice differ from those in young BWF1 mice. However, there are other possible mechanisms for the inability of T_{reg} cells to control the pathogenic autoimmune response in aged BWF1 mice as described below.

There are several reports suggesting a possible effect of T_{reg} cells on T-dependent B-cell responses.[19,39–41] It was, therefore, surprising that T_{reg} cells of BWF1 mice could not suppress the *in vitro* antibody production induced by $CD25^-$ $CD4^+$ T cells despite their intact suppressive activity against the proliferation of T cells *in vitro*. Possible explanations for our result are as follows: first, loss of the sensitivity of $CD25^-$ $CD4^+$ T cells of aged BWF1 mice to T_{reg} cell-mediated suppression; second, reversal of T_{reg} cell-mediated suppression by signaling through co-stimulatory molecules. OX40, 4-1BB, and ICOS have been implicated in the pathogenesis of lupus.[42–44] OX40 and 4-1BB magnify the T-cell response through induction of the proliferation of conventional T cells and inhibition of T_{reg} cell-mediated immune suppression.[37,45] The ICOS-mediated signal is essential for the induction of follicular helper T cells, thus it functions as an enhancer of B-cell response.[46] On the contrary, these molecules as well as ICOS also facilitate the expansion of T_{reg} cells.[36,45,47] B cells of aged BWF1 mice, however, did not show significant expression of ligands for these co-stimulatory molecules (data not shown). This observation implies that reversal of the suppression, if any, might take place through the other pathway(s). Also, $CD25^-$ $CD4^+$ T cells of aged, but not young, BWF1 mice contain $CXCR5^+$ $ICOS^+$ follicular helper T cells whose function may be resistant to T_{reg} cell-mediated suppression. Further studies with regard to the impact of T_{reg} cells on humoral immune response as well as the interaction between T_{reg} cells and their target cells will be required to clarify their role in antibody-mediated autoimmune diseases such as SLE.

Concomitant migratory behavior of T_{reg} cells and conventional T cells was shown to be crucial for the immunoregulatory function of T_{reg} cells.[26,31,32] Chemokines and their receptors, as well as the activation markers CD44, CD62L, CD69, and CD103, are the possible regulators of the migration of T cells. Our present data demonstrating similar localization of T_{reg} cells and conventional T cells with the comparable expression of chemokine receptors and activation markers between these cells suggest that regulation of the migratory behavior of these cells were not impaired; however, BWF1 mice still develop the fatal autoimmune response. This idea, together with our notion of intact suppressive activity, further suggests that failure of T_{reg} cells to control the disease is because of the other factor(s) residing in the microenvironment.

Collectively, we demonstrated that aged BWF1 mice developing lupus nephritis had increased $Foxp3^+$ $CD4^+$ T_{reg} cells with highly activated phenotype and altered localization, but with intact suppressive activity. Our present results may provide a clue to understanding the nature of T_{reg} cells in the lupus and also help to unveil the mechanisms of the failure of T_{reg} cells to control autoimmune responses. Further studies directed at these points would facilitate the development of novel strategies for the treatment of SLE.

Acknowledgments

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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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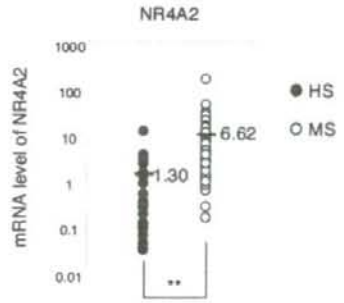

Fig. 1. Quantitative analysis of NR4A2 transcription between MS and controls. $CD3^+$ T cells were isolated from PBMC of 57 MS patients and of 19 healthy donors, and total RNA was extracted. cDNA was synthesized and the expression levels of NR4A2 transcript were analyzed by quantitative RT-PCR. Each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

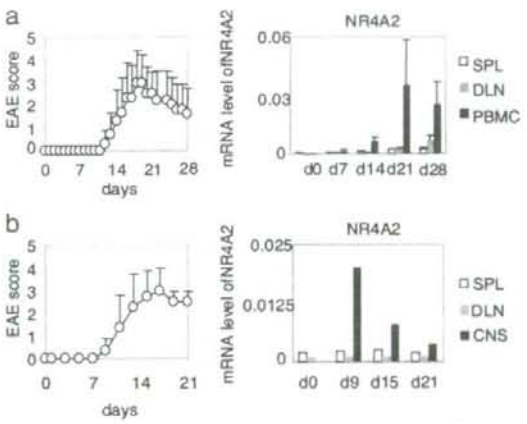

Fig. 2. Kinetic analysis of NR4A2 expression in the disease course of EAE. (a) (*Left*) EAE was induced in B6 mice by immunization with MOG_{35-55} in CFA. Mice were killed on days 7, 14, 21 and 28 after immunization, and T cells were isolated from dLN, SPL, or PBMC, using anti-CD3 magnetic beads. (*Right*)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

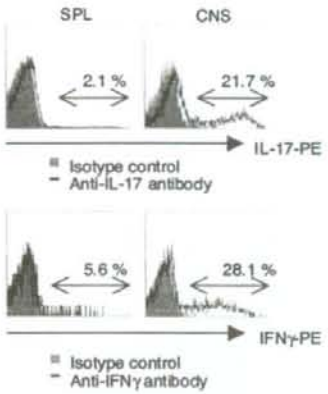

Fig. 3. Accumulation of IL-17 or IFN-γ-producing inflammatory T cells in the CNS. Mononuclear cells were isolated from spleen or CNS on day 17 after immunization and stimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) in the presence of 2 mM monensin for 4 h. Production of IL-17 and IFN-γ was analyzed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

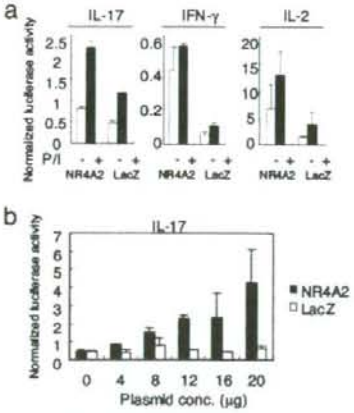

Fig. 4. Promoter activities of cytokine genes in the presence of NR4A2. (*a*) The effect of NR4A2 expression on IL-17, IFN-γ, and IL-2 promoter activity. A reporter plasmid containing promoter of cytokine gene (10 μg) and *Renilla* luciferase plasmid (100 ng) were introduced into EL4 cells by electroporation,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

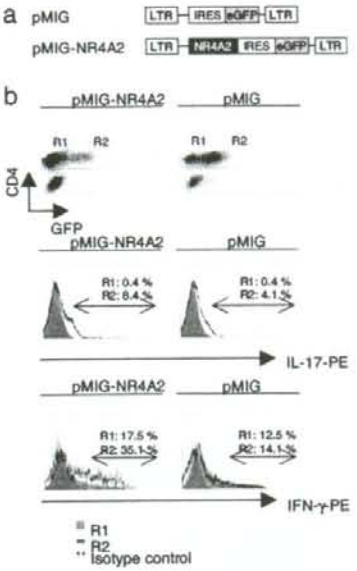

Fig. 5. The effect of retrovirally transduced NR4A2 on cytokine production by primary murine CD4⁺ T cells. (*a*) DNA fragments encoding wild-type NR4A2 were cloned into the pMIG(W) bicistronic retroviral vector. LTR, long terminal repeat; IRES, internal ribosome entry site; eGFP, enhanced green fluorescence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

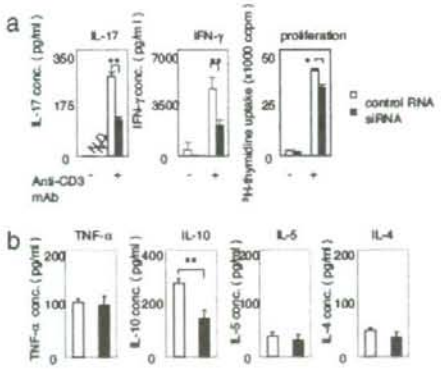

Fig. 6. The effect of NR4A2 gene silencing on T cell cytokine production. (*a*) Specific inhibition of T cell production of IL-17 and IFN-γ by siRNA treatment. Human CD4⁺ T cells derived from PBMC were transfected with siRNA or control RNA and stimulated by immobilized anti-CD3 Ab for 48 h. Cytokine 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.

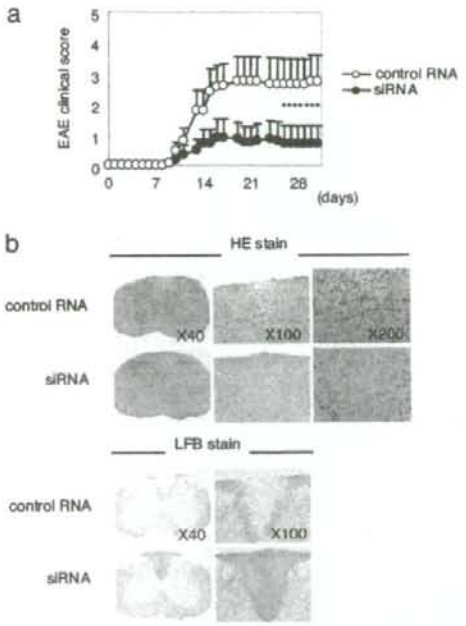

Fig. 7. The effect of T cell silencing of NR4A2 expression on passive EAE. (*a*) Inguinal and popliteal LNs cells were collected from female SJL/J mice 10 days after immunization with $PLP_{139-151}$, and were transfected with siRNA for NR4A2 or control RNA, using HVJ-E vector kit. The cells were cultured 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 [$(A/T)_5AGGTCA$] 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 hemagglutinating 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*.

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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$^+$ 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.

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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

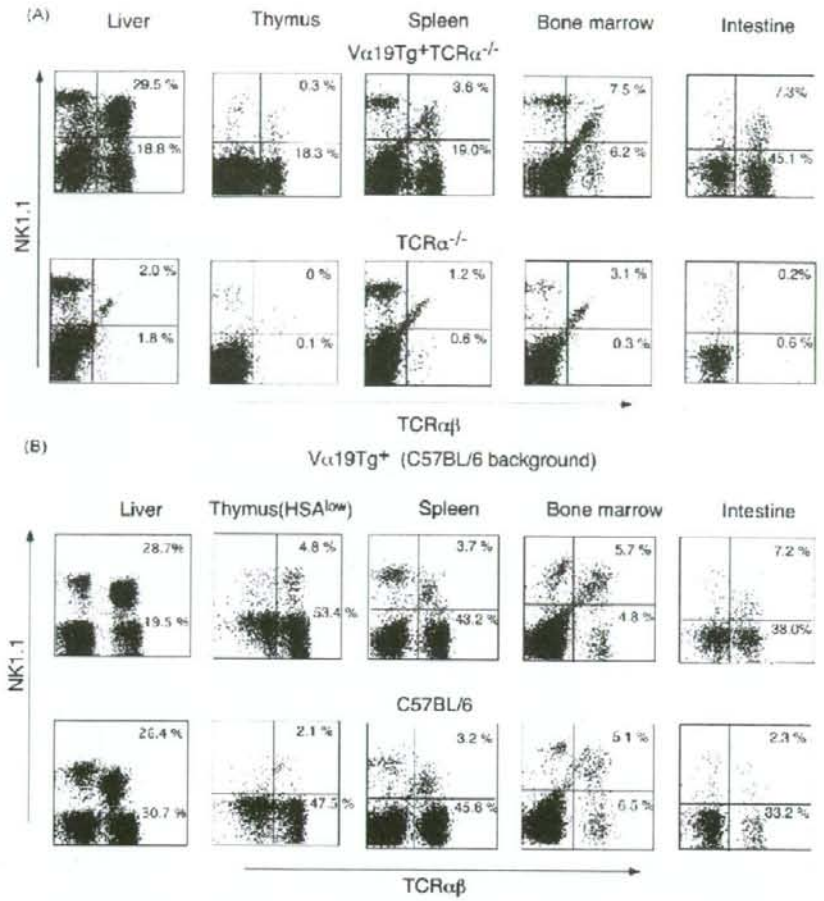

Fig. 1. Development of Vα19 NKT cells in Vα19 Tg mice. MNCs were isolated from liver, thymus, spleen, bone marrow and intestinal lamina propria of mice (at 8–12 weeks of age, female). They were stained with fluorescence-labeled antibodies and analyzed by FACS. (A) Staining profiles of the Vα19 Tg$^+$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

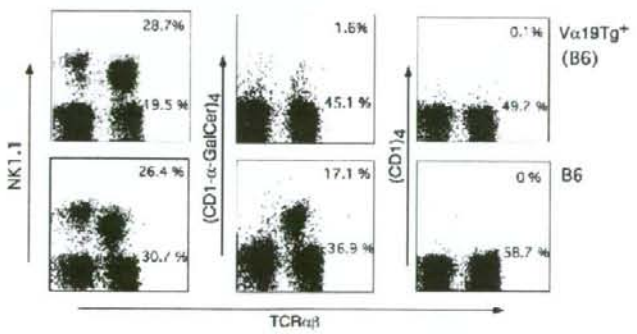

Fig. 2. Staining profiles of Tg- and non-Tg liver MNCs with CD1-α-Gal-Cer tetramers. Liver MNCs isolated from Tg and non-Tg mice on the C57BL/6 background (at 8–10 weeks of age, female, both in the range of 3–7 × 10^6 per animal) were stained with anti-TCR αβ antibody and either anti-NK1.1 antibody,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

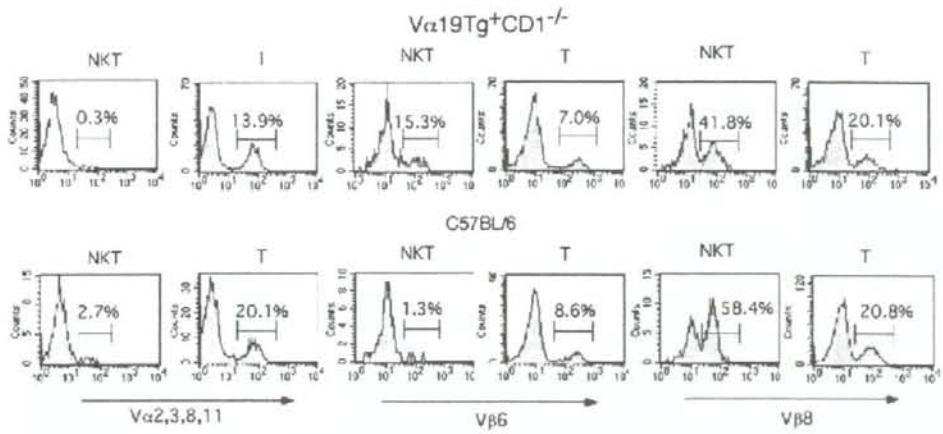

Fig. 3. Preferential expression of the invariant Vα19 TCR transgene by the NK1.1+, TCRα(+ cells in the Tg livers. Liver MNCs from Vα19 TCR Tg (CD1$^{-/-}$ background) and non-Tg mice (at 8 weeks of age, female) were triply stained with an anti-TCRαβ, anti-NK1.1 and anti-TCR Vα mixture (Vα2, 3.2, 8, 11) 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 ± 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.

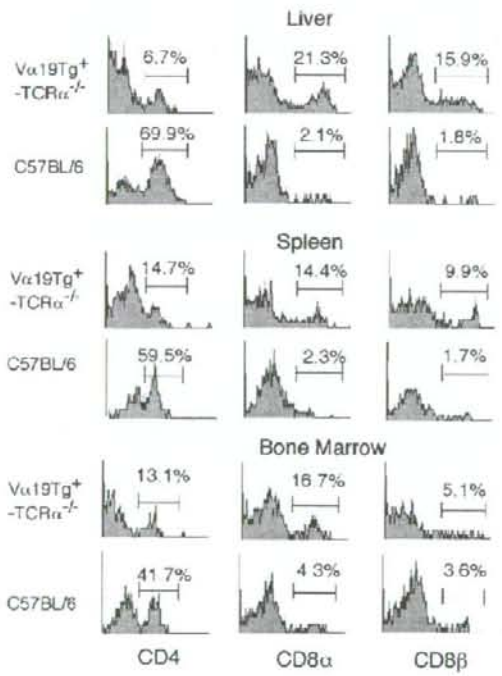

Fig. 4. CD4/CD8 co-receptor expression by NK1.1⁺, TCR αβ⁺ cells of Vα19 Tg⁺ and non-Tg mice. MNCs were isolated from liver, thymus, spleen, and bone marrow of Vα19 Tg⁺ TCRα$^{-/-}$ and C57BL/6 mice (at 8–12 weeks of age). They were immunostained and analyzed by flow cytometry. Profiles of CD4/CD8 co-receptor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

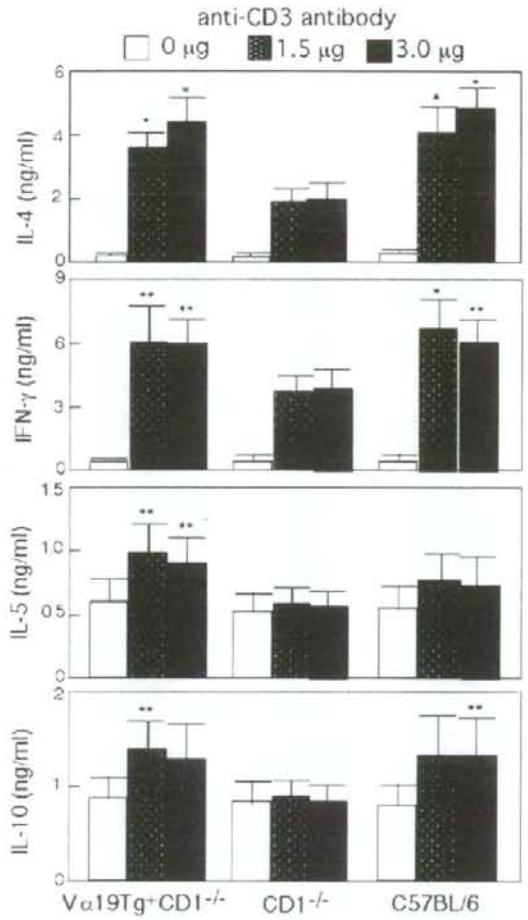

Fig. 5. Cytokine production by Tg and non-Tg mice in response to *in vivo* treatment with anti-CD3 antibody. Liver MNCs prepared from Vα19 Tg$^+$ CD1$^{-/-}$, CD$^{-/-}$, and C57BL/6 mice injected 90 min previously with a different dose of anti-CD3 antibody (2C11) were cultured in DMEM (10% FCS) without 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-

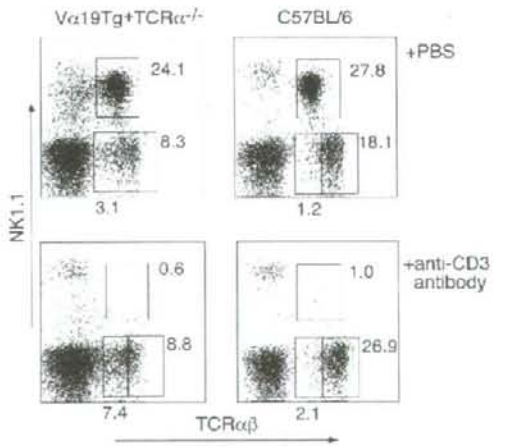

Fig. 6. Surface receptor down-regulation of hepatic Vα19 NKT cells upon TCR engagement in vivo. Liver MNCs were prepared from Vα19 Tg$^+$ TCRα$^{-/-}$ and C57BL/6 mice injected 24 h previously with anti-CD3 antibody (2C11, 2 μg/mouse). Cells were stained with anti-TCRαβ and anti-NK1.1 antibodies and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

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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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Immunopathology and Infectious Diseases

NKT Cell-Dependent Amelioration of a Mouse Model of Multiple Sclerosis by Altering Gut Flora

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Improved hygiene has been suggested to influence certain autoimmune disorders, such as multiple sclerosis. In this study, we addressed whether altering the composition of gut flora may affect susceptibility to experimental autoimmune encephalomyelitis (EAE), an animal model of MS. We administered a mixture of non-absorbing antibiotics, kanamycin, colistin, and vancomycin (KCV), orally to mice induced to develop EAE. The antibiotic treatment, beginning 1 week prior to sensitization, altered the composition of gut flora and, intriguingly, also ameliorated the development of EAE. While this result was associated with a reduced production of pro-inflammatory cytokines from the draining lymph node cells, a reduction of mesenteric Th17 cells was found to correlate with disease suppression. In addition, we found that Vα14 invariant NKT (iNKT) cells were necessary for maintaining the mesenteric Th17 cells. The homologous effects of KCV treatment and iNKT cell depletion led us to speculate that KCV treatment may suppress EAE by altering the function of iNKT cells. Consistent with this hypothesis, KCV treatment did not suppress EAE that was induced in iNKT cell-deficient mice, although it was efficacious in mice that lacked Vα19 mucosal-associated invariant T cells. Thus, gut flora may influence the development of EAE in a way that is dependent on iNKT cells, which has significant implications for the prevention and treatment of autoimmune diseases. *(Am J Pathol 2008, 173:1714–1723; DOI: 10.2353/ajpath.2008.080622)*

The immunopathology of autoimmune diseases is still poorly understood, although comprehensive and multidisciplinary approaches continue to give us new insight into the mechanisms of disease. Previous studies have generally supported a pathogenic role of interferon (IFN)-γ-producing Th1 cells in autoimmune diseases such as multiple sclerosis (MS) that affect the central nervous system (CNS).[1] As Th1 cells are cross-regulated by Th2 cells producing interleukin (IL)-4, IL-5, and IL-13, the counterbalance between Th1 and Th2 cells has been posed as a key issue in understanding the pathogenesis of MS.[2] However, the traditional "Th1/Th2" paradigm is now facing a fundamental challenge since a third class of helper CD4+ T cells, named Th17 cells, have been found to cause autoimmune inflammation.[3–5] Th17 cells are IL-23-dependent cells that are distinct from Th1 and Th2 cells in their ability to produce IL-17[6–8] and their use of the RORγt transcription factor.[9] Although the relationship between Th17 cells and Th1 or Th2 cells remains to be fully characterized, Th17 cells are likely to exert a predominant pathogenic activity in various inflammatory conditions associated with autoimmunity or allergy either independently or collaboratively with Th1 cells.[10]

It is widely accepted that development of autoimmune disease is under control of both genetic and environmental factors. For example, recent whole genome analysis has revealed that several genes including human leukocyte antigen-DR are positively linked with the susceptibility to MS.[11] In contrast, most of our knowledge about environmental factors relies on epidemiological data. Results of migration studies, as well as the reported presence of clusters or outbreaks of MS, have illustrated potential environmental influences on MS, including infection, stress, sunlight exposure, and sex hormone.[12–14] While an altered intestinal microflora has been suggested to be an environmental risk factor for rheumatoid arthritis,[15] inflammatory bowel disease,[16] and human allergy and asthma,[17] the status of gut flora has rarely been evaluated as a potential risk factor for MS.

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Recent studies have shown that animals bred in a germfree environment are characterized by having low densities of lymphoid cells in the gut mucosa, a reduced size of specialized follicle structures, and low concentrations of immunoglobulins in the peripheral blood.[18–21] It is also of note that the intestinal lamina propria (LP) has been identified as a site that is constitutively inhabited by Th17 cells.[9] Thus the dialogue between host and bacteria at the mucosal interface seems to be critical in the development of the competent immune system.

To explore a possible role of intestinal microflora in the development of autoimmune disease, we tested if oral administration of the mixture of non-absorbing antibiotics kanamycin, colistin, and vancomycin (KCV) could modify the development of experimental autoimmune encephalomyelitis (EAE) induced in C57BL/6 (B6) mice sensitized against a myelin oligodendrocyte glycoprotein (MOG) peptide of amino acids 35 to 55 [MOG (35–55)]. Here we report that continuous oral KCV treatment, starting one week before immunization, significantly suppressed the development of EAE along with altering gut flora. Suppression of EAE was accompanied by a reduced production of pro-inflammatory cytokines from the draining lymph nodes (dLNs) in response to MOG (35–55). While the antibiotic treatment suppressed MOG (35–55) reactive Th17 cells within the mesenteric lymph nodes (MLNs), it also reduced the total number of mesenteric Th17 cells in naïve mice. Furthermore, unexpectedly we found that the Th17 cells in the MLNs are greatly reduced in *Cd1*$^{-/-}$ mice or *Jα281*$^{-/-}$ mice, which lack invariant Vα14 natural killer T (iNKT) cells,[22] and that the KCV-induced reduction of the mesenteric Th17 cells was only marginal in the iNKT cell-deficient mice. As such, KCV treatment and iNKT cell deletion showed homologous effects on the mesenteric Th17 cells, which led us to speculate that gut flora may influence the development of CNS autoimmune disease in a way dependent of iNKT cells. Consistently, oral KCV treatment did not alter the development of EAE in iNKT cell-deficient mice. These results indicate that iNKT cells play a critical role in the dialogue between host and commensal flora.

Materials and Methods

Mice and Induction of EAE

Six-week-old female B6 mice were purchased from CLEA Laboratory Animal Corporation (Tokyo, Japan). *Mr1*$^{-/-}$ mice were provided by Dr. Susan Gilfillan, (Washington University School of Medicine, St. Louis)[23] and were backcrossed to B6 mice for ten generations. *β2-microglobulin*$^{-/-}$ mice were purchased from Jackson Laboratories. Cd1 $^{-/-}$[24] and *Jα281*$^{-/-}$[25] mice were provided by Dr. Steve B. Balk (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA) and Dr. Masaru Taniguchi (Riken Research Center for allergy and Immunology, Yokohama, Japan) respectively. These mice were also back-crossed to B6 mice for ten generations. Animals were maintained in specific pathogen-free conditions in accordance with the institutional guidelines. For induction of EAE, B6 mice were injected subcutaneously with 100 μg MOG (35–55) (MEVGWYRSPFSRVVHLYRNGK) (TORAY Laboratory, Tokyo, Japan) and 1 mg heat-killed *Mycobacterium tuberculosis* H37RA (Difco) emulsified in incomplete Freund's adjuvant. 200 ng of pertussis toxin (List Biological Laboratories) in 200 μl PBS was injected i.p. on days 0 and 2 after immunization. Clinical symptoms of EAE were daily evaluated and scored as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, impaired righting reflex; 3, partial hindlimb paralysis; 4, total hindlimb paralysis; 5, moribund or dead.

Antibiotic Treatment of Mice

To treat mice with a mixture of non-absorbing antibiotics, we used a previously described protocol after adding minor modifications.[26] Briefly, to examine the effects of altering gut flora, a group of mice were given *ad libitum* access to drinking water supplemented with kanamycin (1 mg/ml), colistin (2000 U/ml), and vancomycin (0.1 mg/ml). Normal drinking water was given to another group of mice serving as control. For immunological studies of MLNs, LPLs, and splenocytes, the antibiotic-containing water was continuously given for 1 week until individual experiments were conducted. To evaluate the effect of antibiotics on EAE and recall responses, the treatment was started 1 week before immunization, and continued during the entire observation period.

Cell Proliferation and Cytokine Analysis

To measure cell proliferation and cytokine production, we stimulated lymph node cells (1×10^6/well) with anti-CD3 antibody (2C11) at 5 μg/ml for 72 hours in 96-well round-bottomed plates. For evaluating MOG (35–55)-specific recall responses, we stimulated lymph node cells (1×10^6/well) with MOG (35–55) peptide at 1 to 100 μmol/L for 72 hours. The cells were suspended in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/ml of penicillin-streptomycin, 2 mmol/L sodium pyruvate and 50 mmol/L β-mercaptoethanol. T cell proliferation to MOG (35–55) was determined by measuring the incorporation of [^{3}H] thymidine (1 μCi/well) during the last 24 hours of culture in a β-1205 counter (Pharmacia, Uppsala, Sweden). Assays were conducted in triplicate wells and data were expressed as counts per minute (c.p.m.). Culture supernatant was collected 72 hours after stimulation, and cytokines in the supernatant were measured by using cytometric bead array kits for mouse inflammatory cytokines (BD Biosciences) and IL-17 enzyme-linked immunosorbent assay (ELISA) kit (R&D systems).

Surface Marker Analysis, Quantification of CNS Leukocytes and Histology

Cells were stained with fluorescence-labeled specific antibodies after incubation with anti-CD16/32 to avoid non-specific staining and were analyzed with a FACSCalibur

(BD). Except for Foxp3-APC from eBioscience, all of the other antibodies were obtained from BD Pharmingen. For flow cytometric analysis of CNS-infiltrated cells, spinal cords were homogenized, passed through 70-μm nylon mesh and separated by Percoll density-gradient centrifugation to obtain single-cell suspensions. In some experiments, paraffin-embedded spinal cords were stained with either luxol fast blue or H&E for conventional histological analysis.

Intracellular Staining

Cells collected from MLN were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml) and Ionomycin (750 ng/ml) for 5 hours in the presence of GolgiPlug (BD Biosciences). Cells were first stained extracellularly with PerCP-conjugated anti-CD4, APC-conjugated anti-T cell receptor-β and α-GalCer-loaded Dimer X recombinant soluble dimeric mouse CD1 days (BD Pharmingen), and then stained with fluorescein isothiocyanate-conjugated mAb A85-1 specific for mouse IgG1 (BD Pharmingen), and fixed and permeabilized with Fixation/Permiabilization solution (BD Biosciences). Finally, cells were stained intracellulary with phycoerythrin-conjugated anti-IL-17 (BD Biosciences). Samples were acquired on a FACSCalibur (BD Biosciences), and data were analyzed with CELLQuest software (BD Biosciences).

Isolation of Lamina Propria Lymphocytes

Intestines were removed from euthanized mice and placed in ice-cold PBS containing 25 mmol/L HEPES. After removal of residual mesenteric fat tissue, Peyer's patches were carefully excised, and the intestine was opened longitudinally. The intestine was then thoroughly washed in ice-cold PBS and cut into 1.5-cm pieces. The pieces were incubated four times in 5 ml of 5 mmol/L EDTA, in 10% fetal calf serum/25 mmol/L HEPES/PBS for 15 minutes at 37°C with fast rotation (200 rpm). After each round of incubation, the epithelial cell layer, containing the intraepithelial lymphocytes, was removed. After the fourth EDTA incubation, the pieces were washed in PBS, and placed in 25 ml of RPMI containing 20% fetal calf serum, 25 mmol/L HEPES, and 300 U/ml of Collagenase H (Roche). Digestion was performed three times by incubating the pieces at 37°C for 40 minutes with slow rotation (100 rpm). The solution was then vortexed intensely and passed through a 70-mm cell strainer. The pieces were collected and placed into fresh digestion solution. The procedure was repeated three times. Supernatants from all three digestions from a single small intestine were combined, washed once in cold PBS, resuspended in 5 ml of the 40% fraction of a 40:80 Percoll gradient, and overlaid on 2 ml of the 80% fraction in a 15 ml Falcon tube. Percoll gradient separation was performed by centrifugation for 20 minutes at 2800 rpm at room temperature. LPLs were collected at the interphase of the Percoll gradient, washed once, and resuspended in FACS buffer or T cell medium. The cells were used immediately for experiments.

RNA Extraction and Real-Time Reverse Transcription-PCR

The SV Total RNA isolation kit (Promega) was used for isolation of total RNA from mesenteric lymphocytes or splenocytes according to the manufacturer's instruction. First-strand cDNA was generated with the Advantage-RT kit (Clontech). The Light Cycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) was used for quantitative PCR analysis. Gene expression values were normalized to expression of the hypoxanthine guanine phosphoribosyl transferase (*Hprt*) as 'housekeeping' gene. QuantiTect Primer Assay (Qiagen) was used for amplification of IL-21 and IL-23. The other primers used were as follows: HPRT forward, 5'-GTTGGATACAGGCCAGACTTTGTTG-3'; HPRT reverse, 5 '-GAGGGTAGGCTGGCCTATAGGCT-3'; RORγtforward, 5'-TGTCCTGGGCTACC CTACTG-3'; RORγt reverse, 5'-GTGCAGGAGTAGGCCACATT-3'; TGF-β1 forward, 5'-TGCGCTTGCAGAGATTAAAA-3'; TGF-β1 reverse, 5'-GCTGAATCGAAA GCCCTGTA-3'; IL-6 forward, 5'-TTCCATCCAGTTGCCTTCTT-3'; IL-6 reverse, 5'-CAGAATTGCCQATTGCCATTGCACAAC-3'.

Statistics

EAE clinical severity was daily scored as mean ± SEM for each group, and analyzed by the Mann-Whitney U nonparametric ranking test. Differences in cumulative scores of each group of mice were evaluated by Student's *t*-test. Cytokine secretion data were analyzed with Student's *t*-test.

Results

Oral KCV Treatment Suppressed the Development of EAE and Inhibited Pro-Inflammatory Cytokine Production from Draining Lymph Node Cells

With an attempt to modulate the composition of intestinal flora, we treated wild-type B6 mice orally with a combination of antibiotics KCV as described in Materials and Methods. Because these antibiotics are not absorbed through gut mucosa,[27] any effect caused by this treatment is thought to arise from within the gut lumen. To examine whether our treatment protocol would change the composition of intestinal flora, we applied the DNA microarray system referred to as 'FloraArray'[28] and made a comprehensive analysis for intestinal flora derived from KCV-treated mice and control mice. To compare the signal intensities of intestinal flora from the two groups of mice, MA plots were illustrated from the fluorescent images. Although each spot on the FloraArray is derived from a number of different strains in the commensal microflora, this analysis gives us useful information regarding the composition of gut flora. The MA-plot analysis revealed that 722 out of 1536 spots showed more than twofold increase in the fecal DNA sample from KCV-treated mice as compared with those from control mice. By contrast, 894 spots showed more than twofold in-

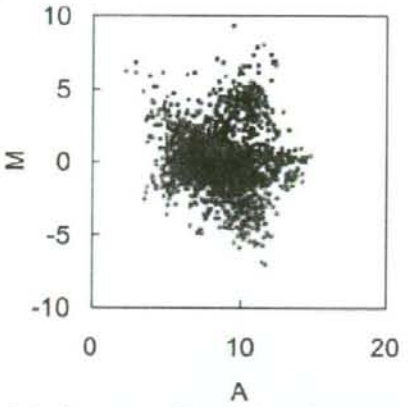

Figure 1. Altered composition of the intestinal microflora by oral administration of antibiotics KCV. A custom DNA microarray named FloraArray[27] was used for evaluating the gut flora of mice. Briefly, genomic DNA was extracted from freshly collected fecal samples and fragmented by physical force. DNA fragments of approximately 2.0 kb were inserted into the pUC vector to construct a shotgun library. Plasmid DNA was then extracted from this library. A DNA microarray was fabricated by spotting the randomly selected plasmid DNA without amplification on a glass slide. For analysis of sample DNA by the array, genomic DNA was extracted from fecal content of either control or KCV-treated mice after 1-week treatment with antibiotics KCV, and purified DNA was labeled with Cy3 or Cy5, respectively. Then fluorescent images were analyzed by scanning the array after performing competitive hybridization with mixed labeled DNA on the array. To compare the signal intensities between the two samples with or without antibiotics treatment, the data spots were displayed as MA plots. Red circles and blue circles represent data of samples from control and KCV-treated mice, respectively.

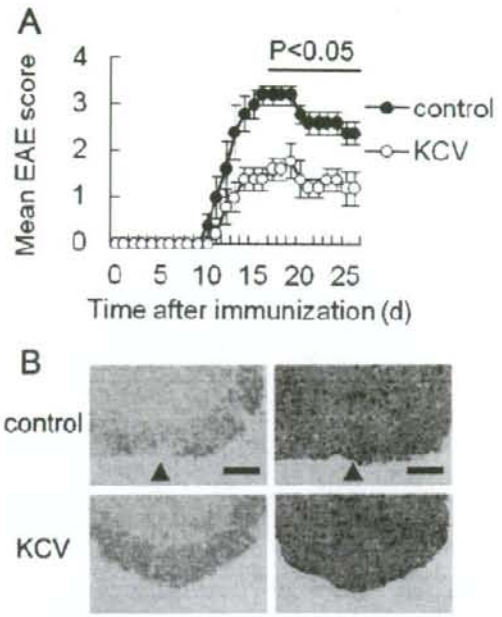

Figure 2. Suppression of EAE by oral KCV treatment. **A:** Clinical score of EAE. After immunized with MOG (35–55) mice were treated with KCV as described in Materials and Methods. Clinical EAE scores of KCV-treated mice (KCV) and of control mice (control) are shown. Data represent mean score ± SEM from a representative of three experiments ($n = 5$ for each group of mice). The bar indicates the duration during which a significant difference was observed between KCV and control; $^*P < 0.05$ (Mann-Whitney *U*-test). **B:** Histopathological assessment of the CNS region in EAE-induced mice. Shown are cellular infiltration and demyelination (**arrowheads**) of the lumbar spinal cord of control or KCV-treated mice on day 18. Paraffin-embedded spinal cords were stained with luxol fast blue (left panels) or H&E (right panels). Representative figures from two separate experiments are demonstrated. Scale bar = 100 μm.

crease in fecal DNA from control mice as compared with the mice treated with antibiotics (Figure 1). We additionally performed quantitative PCR analysis and revealed that the antibiotic treatment caused differential and reciprocal changes in the quantity of each bacterium species. For example, a great reduction of *Lactobacillus murinus* and *Bacteroides fragilis* was seen in the feces from KCV-treated mice, whereas *Bacteroides thetaiotaomicron* was significantly increased in the same samples of feces (data not shown). These results demonstrate that the protocol of the antibiotic treatment significantly affects the content of intestinal flora.

We next addressed whether the change of intestinal flora could also modulate the progression of EAE, an animal model of MS. When we continuously treated the mice with KCV-containing drinking water from 1 week before immunization, clinical signs of MOG (35–55)-induced EAE were significantly suppressed in comparison with control mice (Figure 2A). Accordingly, histological examination showed a reduced infiltration of mononuclear cells and less noticeable demyelination at the lumbar region of the spinal cord of the treated mice (Figure 2B). Moreover, we observed a lower number of total CNS infiltrating cells at an active stage of EAE (day 18) in KCV-treated mice than in control mice when we isolated mononuclear cells from CNS of those mice (data not shown). In parallel, we examined the recall responses of the dLNs to MOG (35–55) on day 11 after immunization. Although proliferation rates of the dLNs in response to MOG (35–55) were comparable between KCV-treated mice and control mice (Figure 3A), the dLN cells from the treated mice produced significantly lower amounts of pro-inflammatory cytokines IFN-γ, TNF-α, IL-6, and IL-17 in response to MOG (35–55) (Figure 3B), consistent with the suppressed signs of EAE. We also measured the recall response of the MLNs to MOG (35–55). The MLN cells from control mice immunized with MOG (35–55) showed significant responses to the MOG peptide in the proliferative responses as well as IL-17 production (Figure 3C). However, those from KCV-treated mice showed only marginal responses, indicating that induction of MOG (35–55) specific encephalitogenic Th17 cells in both dLNs and MLNs is impaired by an alteration of intestinal contents caused by the antibiotic treatment.

Mesenteric Lymphocytes from Naive Mice Produce a Lower Amount of IL-17 after KCV Treatment

MLNs are thought to offer an important site for the functional cross talk between intestinal microflora and gut immunity.[29,30] Next we investigated whether the antibiotic treatment induced an alteration of the MLN cell functions