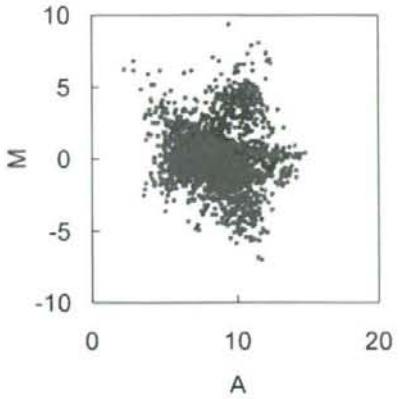

Figure 1. Altered composition of the intestinal microflora by oral administration of antibiotics KCV. A custom DNA microarray named 'FloraArray'[27] was used for evaluating the gut flora of mice. Briefly, genomic DNA was extracted from freshly collected fecal samples and fragmented by physical force. 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.

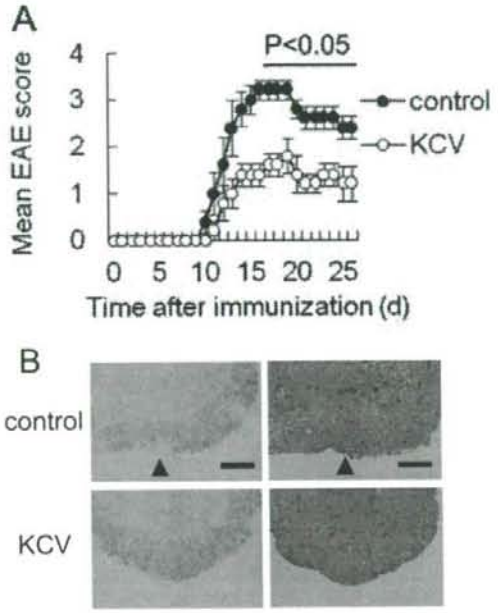

Figure 2. Suppression of EAE by oral KCV treatment. **A:** Clinical score of EAE. After immunized with MOG (35–55) mice were treated with KCV as described in Materials and Methods. Clinical EAE scores of KCV-treated mice (KCV) and of control mice (control) are shown. Data represent mean score ± SEM from 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

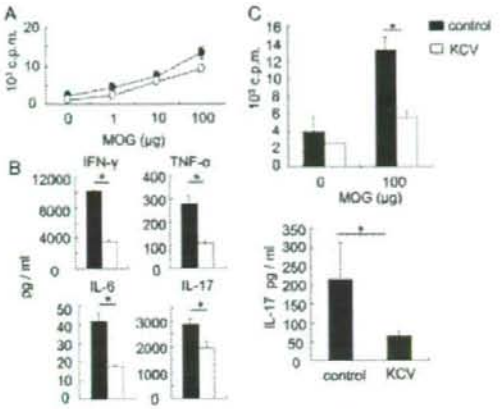

Figure 3. Reduced MOG-specific responses in dLN and MLN cells from KCV-treated mice. **A:** Effect of KCV on the lymphocyte proliferative responses. Draining lymph nodes (dLNs) were removed from control or KCV-treated mice 11 days after immunization with MOG (35–55) and the total lymphoid cells (1×10^6) were stimulated with varying doses of MOG (35–55) peptide for 72 hours. Proliferative responses were assessed by [^{3}H] thymidine incorporation. Data are from one of three independent experiments, showing the mean of triplicate samples. **B:** Effect of KCV treatment on MOG (35–55)-reactive T cells in the dLN. Supernatants were collected after stimulating the dLN cells of day 11 with 100 μmol/L MOG (35–55) peptide *in vitro* for 72 hours. Cytokine concentration was measured by cytometric bead array or ELISA as described in Materials and Methods. Data represent the mean ± SEM of duplicated samples from one of three separate experiments ($n = 2$ mice). *$P < 0.05$ (two-tailed Student's *t*-test.) **C:** Effect of KCV treatment on MOG (35–55)-reactive T cells in the MLN. Whole MLN cells were isolated from control or KCV-treated mice ($n = 2$) 11 days after EAE induction. The cells were stimulated with MOG (35–55) as conducted for dLN cells and proliferative responses (upper panel) and IL-17 production (lower panel) were measured. IL-17 was measured by using ELISA. Data represent the mean ± SEM of triplicate samples from one of two independent experiments ($n = 2$ mice). *$P < 0.05$ (two-tailed Student's *t*-test).

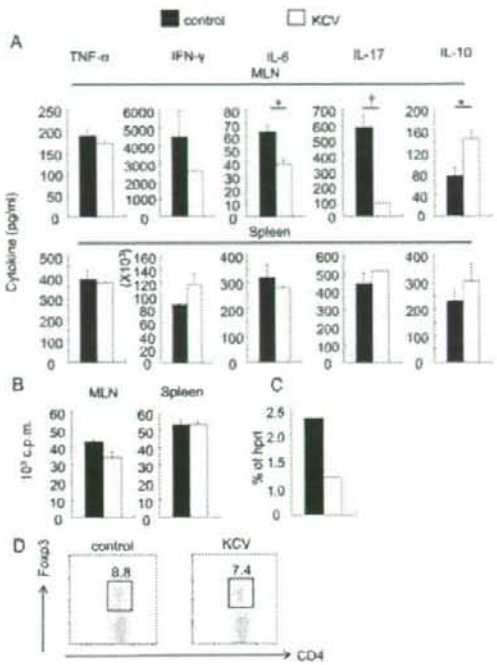

Figure 4. Decreased production of inflammatory cytokines from MLN cells after oral KCV treatment. **A:** Cytokine production from MLN T cells of naïve mice after KCV treatment. Mice were continuously given KCV-containing or control water for 7 days. Then MLN cells and splenocytes were isolated and stimulated by immobilized anti-CD3. MLNs (top panels) or splenocytes (bottom panels) from control or KCV-treated mice (unprimed) were stimulated with immobilized anti-CD3 antibody for 72 hours. Cytokines in the supernatants were measured by using cytometric bead array or ELISA. Data are from a representative out of three independent experiments ($n = 2$ mice). *$P < 0.05$, †$P < 0.001$, (Student's *t*-test). **B:** Proliferative responses of MLN cells after anti-CD3 stimulation. MLN cells and splenocytes were prepared as described in (**A**). Proliferative responses were assessed by [^{3}H] thymidine incorporation. Data represent the mean ± SEM of triplicate samples from one out of three independent experiments ($n = 2$ mice). *$P < 0.05$ (Student's *t*-test). **C:** Reduction of RORγt expression after KCV treatment. Total RNA was isolated from pooled MLN cells prepared from control mice or from mice given oral KCV treatment for 7 days. RORγt mRNA was estimated by quantitative RT-PCR and all data were normalized to hprt ($n = 2$ mice). **D:** Intracellular expression of Foxp3 for gated CD4$^+$ T cells derived from MLNs. Mice were given control or KCV-containing water for 1 week. Dot plots are gated on CD4$^+$ T cells. Data are representative of three independent experiments showing similar results.

in naïve wild-type mice. First we compared the ability of the MLN cells to produce pro-inflammatory cytokines on stimulation with plate-bound anti-CD3 antibody. Proliferative responses of the MLN cells were not affected or slightly suppressed at most by KCV treatment. Interestingly, MLN cells from KCV-treated mice secreted significantly lower amounts of IL-6 and IL-17 compared with those from control mice, whereas production of TNF-α and IFN-γ was not significantly suppressed (Figure 4, A and B). In contrast, splenocytes from both groups of mice showed essentially similar result following stimulation with anti-CD3 (Figure 4, A and B). Recently, Ivanov et al showed that an orphan nuclear receptor RORγt is the key transcription factor that orchestrates the differentiation of the Th17 cell lineage.[9] They also showed that Th17 cells tend to accumulate in the mucosa of the small intestine. Quantitative RT-PCR analysis revealed a lower expression of RORγt in the MLN cells from KCV-treated mice as compared with control mice (Figure 4C). We also found that the MLN cells from KCV-treated mice secreted significantly greater amounts of IL-10 than those from control mice (Figure 4A), suggesting that the mesenteric T cells would acquire less inflammatory properties after the antibiotic treatment.

Next we examined whether this treatment may alter the composition of lymphocytes in the MLN. We found that the total number of MLN cells was almost equal in KCV-treated and control mice (data not shown). Furthermore, flow cytometric analysis demonstrated that the proportion of dendritic cells, macrophage/monocytes, B cells, conventional CD4$^+$ and CD8$^+$ T cells, NK cells, and NKT cells in the MLN did not change after treatment with KCV (data not shown). These data indicate that the antibiotic treatment protocol does not exhibit any cytotoxic effect on the mesenteric lymphocyte populations, although it remarkably alters the cytokine profile of T cells. We also examined the frequency of Foxp3$^+$ regulatory CD4$^+$ T cells in the MLN. Although recent studies have revealed the presence of reciprocal developmental pathways between Th17 cells and Foxp3$^+$ regulatory T cells,[31] we

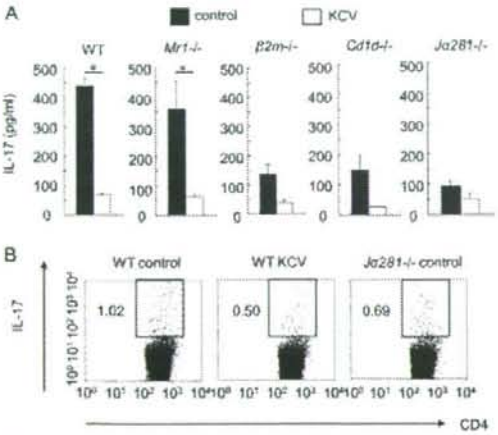

Figure 5. A role of Vα14 iNKT cells in the regulation of mesenteric Th17 cells. **A:** IL-17 production by the MLN T cells of mice lacking invariant iNKT or MAIT cells. After 1 week of KCV treatment, MLN cells were isolated from control or KCV-treated mice, including wild-type (WT), *Mr1*$^{-/-}$, *β2m*$^{-/-}$, *Cd1*$^{-/-}$, or *Jα281*$^{-/-}$ mice. The cells were stimulated with immobilized anti-CD3 antibody for 72 hours. IL-17 in the supernatant was measured using ELISA. Data are a representative of two independent experiments ($n = 2$ mice). *$P < 0.05$ (Student's *t*-test). **B:** Th17 cells in MLNs in KCV-treated or iNKT deficient mice. MLN cells were isolated from wild-type mice (WT control), KCV-treated wild-type mice (WT KCV), or iNKT cell-deficient *Jα281*$^{-/-}$ mice and stimulated for 5 hours with phorbol 12-myristate 13-acetate and ionomycin in the presence of GolgiPlug. We conducted surface labeling with the indicated antibody and αGalCer-loaded CD1 days dimer as well as intracellular IL-17 staining. Dot plots are gated on CD4$^+$ T cells devoid of iNKT cells. Data are representative of two independent experiments ($n = 2$ mice).

could not detect any increase of CD4$^+$Foxp3$^+$ T cells in the MLN cells after KCV treatment (Figure 4D).

A Role of Vα14 iNKT Cells in the Regulation of Mesenteric Th17 Cells that Are Vulnerable to KCV Treatment

Recent studies have revealed that MR1-restricted invariant Vα19-Jα33 T cells, also referred to as mucosal associated invariant T (MAIT) cells, are preferentially distributed to gut LP and are strikingly influenced by the presence of gut flora.[23,32] We have recently shown that the MAIT cells could play a regulatory role in EAE.[33] Because of their dependence on commensal flora[23,32] we speculated that the antibiotic treatment might suppress the Th17 cell-mediated EAE disease by using the regulatory function of MAIT cells triggered by a change of flora. To verify this idea, we treated MAIT cell-deficient *Mr1*$^{-/-}$ mice as well as wild-type B6 mice with oral KCV, and examined the ability of the MLN cells to produce IL-17 after anti-CD3 stimulation. Contrary to our speculation, the results showed that the MLN cells from *Mr1*$^{-/-}$ mice and wild-type mice produced an equivalent amount of IL-17 either before or after KCV treatment (Figure 5A), indicating that MAIT cells do not play a major role in the suppression of Th17 cells by KCV treatment. However, in additional experiments using *β2-microglobulin*$^{-/-}$ (*β2m*$^{-/-}$) mice, we found that the baseline production of IL-17 by the MLN T cells after anti-CD3 stimulation was remarkably diminished in the mice, whereas the mesenteric T cells from *β2m*$^{-/-}$ mice and wild-type mice produced a similar amount of IL-17 after KCV treatment. Accordingly, oral KCV causes only a marginal reduction of IL-17 in *β2m*$^{-/-}$ mice, indicating that class I-restricted T cells other than MAIT cells play a critical role in the KCV-induced suppression of the Th17 cells within MLN.

Then we explored a possible role of Vα14 iNKT cells restricted by CD1 days, an MHC class 1b molecule. As is widely known, iNKT cells produce a variety of regulatory cytokines after recognizing glycolipid antigens such as α-galactocylceramide (α-GalCer) in association with CD1 days. Numerous reports have supported the role of iNKT cells in the regulation of autoimmunity.[22,34,35] We, therefore, repeated our above experiments using *Cd1*$^{-/-}$ mice,[24] which do not express either iNKT cells or non-invariant type II NKT cells,[36] as well as with *Jα281*$^{-/-}$ mice,[25] in which iNKT cells alone are specifically deleted. In these iNKT cell-deficient mice, we again found a great reduction in the baseline production of IL-17 from the MLNs after anti-CD3 stimulation. Furthermore, effects of oral KCV on the Th17 cells were only marginal, if any, in the mice (Figure 5A), raising a possibility that the host immune system may sense the change of gut flora by using iNKT cells.

It is now known that IL-17 secreting CD4$^+$ MLN cells comprise not only Th17 cells but also CD4$^+$ Vα14 iNKT cells.[37] To evaluate the alteration of mesenteric Th17 cells with accuracy, we next evaluated the proportion of IL-17$^+$ CD4$^+$ T cells after excluding iNKT cells by gating. By analyzing the MLN cells from wild-type mice (WT control), KCV-treated wild-type mice (WT KCV), or iNKT cell-deficient *Jα281*$^{-/-}$ mice (Figure 5B), we have confirmed that the number of IL-17$^+$ CD4$^+$ T cells corresponding to Th17 cells is reduced in the KCV-treated wild-type mice and in the iNKT cell-deficient *Jα281*$^{-/-}$ mice. We also noticed that IL-17$^+$ iNKT cells are 15 times lower than IL-17$^+$ CD4$^+$ T cells in wild-type mice (data not shown).

Oral KCV Treatment Inhibits Production of Th17-Promoting Cytokines in the Intestinal Lamina Propria

Next we sought to identify a primary event that would take place in the intestinal immune system following oral KCV treatment. Because the vast majority of Th17 cells in the MLNs appear to depend on iNKT cells (Figure 5A), we evaluated the number and function of iNKT cells in the MLNs. However, neither reduction nor increase of iNKT cells was found in the MLNs after the antibiotic treatment (data not shown). In addition, the MLN cells from KCV-treated mice and from control mice produced similar levels of cytokines in response to α-GalCer (data not shown). These results indicate that as seen with CD4$^+$Fox3$^+$ T cells (Figure 4D), iNKT cells in the MLN are not significantly influenced by the status of gut flora. Therefore, we postulate that local accumulation of regulatory cells is probably not the mechanism for the reduction of Th17

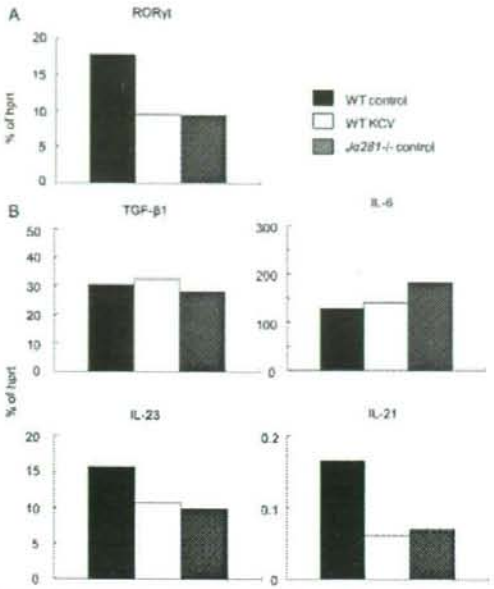

Figure 6. Reduced expression of Th17-promoting cytokines in the intestinal lamina propria lymphocytes from KCV-treated mice as well as iNKT-deficient mice. **A:** RORγt expression in the intestinal LP after treatment with oral KCV. Total RNA was isolated from pooled LPLs prepared from wild-type control B6 mice (WT control), KCV-treated wild-type B6 mice (WT KCV), and control $J\alpha281^{-/-}$ mice ($J\alpha281^{-/-}$ control) ($n = 2$). Wild-type KCV were given oral KCV for 7 days before the analysis. RORγt mRNA was estimated by quantitative RT-PCR and all data were normalized to hprt. Data are representative of two independent experiments. **B:** Expression of Th17-promoting cytokines in the intestinal LPL. Total RNA was isolated from LPLs of the three groups of mice as described in (**A**). Expression of TGFβ−1, IL-6, IL-23, and IL-21 mRNA was estimated by quantitative RT-PCR and all data were normalized to hprt. Data are a representative of two independent experiments.

cells in the MLN of KCV-treated mice (Figures 3 and 4). By using quantitative RT-PCR, we also measured mRNA expression of TGF-β, IL-6, IL-21, and IL-23 in the MLNs, which play key roles in the development or maintenance of Th17 cells in the intestine.[7,31,38,39,40] However, expression of these Th17-promoting cytokines did not change after KCV treatment (data not shown). Taking these results together, we assumed that the reduction of Th17 cells in the MLNs might result from a primary event that takes place upstream to the MLNs. Therefore, we shifted our attention from MLNs to intestinal LPLs.

Notably, Th17 cells constitutively inhabit LP,[9] and more iNKT cells are detected in LP than in MLN (our unpublished data). We first confirmed that RORγt expression was significantly reduced in the LPLs from KCV-treated wild-type mice as compared with those from control wild-type mice (Figure 6A), indicating that a reduced number of Th17 cells could be traced upstream to the LP. Moreover, the LPLs from iNKT cell deficient $J\alpha281^{-/-}$ mice showed a reduced expression of RORγt, again indicating the importance of iNKT cells for the maintenance of Th17 cells. We further quantified mRNAs of TGF-β1, IL-6, IL-23, and IL-21 expressed by LPLs by RT-PCR. Compared with the LPLs from control wild-type mice, those from KCV-treated wild-type mice and from $J\alpha281^{-/-}$ mice showed

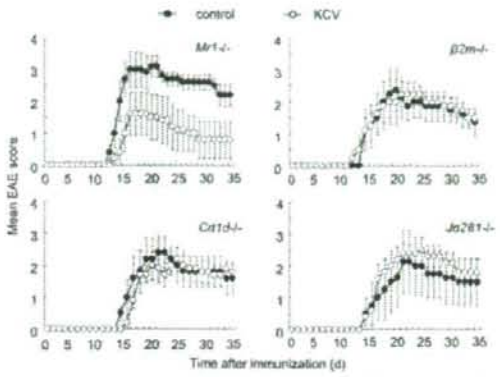

Figure 7. Suppressive effect of oral KCV treatment on EAE is abolished in Vα14 iNKT-deficient mice. $Mr1^{-/-}$, $\beta2m^{-/-}$, $Cd1^{-/-}$, and $J\alpha281^{-/-}$ mice were treated with KCV as described in Materials and Methods. After immunization of mice with MOG (35–55) clinical EAE scores of mice were assessed. Data represent mean score ± SEM from two independent experiments ($n = 4$ or 5 mice).

a reduced expression of IL-21 (Figure 6B). Expression of IL-23 was also reduced in KCV-treated wild-type mice as well as in $J\alpha281^{-/-}$ mice. These results support our postulation that LPLs are primarily influenced by the antibiotic treatment, resulting in a downstream decrease in the number of Th17 cells.

Suppressive Effect of KCV Treatment on EAE Is Abolished in iNKT-Cell Deficient Mice

The *ex vivo* experiments have demonstrated that Th17 cells in the MLN and LP are affected by KCV treatment in association with suppressed signs of EAE. Moreover, we showed that the KCV effects on Th17 cells could not be seen in the absence of iNKT cells. Although the results indicate an intimate relationship between Th17 cells and iNKT cells in the intestinal immune system, it does not necessarily imply that altering gut flora would suppress the development of EAE in a way dependent of iNKT cells. To make this point clear, we examined the effects of oral KCV treatment on the development of EAE induced in iNKT cell-deficient mice ($\beta2m^{-/-}$, $Cd1^{-/-}$, $J\alpha281^{-/-}$) as well as in MAIT cell-deficient mice ($Mr1^{-/-}$) (Figure 7). First, we noted that clinical EAE induced in $Mr1^{-/-}$ mice was significantly suppressed by KCV treatment, which coincides with the fact that the mesenteric Th17 cells are not affected by the absence of MAIT cells (Figure 6A). In contrast, suppressive effects of oral KCV was almost completely abolished in $\beta2m^{-/-}$, $Cd1^{-/-}$, and $J\alpha281^{-/-}$ mice (Figure 7), allowing us to conclude that iNKT cells play a key role in the KCV-induced suppression of EAE.

Health Status of KCV-Treated Mice

We have observed that antibiotic treatment tended to cause loose stool in the KCV treated mice. However, this happened in both wild-type mice and iNKT cell-deficient mice, which does not validate speculation on any relation

with the EAE disease suppression by KCV. Furthermore, KCV-treatment did not cause a significant change in body weight. We also examined the histology of gut lumen, and found that KCV treatment did not cause any pathological changes.

Discussion

The present study has experimentally demonstrated that altering gut flora by non-absorbing antibiotics could lead to protection against autoimmune disease EAE. Although the suppressive effect of antibiotics on EAE has been previously described,[41] the prior study did not address the possible contribution of the altered gut flora and has correlated the EAE suppression with an altered Th1/Th2 balance. In contrast, the present study has linked the antibiotic effects with a reduced number of Th17 cells in the gut-associated immune system. Most notably, the immunomodulatory effects of KCV could not be seen in iNKT cell-deficient mice, as assessed by the number of mesenteric Th17 cells or by severity of EAE. Comparison of wild-type and iNKT cell-deficient mice revealed that iNKT cells in the wild-type mice are able to promote the maintenance of mesenteric Th17 cells in the steady state, whereas the disease promoting ability of iNKT cell is impaired by KCV treatment. Given that oral administration of synthetic glycolipid ligands stimulatory for iNKT cells could alter the manifestation of autoimmune diseases,[22,42] one may speculate that oral KCV treatment leads to the appearance or disappearance of glycolipid ligands in the intestinal content that critically influence the function of iNKT cells.

The mucosal sites continuously sample foreign materials mainly via M cells in Peyer's patch and dendritic cells (DCs) in the LP.[43] The DCs in the LP would present orally applied antigens, migrate and enter the MLN.[44,45] Therefore, we wondered if the MLN might serve as the primary site where a contraction of Th17 cells takes place via mechanisms involving regulatory cells or changes of local cytokine milieu. However, the antibiotic treatment did not influence iNKT cells or Foxp3$^+$ regulatory T cells in the MLNs. Cytokines needed for promoting Th17 cell development and survival were not altered either, indicating that a critical event causing a reduction of Th17 cells probably takes place upstream. Consistent with this idea, we showed that expression of IL-21 and IL-23 in the LPLs was significantly suppressed in KCV-treated mice and iNKT cell-deficient mice. The role of IL-21 in the development of Th17 cells[39] has been demonstrated in mice lacking IL-6, the cytokine originally identified as a crucial promoter of Th17 cells. Intriguingly, it has recently been reported that IL-21 plays a critical role in the regulation of Th17 cells involved in gut inflammation.[38] Taken together, we suggest that the suppression of IL-21 and IL-23 may be a primary event after KCV treatment, which leads to the reduction of mesenteric Th17 cells. It is known that both iNKT cells and Th17 cells are able to produce IL-21.[46,47] Given that iNKT cells in the MLNs were not altered after KCV treatment, we speculated that iNKT cells within LP may numerically or functionally be altered, which could account for the reduced IL-21 in the LPLs. However, because of technical limitations, we have not definitively demonstrated that this is the case. Although a recent report using IL-21 knockout mice showed that IL-21 is not essential for the development of Th17 cells *in vitro* and *in vivo*,[48] it does not exclude the role of IL-21 in wild-type mice.

It is arguable that the reduced Th17 cells in the MLN cells from KCV-treated mice may result from a direct or indirect effect of KCV on DCs. However, flow cytometric analysis did not reveal any difference between KCV-treated and control mice with regard to the surface levels of MHC class II, CD80 or CD86 on the MLN-DCs (data not shown). In addition, there was no alteration of CD103 on the MLN-DCs that is described as an inducer of Foxp3$^+$ regulatory T cells.[49]

Although we have so far focused on analysis of Th17 cells and iNKT cells in the gut immune system, we cannot overlook that dLN cells from KCV-treated mice produced a lower amount of IFN-γ in response to MOG (35–55) indicating that Th1 cells in the dLNs could be also affected by KCV treatment. Interestingly, a concomitant reduction of Th1 cells and Th17 cells has recently been demonstrated in EAE mice treated with anti-IL-6 receptor antibody, which was used for aiming at specific suppression of Th17 cells.[50] These homologous results suggest the possible induction of regulatory T cells in the dLN that may regulate both Th1 and Th17 cells. Although Foxp3$^+$ regulatory T cells are qualified suppressors, total number of the CD4$^+$Foxp3$^+$ T cells in dLN was not altered after KCV treatment. It is possible that MOG (35–55) specific regulatory T cells might be selectively induced by altering gut flora. It is obvious that further studies are needed to clarify the total picture of NKT cell-dependent suppression of EAE by altering gut flora.

There is a clear tendency for an increased incidence of immune-mediated disorders in developed countries.[51] Although this increase has often been linked with improved hygiene, a number of studies have suggested a role for commensal flora affected by life style.[52] This is an attractive idea, in particular for inflammatory bowel disease, where the target is the gut and is inhabited by pathogenic Th17 cells as well as regulatory cells such as MAIT cells. In contrast, much less attention has been paid on the role of commensal flora in the development of the CNS autoimmune disease MS. The present study emphasizes that the repertoire of the immune system is greatly regulated by gut flora, which has broad implications for understanding the pathogenesis of autoimmune disease and allergy, and could be applied for future studies. However, it is too early to suggest that antibiotic treatment will be beneficial for MS. Indeed, altering gut flora could trigger or prevent the development of autoimmune conditions. Future studies coping with such variables as timing, duration, choice of antibiotics used for treatment will not only give us deeper understanding on the interaction between gut flora and Th17 cells, but also provide important information related to the human health.

Acknowledgments

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＜特集3：中枢神経系脱髄疾患の病態＞

腸管免疫からみた自己免疫性脳炎の発症と制御

山村　隆，横手　裕明，三宅　幸子

Development and regulation of autoimmune encephalomyelitis mediated by gut immune system

Takashi Yamamura, Hiroaki Yokote, and Sachiko Miyake

Abstract

An influence of altered hygiene has been indicated for autoimmune disorders such as multiple sclerosis. However, most works are based on epidemiological analysis and the lack of appropriate experimental systems appears to hamper the development of the study. Here we report that altering gut flora by a combination of non-absorbing antibiotics (kanamycin, colistin, vancomycin) would significantly suppress the development of EAE by altering gut flora. The suppression of EAE was associated with an inhibited Th17 responses in the gut immune system. Interestingly, Th17 cells in the gut lamina propria or mesenteric lymph node cells were also inhibited in the mice whose NKT cells are defective. Furthermore, KCV treatment did not modulate the EAE induced in the NKT cell-deficient mice. We would propose that NKT cells play a critical role in sensing the gut environment, which leads to regulation of autoimmune diseases.

Key words: mucosal immunity, multiple sclerosis, autoimmune encephalomyelitis, immunoregulation

はじめに

従来の免疫学では、T細胞産生の場である胸腺とB細胞産生の場である骨髄を中心に据え、腸管を免疫組織として捉えるという意識には乏しかった。しかし、体内のリンパ球の60－70％が腸管に存在するという事実などに基づき、腸管が重要な免疫臓器であることが徐々に認識され、今日では腸管免疫（gut immunity）に関する研究が免疫学のmain streamの一つになっている。腸管免疫系（gut immune system）は、パイエル板（Peyer's patch）、小腸上皮間リンパ球（intraepithelial lymphocytes; IEL）、粘膜固有リンパ球（lamina propria lymphocytes; LPL）、腸間膜リンパ節、cryptopatchなどから構成される[1)2)]。

腸管免疫系は腸管局所における感染病原体の認識と排除などの生理的免疫応答や、炎症性腸疾患（inflammatory bowel disease）の発症や制御において決定的な役割を果たす。また、腸管リンパ球が全身性免疫応答の誘導や制御、あるいは遠隔臓器の自己免疫病（EAE、1型糖尿病）やアレルギー性炎症の制御にも関わることが報告された[3)4)5)]。また、自己免疫病の炎症惹起性細胞（エフェクターT細胞）、およびその制御細胞（MAIT細胞、NKT細胞など）の両者が腸管免疫系に分布し、腸内細菌叢（gut flora）の支配を受けることも示されている。このように、腸管免疫と自己免疫疾患の密接な関連を推測させる証拠が揃いつつある。

MSの発症には遺伝的要因と環境因子（後天的因子）の両者が関与する。遺伝的要因については、近年のゲノムワイド解析によってMS感受性に関連する複数の遺伝子

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が同定されている[6]。しかし、環境因子の研究は、主に疫学調査の結果に依存しており[7][8][9]、細胞生物学的な解析は、まだこれからという状況である。我が国においてMSはこの30－40年間で顕著な増加傾向を示しているが[10]、最近では年間の新規発症率がカナダの約6分の1に達し、日本人と西洋人の発症率の差が減少して来ていることは確かである。日本人MS患者が増加した背景には後天的な因子の存在することは明白であるが、我々は食生活を中心とする生活習慣の欧米化が深く関わっている可能性を考え[11]、その中心に腸内細菌叢の変化が関係しているのではないかと考えている("MSの生活習慣病仮説")。

腸内細菌と免疫疾患の関連については、これまでに様々な実験系において検証されているが、MSの発症の背景に腸内細菌の変化を仮想した研究はない。本稿では、最近我々の研究室で行った、抗生物質投与による腸内細菌偏倚とEAE抑制の実験結果を紹介する[5]。興味深いことに、抗生物質によるEAEの抑制はNKT細胞の欠損するマウス(Jα281$^{-/-}$、CD1d$^{-/-}$マウス)では観察されなかった。一方、MAIT細胞の欠損するMR1-/-マウスでは、野生型マウスと同じように抗生物質投与によるEAE抑制が観察された。免疫系は恒常的に腸内環境をモニターしていることは定説となっているが、そのメカニズムにおいてNKT細胞が重要な役割を果たすことが示されたと考えている。本稿がMSの発症と腸内環境の関係を理解する一助となれば幸いである。

1. EAE誘導と制御に関与するリンパ球

EAEはミエリン抗原に反応するCD4陽性の脳炎惹起性T細胞によって誘導される動物モデルである。脳炎惹起性T細胞はIFN-γを産生するTh1細胞であると永年にわたって信じられて来たが、最近ではIL-17を産生するTh17細胞の決定的な関与が示された[12]。EAEの発症にはTh17細胞のみが関与するという極論も現れたが、実際はTh17細胞とTh1細胞それぞれが重要な役割を果たし、状況によって異なるバランスを取ってEAE病態に関与してくるように思われる[13][14]。最近の論文ではTh1細胞によって誘導されるEAEでは活性化マクロファージの浸潤が顕著であるのに対し、Th17細胞によって誘導されるEAEでは好中球浸潤が優勢であると報告されている。Th17細胞の分化・誘導に必須である転写因子RORγt陽性のTh17細胞は腸管(回腸など)のlamina propria (LP)に恒常的に分布しているが[15]、無菌マウス(germfreeマウス)では激減するので、腸内細菌叢が腸管Th17細胞の誘導に必須であると考えられる[16]。他方、EAEの誘導を制御するリンパ球としては、CD25^{+}CD4^{+}T細胞、NKT細胞[17]、MAIT細胞[3]、NK細胞[18]などが報告されている。MAIT細胞はMR1分子に拘束されるインバリアントT細胞で、腸管LPに分布する[19]。腸管Th17細胞と同様、無菌マウスでは減少しており、腸内細菌に依存性の細胞と考えられている。

2. 抗生物質投与による腸内細菌叢の偏倚

我々は、腸内細菌依存性のMAIT細胞がEAEの制御細胞として働くことを発見して以来[3]、腸内細菌を変化させることによってEAEの病態が変化するのではないかという仮説を抱いてきた。その妥当性を検証するために、腸内細菌を偏倚させたマウスにEAEを誘導することにした。方法としては、Bashirら[20]に準拠し、腸管非吸収性の抗生物質カナマイシン(kanamycin)、コリスチン(colistin)、バンコマイシン(vancomycin)(KCVと略す)を飲用水に持続的に添加した(1－4週間)。この方法によって腸内細菌叢が偏倚することを確認するために、マウス糞便中の腸内細菌ゲノムのフラグメントをスポットしたDNA microarray (FloraArray)[21]を用いて、KCV投与群と対照群の糞便サンプルを比較した。その結果、KCV投与によって腸内細菌叢が比較的短期間(7－10日)で著明に偏倚することがわかった[5]。

3. KCV投与によるEAE抑制と所属リンパ節細胞のサイトカイン産生低下

つぎにKCV投与マウスにMOG35-55ペプチド感作によりEAEを誘導した。その結果、C57BL/6(B6)マウスにMOG 35-55ペプチド感作の1週間前より抗生物質を持続的に投与すると、EAEの誘導が有意に抑制されることがわかった(図1)。しかしEAE発症前あるいは発症後にKCV投与を中止すると、EAEの抑制は解除された。EAEピーク時に脊髄の病理所見を検討したが、KCV投与群では炎症細胞浸潤の低下および脱髄病変の減少が見られた。また、脊髄浸潤細胞の総数も減少していた。併せて所属リンパ節細胞をMOG35-55で刺激し、増殖反応とサイトカイン産生を評価したところ、KCV投与によって炎症性サイトカインであるIFN-γ、TNF-α、IL-6およびIL-7の産生が、有意に抑制されることがわかった。

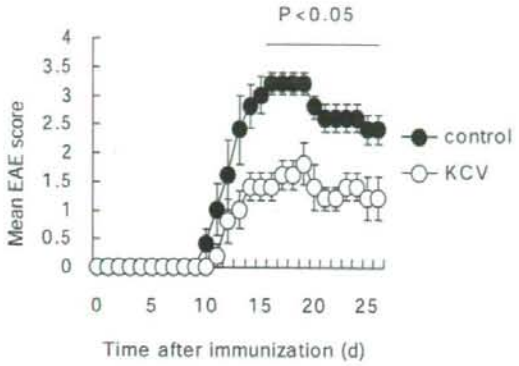

図1. 抗生物質KCVの経口投与によるEAEの抑制
EAEはB6マウスにMOG35-55感作によって誘導した。EAE誘導を行う1週間前より飲用水にKCVを加え(KCV)、EAEの臨床経過を対照群のそれと比較した(文献5の図を一部改変)。

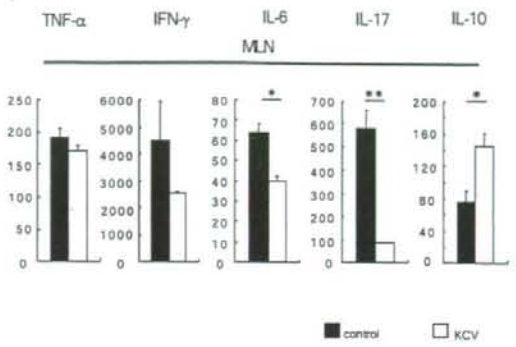

図2. KCV投与による腸間膜リンパ節細胞のサイトカイン産生偏倚
B6マウスに1週間KCVを与え、腸間膜リンパ節(MLN)よりリンパ球を分離して固相化した抗CD3抗体で刺激した。培養上清中のサイトカインレベル(pg/ml)をELISAまたはcytometric bead arrayで測定した。
*;P < 0.05, **;P < 0.001 (Student's t-test)

これは、所属リンパ節におけるMOG35-55特異的Th1細胞およびTh17細胞の誘導が阻害されることを意味し、KCVによるEAE抑制を説明する変化であると考えた。

4. KCV投与マウスにおける腸間膜リンパ節Th17細胞の減少

さらに腸管関連リンパ節細胞を解析することにした。最初にEAE誘導実験で用いたマウスの腸間膜リンパ節(mesenteric lymph node; MLN)から分離したリンパ球をMOG35-55ペプチドで刺激し、培養上清中のサイトカイン濃度を測定した。その結果、IL-17の産生量は抗生物質投与群で有意に低下していた。つぎに感作をしないで抗生物質KCVを投与したマウスのMLN細胞を抗CD3抗体で刺激し、培養上清中のサイトカインを測定したが、IL-17とIL-6の産生が特異的に低下しており(図2)、腸内細菌層の偏倚によって、腸管Th17細胞の活性が抑制されることが明らかになった。一方、IL-10産生はKCV投与により上昇した。平行して脾臓細胞を用いて検討したが、KCV投与群と対照群には差はなかった。なお、MLN中のCD25$^+$CD4$^+$制御性T細胞数やNKT細胞数には有意な変化はなかった。

5. NKT細胞欠損マウスにおける腸間膜リンパ球Th17細胞の減少

MAIT細胞は腸管LPに偏在するユニークなリンパ球で、MR1に結合したα-mannosylceramideに反応することが報告されている[22]。MAIT細胞が腸管でTh17細胞の生存・維持に関与する可能性を考え、MAIT細胞を欠損するMR1$^{-/-}$マウスのMLNよりリンパ球を分離し、抗CD3抗体で刺激した。その結果、野生型(WT)マウスのMLNリンパ球を刺激した場合と同じレベルのIL-17産生が誘導された。したがって、Th17細胞がMAIT細胞に依存するという仮説は妥当でないと考えられた。しかし、クラスI拘束性T細胞(MAIT細胞やNKT細胞を含む)を広く欠損するβ2-microglobulin欠損マウス(β2m$^{-/-}$)では、MLNリンパ球のIL-17産生が顕著に低下していた。そこで、NKT細胞を欠損するJα281$^{-/-}$マウスおよびCD1d$^{-/-}$マウスを用いて同じ実験を行ったところ、これらのマウスにおいて抗CD3抗体刺激によるMLN細胞のIL-17産生が顕著に低下していることがわかった(図3)。また、抗生物質KCVの投与を受けたマウスMLN細胞のIL-17産生は、野生型マウスでも、MAIT細胞やNKT細胞の欠損マウスでも大きな差はなかった。以上の結果から、NKT細胞は腸管免疫系のTh17細胞の維持にきわめて重要な役割を果たすことが示唆された。免疫系が腸管内環境の変化を感知するセンサーとして

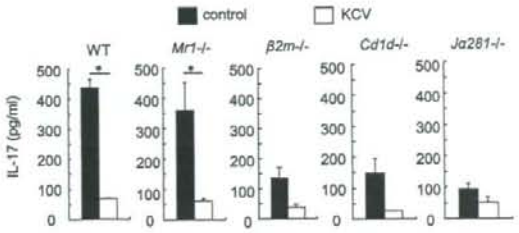

図3. NKT細胞欠損マウスにおける腸間膜リンパ節IL-17産生T細胞(Th17)の減少
野生型マウス(WT)、MR1ノックアウトマウス(Mr1$^{-/-}$)、β2-microglobulin欠損マウス(β2m$^{-/-}$)、CD1d欠損マウス(Cd1d$^{-/-}$)、Jα281欠損マウス(Jα281$^{-/-}$)に1週間KCVまたはKCV-free water(control)を与え、腸間膜リンパ節(MLN)よりリンパ球を分離して固相化した抗CD3抗体で刺激し、培養上清中のIL-17濃度を測定した。
*;$P < 0.05$ (Student's t-test)

NKT細胞が機能する可能性や、腸内環境の変化を伝達するシグナルをNKT細胞が増強する可能性が考えられた。

6. KCV投与マウスにおけるLPリンパ球のIL-17産生低下

我々はさらに、MLN内のNKT細胞の数や機能を評価したが、抗生物質投与による大きな影響は見られなかった。この結果は、NKT細胞の関与する生体反応が、MLNよりも'上流'で起こっている可能性を示唆した。そこで、つぎにlamina propriaのリンパ球(LPL)の解析に着手した。LPではTh17細胞が恒常的に検出され、NKT細胞の頻度も比較的多いとされる(～2%)。細胞数が少ないため、Th17細胞の定量評価には、転写因子ROR-γtの発現をRT-PCRで測定することにした。その結果、ROR-γtは抗生物質投与マウスのLPLで野生型マウスのそれに比べて有意に低下しており、抗生物質投与によるTh17細胞の低下がLPLまでさかのぼれることがわかった。また抗生物質治療をしていないNKT細胞欠損マウスのLPLにおいても、野生型マウスに比べてROR-γt発現が顕著に低下していた。抗生物質投与とNKT細胞欠損が同じような効果を与えることから、抗生物質投与の影響がNKT細胞を介して現れる可能性が推測された。

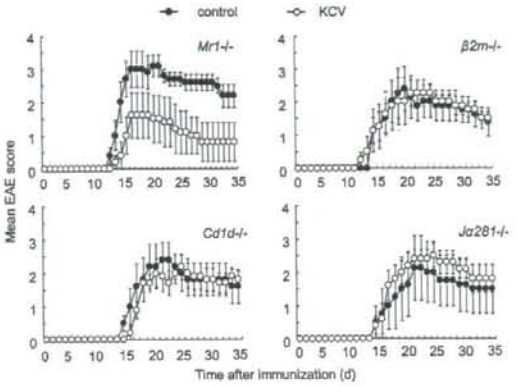

図4. NKT細胞欠損マウスにおけるKCV-EAE抑制効果の消失

7. 抗生物質投与によるEAE抑制にはNKT細胞が必須である

最後に抗生物質投与によるEAE抑制実験を、MAIT細胞欠損マウス(MR1$^{-/-}$)およびNKT細胞欠損マウス(β2-microglobulin$^{-/-}$、Jα281$^{-/-}$、CD1d$^{-/-}$)で行った。In vitroの実験結果からなかば予測できたことであるが、抗生物質投与によるEAE抑制はMAIT細胞欠損マウスでは観察されたが、NKT細胞欠損マウスでは見られなかった(図4)。

考　察

1－4週間のKCV投与実験において、腸内細菌の偏倚がTh17細胞の低下とEAEの抑制を招き、それがNKT細胞を介した免疫制御であることを明らかにできた。しかし、異なる抗生物質の組み合わせ、異なる投与量(投与期間)、マウス系統差によって、異なる結果が得られる可能性は高い。具体的には、EAEが重症化する、あるいは遷延するという結果も得られる可能性がある。いずれにせよ腸内細菌と免疫系の関係を細胞・分子レベルで解析できる有用な実験系の構築に成功したと考えている。

NKT細胞は糖脂質抗原を認識し、経口的に投与されたNKT細胞糖脂質リガンドがEAEの臨床症状を軽減することがわかっている[23]。また糞便成分は多量の糖脂質抗原を含む。こういう状況から考えて、KCV投与は腸内細菌の偏倚を介して腸内のEAE抑制的な糖脂質リガンド産生を促し、その結果、EAEが抑制される可能性

が推測される。しかし他の可能性も含めて、これから詳細に検討を加えなければならない。

まとめ

腸管免疫の変調がMS増加の遠因である可能性を検証する研究は重要である。抗生物質投与によってMSの動物モデルEAEが軽症化したことは、腸内細菌の変化が自己免疫疾患の促進あるいは抑制に結びつくことを意味し、「MSの生活習慣病仮説」を支持する結果である。

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要　旨

免疫系が正常に発達するには腸内細菌の存在が必須であり、腸内細菌叢の量的、質的偏倚によって腸管免疫はもとより全身免疫系にまで変化が生じ、ひいては炎症性腸疾患のような免疫病の発症に至ることが明らかになって来た。一方、神経免疫疾患の代表的動物モデルである自己免疫性脳炎(EAE)の病態に関わるTh17細胞や制御性MAIT(mucosal associated invariant T)細胞が腸内細菌による支配を受けることが証明され、多発性硬化症(MS)と腸管免疫の関連が推測されるに至った。本稿では、抗生物質投与による腸内細菌叢の修飾によりEAE発症が抑制されたという興味ある実験結果を紹介するとともに、その意義、今後の研究の展開、MSが日本人の生活習慣の欧米化によって増加した可能性などを議論する。

キーワード: 粘膜免疫，多発性硬化症，自己免疫性脳炎，免疫制御機構

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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

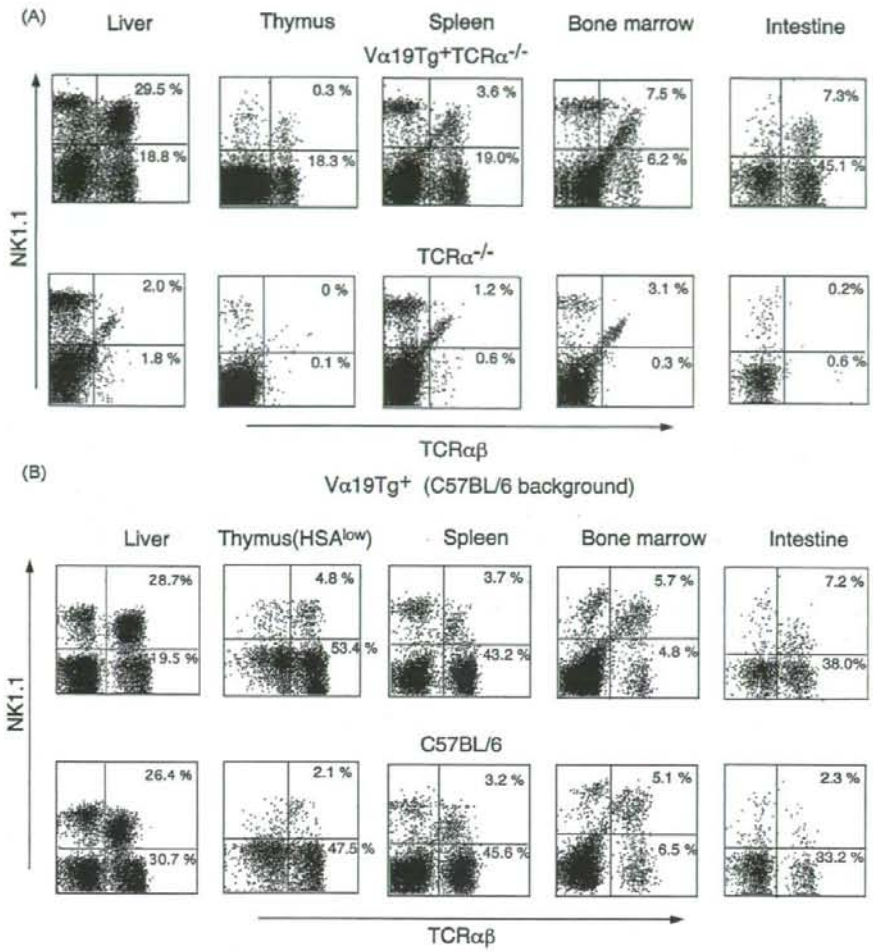

Fig. 1. Development of Vα19 NKT cells in Vα19 Tg mice. MNCs were isolated from liver, thymus, spleen, bone marrow and intestinal lamina propria of mice (at 8–12 weeks of age, female). They were stained with fluorescence-labeled antibodies and analyzed by FACS. (A) Staining profiles of the Vα19 Tg$^+$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

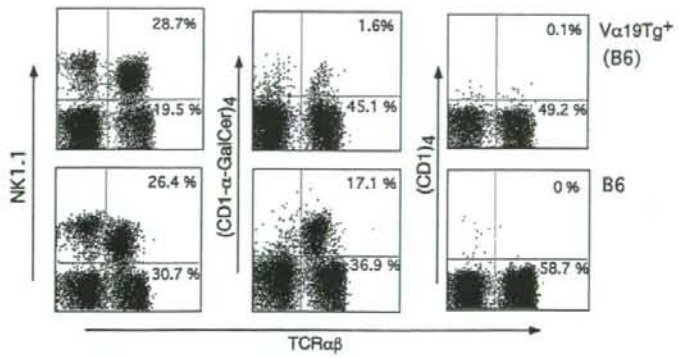

Fig. 2. Staining profiles of Tg- and non-Tg liver MNCs with CD1-α-Gal-Cer tetramers. Liver MNCs isolated from Tg and non-Tg mice on the C57BL/6 background (at 8–10 weeks of age, female, both in the range of 3–7 × 10^6 per animal) were stained with anti-TCR αβ antibody and either anti-NK1.1 antibody,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

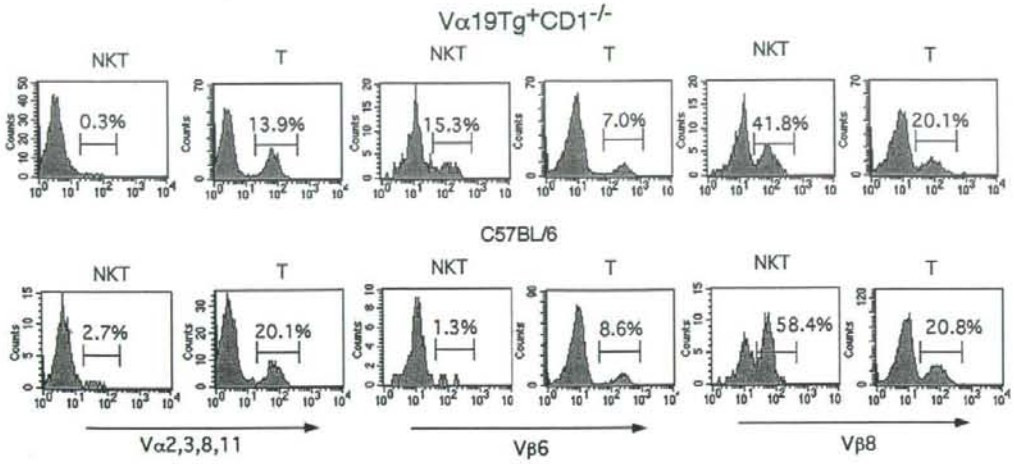

Fig. 3. Preferential expression of the invariant Vα19 TCR transgene by the NK1.1$^+$, TCRα(* cells in the Tg livers. Liver MNCs from Vα19 TCR Tg (CD1$^{-/-}$ background) and non-Tg mice (at 8 weeks of age, female) were triply stained with an anti-TCRαβ, anti-NK1.1 and anti-TCR Vα mixture (Vα2, 3.2, 8,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

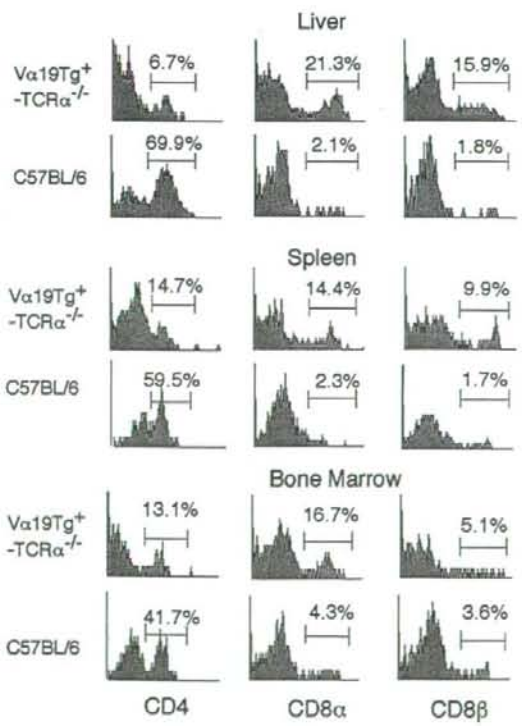

Fig. 4. CD4/CD8 co-receptor expression by NK1.1$^+$, TCR αβ$^+$ cells of Vα19 Tg$^+$ and non-Tg mice. MNCs were isolated from liver, thymus, spleen, and bone marrow of Vα19 Tg$^+$ TCRα$^{-/-}$ and C57BL/6 mice (at 8–12 weeks of age). They were immunostained and analyzed by flow cytometry. Profiles of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

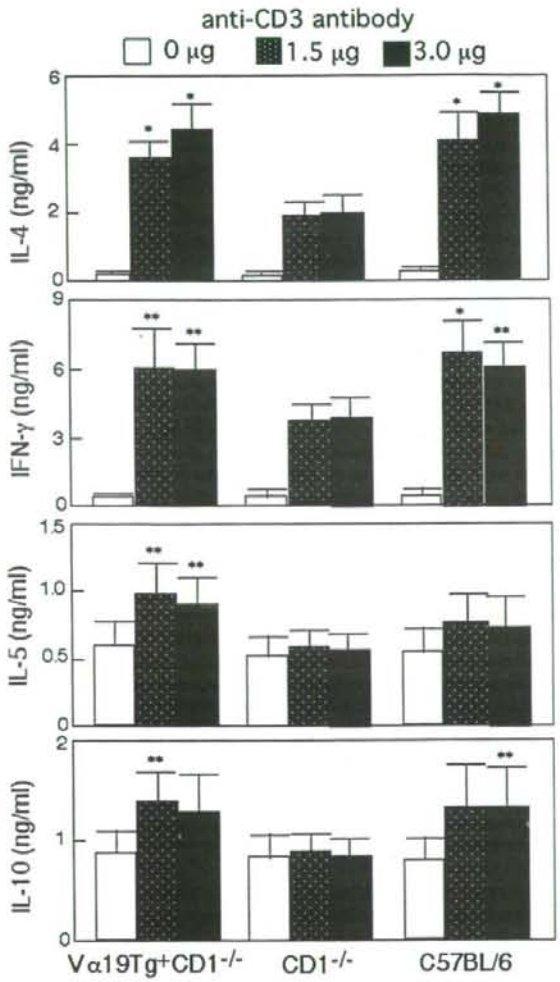

Fig. 5. Cytokine production by Tg and non-Tg mice in response to *in vivo* treatment with anti-CD3 antibody. Liver MNCs prepared from Vα19 Tg$^+$ CD1$^{-/-}$, CD$^{-/-}$, and C57BL/6 mice injected 90 min previously with a different dose of anti-CD3 antibody (2C11) were cultured in DMEM (10% FCS) without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

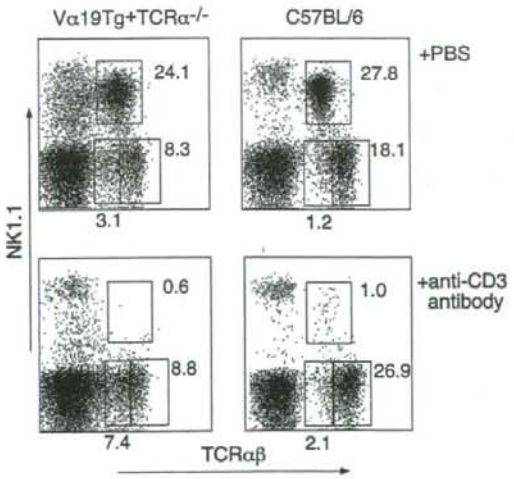

Fig. 6. Surface receptor down-regulation of hepatic Vα19 NKT cells upon TCR engagement in vivo. Liver MNCs were prepared from Vα19 Tg$^+$ TCRα$^{-/-}$ and C57BL/6 mice injected 24 h previously with anti-CD3 antibody (2C11, 2 μg/mouse). Cells were stained with anti-TCRαβ and anti-NK1.1 antibodies and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

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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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