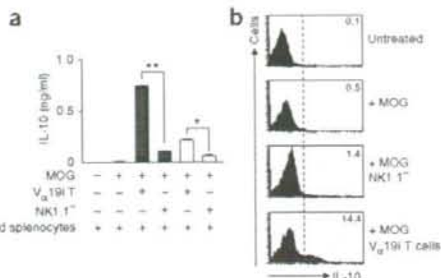

Figure 4 Interactions of $V_\alpha 19i$ T cells and splenocytes induce IL-10. (a) Cytometric bead assay of IL-10 in the supernatants of liver $V_\alpha 19i$ T cells from naive $V_\alpha 19iTgCd1d1^{-/-}$ mice, cultured for 72 h with MOG(35–55)-specific splenocytes and MOG(35–55) (filled bars). In some cases, $V_\alpha 19i$ T cells were separated from splenocytes by transwell inserts (open bars). Controls received NK1.1⁻ liver cells from $V_\alpha 19iTgCd1d1^{-/-}$ mice. Data represent ± s.e.m. from duplicate samples from three independent experiments. *, $P < 0.01$, and **, $P < 0.001$, compared with control (two-tailed Student's t-test). (b) Intracellular flow cytometry of IL-10 production by total cells from a. Areas to the right of dashed lines indicate positive cellular staining; numbers in histograms indicate percentages of IL-10-producing cells. Data are representative of three separate experiments.

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IFN-γ secretion was more susceptible to the inhibitory effects of $V_\alpha 19i$ T cells than was IL-17 (**Fig. 3b**). Splenocytes acted like lymph node cells (data not shown).

Overexpression of the $V_\alpha 19i$ TCR might compromise the ability of conventional T cells to recognize myelin-derived peptides. However, the proliferative responses of MOG(35–55)-reactive T cells were not lower in $V_\alpha 19iTgCd1d1^{-/-}$ mice, despite the inhibition of T_H1 cytokine production (**Fig. 3c**). Therefore, it is unlikely that the degree of EAE suppression seen in $V_\alpha 19iTgCd1d1^{-/-}$ mice was the result of alterations in the MOG(35–55)-specific T cell repertoire. However, to exclude that possibility, we did adoptive transfer experiments. We transferred 1×10^6 $V_\alpha 19i$ T cells isolated from $V_\alpha 19iTgCd1d1^{-/-}$ mice into nontransgenic mice on the day of EAE induction. Mice that received $TCR\beta^+$ T cells were significantly protected from EAE (**Fig. 3d**) and the onset of clinical disease was significantly delayed (**Table 1**) compared with that of mice that received $V_\alpha 19i^-$ NK1.1⁻ T cells.

Next we sought to determine if $V_\alpha 19i$ T cell deficiency could also influence clinical EAE. As no $V_\alpha 19i$-specific TCR antibody is available to deplete mice of $V_\alpha 19i$ T cells *in vivo*, we used $Mr1^{-/-}$ mice, which lack $V_\alpha 19i$ T cells[7]. As wild-type nontransgenic mice have about four times more $V_\alpha 14i$ NKT cells than $V_\alpha 19i$ T cells and $Cd1d1^{-/-}$ mice did not show protection from EAE (**Fig. 2a**), we sought to determine whether the deletion of small numbers of MR1-restricted T cells could alter the clinical course of EAE. Compared with wild-type nontransgenic controls, $Mr1^{-/-}$ mice showed a significantly more severe form of EAE with an earlier onset ($P < 0.05$; **Fig. 3e** and **Table 1**). Furthermore, T cells from $Mr1^{-/-}$ mice proliferated more and produced more T_H1 cytokines and less IL-10 (data not shown). These experiments collectively suggest that $V_\alpha 19i$ T cells have a regulatory function in a T_H1-mediated autoimmune disease.

$V_\alpha 19i$ T cells induce B cell IL-10 production

MOG(35–55)-primed $V_\alpha 19iTg$ lymph node cells and splenocytes secreted IL-10, which potently inhibits EAE[27–30] (**Fig. 3a**). Therefore, we sought to determine whether an increase in $V_\alpha 19i$ T cells augmented general IL-10 production. To address that, we developed a mixed-lymphocyte assay in which we cultured NK1.1⁺ or NK1.1⁻ T cells from $V_\alpha 19iTgCd1d1^{-/-}$ mice together with MOG(35–55)-primed nontransgenic splenocytes (**Fig. 4a**). Neither NK1.1⁺ or NK1.1⁻ T cells inhibited the proliferation of MOG(35–55)-primed splenic T cells restimulated with MOG(35–55) (data not shown). Cytokine analysis showed that the coculture supernatant contained considerable IL-10 (after stimulation with MOG(35–55)) in the presence of NK1.1⁺ but not NK1.1⁻ T cells from $V_\alpha 19iTgCd1d1^{-/-}$ mice (**Fig. 4a**). NK1.1⁺ T cells from $V_\alpha 19iTgCd1d1^{-/-}$ mice induced IL-10 production even in the absence of MOG(35–55) ($P < 0.05$; **Supplementary Fig. 2** online). However, IL-10 secretion was significantly enhanced in the presence of exogenous MOG(35–55) ($P < 0.01$; **Supplementary Fig. 2**). Intracellular cytokine analysis confirmed that IL-10 production was induced by the addition of NK1.1⁺ but not NK1.1⁻ T cells from $V_\alpha 19iTgCd1d1^{-/-}$ mice (**Fig. 4b**). However, in the presence of transwell inserts, IL-10 production was inhibited, indicating that $V_\alpha 19i$ T cell–mediated IL-10 production depends mainly on cell-cell contact (**Fig. 4a**). IL-4 and IL-5 were below the limit of detection (less than 5 pg/ml), and IFN-γ and TNF were slightly upregulated in the presence of $V_\alpha 19i$ T cells (data not shown).

To determine which cells produced IL-10, in the same coculture experiment we analyzed IL-10 production by CD19⁺, CD4⁺, CD8⁺,

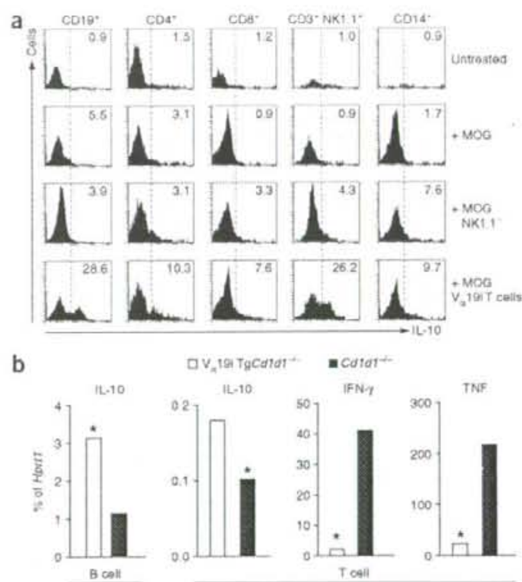

Figure 5 $V_\alpha 19i$ T cells induce B cells to secrete IL-10. (a) Intracellular flow cytometry of IL-10 production by liver $V_\alpha 19i$ T cells from naive $V_\alpha 19iTgCd1d1^{-/-}$ mice, cultured for 72 h with MOG(35–55)-specific splenocytes and MOG(35–55). Areas to the right of dashed lines indicate positive cellular staining; numbers in histograms indicate percentage of IL-10-producing cells expressing various surface markers (above plots). Data are representative of two separate experiments. (b) Real-time RT-PCR of the expression of transcripts encoding various cytokines (above graphs) by splenic CD19⁺ B cells or CD4⁺ T cells sorted from mice with EAE. Data are expressed as a percentage of expression of *Hprt1* and are representative of two separate experiments. *, $P < 0.05$ (two-tailed Student's t-test).

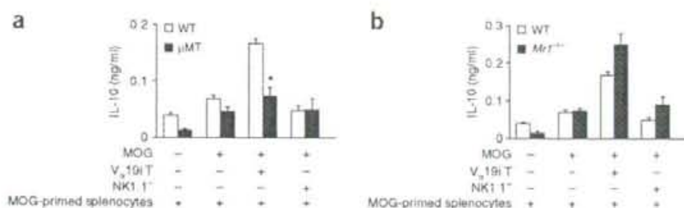

Figure 6 V_α19i T cell–induced IL-10 production is partially B cell dependent but completely MR1 independent. Cytometric bead assay of IL-10 in the supernatants of liver V_α19i T cells from naive V_α19iTg*Cd1d1*$^{-/-}$ mice, cultured for 72 h with MOG(35–55) plus MOG(35–55)-specific splenocytes from wild-type nontransgenic or B cell–deficient μMT mice (a) or from wild-type nontransgenic or MR1-deficient mice (b). Data represent mean ± s.e.m. of duplicate samples from three independent experiments. *, $P < 0.05$, compared with control (two-tailed Student's *t*-test).

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CD3$^+$NK1.1$^+$ or CD14$^+$ cells using intracellular cytokine flow cytometry. The addition of V_α19i T cells greatly increased IL-10 production by CD19$^+$ B cells and CD3$^+$ NK1.1$^+$ NKT cells (**Fig. 5a**). CD4$^+$ and CD8$^+$ T cells also showed slight increases in IL-10 production in the presence of V_α19i T cells. To demonstrate that B cells were the main IL-10 producing cells *in vivo*, we extracted RNA from sorted splenic CD4$^+$ T cells or CD19$^+$ B cells from V_α19iTg*Cd1d1*$^{-/-}$ or nontransgenic mice with EAE (**Fig. 5b**). In agreement with the results of the *in vitro* coculture system, we found that B cells isolated from V_α19iTg*Cd1d1*$^{-/-}$ mice had higher expression of mRNA transcripts encoding IL-10 than did T cells (**Fig. 5b**). In addition, B cells from V_α19iTg*Cd1d1*$^{-/-}$ mice had higher expression of *Il10* transcripts than did B cells from *Cd1d1*$^{-/-}$ mice (**Fig. 5b**). In contrast, CD4$^+$ T cells from V_α19iTg*Cd1d1*$^{-/-}$ mice had lower expression of T_H1 cytokine-encoding mRNA transcripts than did CD4$^+$ T cells from *Cd1d1*$^{-/-}$ mice (**Fig. 5b**).

To determine if V_α19i T cell–B cell interactions are essential for IL-10 production in the coculture system, we immunized B cell–deficient (μMT) mice with MOG(35–55) to obtain a source of MOG-primed spleen cells lacking B cells. After culture together with V_α19i T cells, B cell–deficient splenocytes produced less IL-10 than did wild-type nontransgenic splenocytes (**Fig. 6a**). As μMT knockout mice may have unusual follicular architecture, to exclude potential indirect effects we repeated these coculture experiments using B cell–depleted wild-type nontransgenic splenocyte samples. B cell–depleted splenocyte samples produced less IL-10 than did nondepleted splenocyte samples whereas the readdition of wild-type B cells to B cell–depleted splenocyte samples restored IL-10 production (56.3 ± 1.2 pg/ml for B cell–depleted splenocyte samples; 126.0 ± 4.4 pg/ml for B cell–depleted splenocyte samples with B cells 'added back'; and 170.4 ± 0.8 pg/ml for nondepleted splenocyte samples).

We hypothesized that an interaction between MR1 on B cells and the V_α19i TCR on T cells could induce IL-10 secretion from both cell types. To test that, we immunized *Mr1*$^{-/-}$ mice with MOG(35–55), followed by coculture experiments. In the absence of MR1, V_α19i T cell–mediated IL-10 production was not reduced (**Fig. 6b**). These results suggest that V_α19i T cell–induced IL-10 production can occur at least in part through MR1-independent interaction with B cells. However, non–B cells also seem to contribute to V_α19i T cell–induced IL-10 production.

Costimulation in V_α19i T cell–induced IL-10 production

Naive V_α19i T cells from V_α19iTg*Cd1d1*$^{-/-}$ mice expressed more of the costimulatory molecules CD278 (ICOS), CD86 (B7-2), CD154 (CD40L) and CD28 than did naive splenic T cells (**Fig. 7a**). V_α19i T cells also expressed CD44 more 'brightly' than did naive T cells (data not shown). These results indicate that V_α19i T cells have an activated or memory phenotype, similar to that of V_α14i NKT cells[1] and 'mucosal-associated invariant T cells' isolated from gut mucosa[2].

Given that MR1 is not required for IL-10 production, we hypothesized that costimulatory interactions may provide the stimulus for IL-10 production. To test that, we repeated the coculture experiments in the presence of blocking antibodies specific for the costimulatory molecules B7RP-1, CD80, CD86 and CD40L. We found that blockade of each costimulatory pathway resulted in significantly lower IL-10 secretion than that of control cocultures treated with control immunoglobulin (**Fig. 7b**). However, blockade of the ICOS–B7RP-1 pathway inhibited IL-10 production most substantially. To extend those

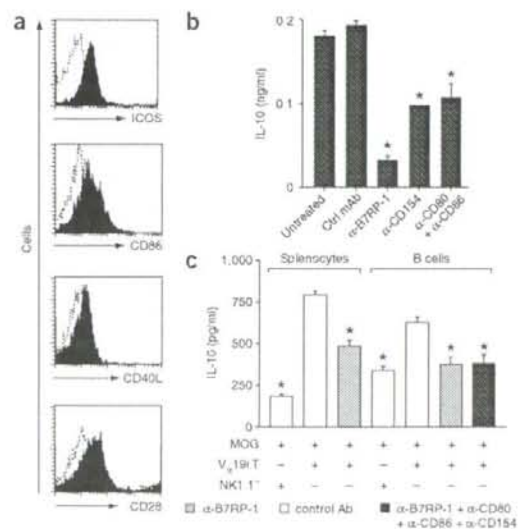

Figure 7 ICOS–B7RP-1 costimulation contributes to V_α19i T cell–induced B cell IL-10 production. (a) Flow cytometry of costimulatory molecule expression on the surface of liver V_α19i T cells (filled histograms) and naive splenic T cells from C57BL/6 mice (dotted lines). Data are representative of three separate experiments. (b) Cytometric bead assay of IL-10 in the supernatants of liver V_α19i T cells from naive mice, cultured with MOG(35–55) and MOG(35–55)-specific splenocytes from wild-type nontransgenic EAE mice in the presence of isotype-matched control antibody (Ctrl mAb) or of blocking antibodies specific to various costimulatory molecules (α-; below graph), measured after 72 h of incubation. Data are representative of two separate experiments. (c) Cytometric bead assay of IL-10 in the supernatants of liver V_α19i T cells from naive V_α19iTg*Cd1d1*$^{-/-}$ mice, cultured with MOG(35–55) and MOG(35–55)-specific splenocytes or sorted B cells from wild-type nontransgenic EAE mice in the presence of various antibodies (key), measured after 72 h of incubation. *, $P < 0.001$, compared with control groups (analysis of variance). Data represent mean ± s.e.m. of triplicate samples from two separate experiments.

findings further, we cultured $V_{\alpha}19i$ T cells together with purified B cells. This resulted in B7RP-1-dependent IL-10 production (**Fig. 7c**). B7RP-1 blockade partially inhibited IL-10 production in cocultures of $V_{\alpha}19i$ T cells and splenocytes and fully inhibited IL-10 production in cocultures of $V_{\alpha}19i$ T cells and purified B cells (**Fig. 7c**). These results suggest that although B cells are a chief producer of IL-10 in this system, other cell types also contribute to $V_{\alpha}19i$ T cell–induced IL-10 production. Furthermore, the ICOS–B7RP-1 pathway is vital for $V_{\alpha}19i$ T cell–induced, B cell–mediated IL-10 production, as blockade with a combination of antibodies to costimulatory molecules (B7RP-1, CD80, CD86 and CD40L) inhibited IL-10 to the same degree as anti-B7RP-1 alone (**Fig. 7c**). However, other costimulatory molecules are involved in $V_{\alpha}19i$ T cell–induced IL-10 production from whole splenocytes (**Fig. 7b**).

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DISCUSSION

Although T cells expressing the invariant $V_{\alpha}19\text{-}J_{\alpha}33$ TCR chain were first identified in 1993 (ref. 22), knowledge of the immunological function of this invariant T cell population is still limited. Nevertheless, important characteristics of this lymphocyte subset have been characterized, including their restriction by MR1, their TAP (transporter associated with antigen processing)–independent development in rodents, humans and cattle, and the notable interspecies conservation of this invariant TCR. Because CD1d-restricted $V_{\alpha}14i$ NKT cells, which influence autoimmunity, have similar properties, we speculated that MR1-resricted T cells would also be capable of modifying autoimmunity. However, $V_{\alpha}19i$ T cells are distinct from $V_{\alpha}14i$ CD1d-restricted T cells in their 'preferential' distribution in the gut mucosa and their dependence on the presence of B cells and gut flora.

$V_{\alpha}7.2i$ T cells, the human homolog of $V_{\alpha}19i$ T cells, are present in lesions of patients with multiple sclerosis[25]. As multiple sclerosis is a demyelinating disease involving autoimmune T cells, B cells, macrophages and various inflammatory mediators, it is possible that MR1-restricted T cells may regulate ongoing disease activity in the CNS. Using an animal model of multiple sclerosis, we examined the effect of overexpression or deletion of MR1-restricted T cells on disease course and severity. Our study suggests that $V_{\alpha}19i$ T cells can suppress autoimmune inflammation. In addition, we have shown that $V_{\alpha}19i$ T cells have a memory or activated surface phenotype and are able to produce large amounts of T_H1 and T_H2 cytokines. $NK1.1^+$ T cells from $V_{\alpha}19i$Tg mice produced more cytokines than did $NK1.1^+$ T cells from $V_{\alpha}19i\text{Tg}Cd1d1^{-/-}$ mice, indicating a possible interaction between CD1d- and MR1-restricted lymphocytes.

We undertook several approaches to determine whether $V_{\alpha}19i$ T cells regulate EAE pathogenesis. Overexpression of $V_{\alpha}19i$ T cells protected mice from clinical EAE. Inhibition of EAE was associated with reduced infiltration and demyelination of the spinal cord as well as a decrease in the production of disease-promoting T_H1 cytokines in the draining lymph nodes and spleen and a reciprocal increase in IL-10, a well established inhibitor of EAE[27–30]. IL-17-secreting cells, which function independently of T_H1 cells, may promote EAE[31]. Here we determined that the inhibitory effect of $V_{\alpha}19i$ T cells is biased toward prevention of secretion of T_H1 cytokines rather than IL-17.

A potential limitation of TCR-transgenic models is the possible disruption of conventional TCR diversity, which could skew TCR recognition of MOG. However, this is unlikely, as anti-MOG T cell proliferative responses were similar in wild-type nontransgenic and $V_{\alpha}19i$Tg mice. Furthermore, we adoptively transferred liver $V_{\alpha}19i$ T cells from naive $V_{\alpha}19i\text{Tg}Cd1d1^{-/-}$ mice into wild-type nontransgenic mice with EAE, which express natural TCR diversity. In those experiments, $V_{\alpha}19i$ T cells effectively inhibited EAE, suggesting that $V_{\alpha}19i$ T cells have a regulatory function during EAE. However, a potential limitation of our model is the difficulty of obtaining pure $V_{\alpha}19i$ T cell preparations because of the lack of a $V_{\alpha}19$ TCR–specific antibody. Therefore, experiments using sorted $CD3^+$ $NK1.1^+$ cells from $V_{\alpha}19i\text{Tg}Cd1d1^{-/-}$ mice may also contain small numbers of non–$V_{\alpha}14i$ TCR $NK1.1^+$ T cells of other TCR specificities. To ascertain whether normal numbers of $V_{\alpha}19i$ T cells in wild-type nontransgenic mice could be involved during EAE, we induced EAE in $Mr1^{-/-}$ mice and found that the absence of $V_{\alpha}19i$ T cells resulted in a more severe clinical disease than that of wild-type nontransgenic mice.

$V_{\alpha}19i$ T cells most likely exert their main effects in the peripheral lymphoid tissue, as the reduction in proinflammatory cytokines and increase in IL-10 was in the draining lymph nodes and spleen. We also demonstrated that the protective effect of $V_{\alpha}19i$ T cells was independent of $V_{\alpha}14i$ NKT cells by using $V_{\alpha}19i$Tg mice on a CD1d-deficient background. Notably, we found reduced adhesion molecule expression on effector T cells from $V_{\alpha}19i\text{Tg}Cd1d1^{-/-}$ mice, which correlated with reduced T cell infiltration of the CNS. However, we did note low numbers of $V_{\alpha}19i$ T cells ($CD3^+NK1.1^+$ from $V_{\alpha}19i\text{Tg}Cd1d1^{-/-}$mice) and B cells in the CNS of mice with EAE, suggesting that $V_{\alpha}19i$ T cells may also regulate EAE in the CNS.

Coculture experiments suggested that IL-10-producing B cells are involved in the amelioration of EAE in $V_{\alpha}19i\text{Tg}Cd1d1^{-/-}$ mice. Notably, that finding is consistent with published studies demonstrating that IL-10-producing B cells are involved in spontaneous remission from EAE and could limit clinical disease when adoptively transferred into mice with EAE[32] or a model of collagen-induced arthritis[33]. However, those results do not exclude the possibility that *in vivo*, other cell types are also involved in $V_{\alpha}19i$ T cell–mediated immune regulation. B cells express MR1 (ref. 34), and $V_{\alpha}19i$ T cells are MR1 restricted[7], but IL-10 production was unaffected in coculture experiments with lymphocytes from MR1-deficient mice, suggesting that MR1, although necessary for $V_{\alpha}19i$ T cell selection, is not essential for $V_{\alpha}19i$ T cell–induced B cell IL-10 production.

T cell activation requires TCR stimulation as well as costimulatory signals. Many costimulatory molecules that regulate cell activation and cytokine secretion have been identified: ICOS and its ligand B7RP-1, CD40-CD40L and CD28-CD80 and CD28-CD86 (refs. 35–38). ICOS costimulation induces IL-10 production as well as help for B cell maturation and CD40L expression[39,40]. The expression of costimulatory molecules on $V_{\alpha}19i$ T cells was unknown before; we have demonstrated here that $V_{\alpha}19i$ T cells express ICOS, CD28, CD86 and CD40L. To determine the contribution of each of these costimulatory signaling pathways on the production of IL-10 after $V_{\alpha}19i$ T cell–B cell interactions, we repeated the coculture experiments using blocking monoclonal antibody to each of the costimulatory pathways. We found that blockade of the ICOS–B7RP-1 pathway inhibited IL-10 production. Furthermore, blockade of the CD40-CD40L, CD28-CD80 or CD28-CD86 pathway also blocked IL-10 production, although not to the extent seen with ICOS blockade.

Commensal flora in the gut are important for the selection of $V_{\alpha}19i$ T cells[7]. $V_{\alpha}19i$ T cells may also control gut production of immunoglobulin A from B cells, suggesting involvement of $V_{\alpha}19i$ T cells in intestinal B cell regulation[7]. Additionally, IL-10 is important for inhibiting excessive inflammation toward gut flora[41], and it has been shown that IL-10 and transforming growth factor-β are involved in immunoglobulin A synthesis and secretion[42]. In the presence of IL-10 and CD40-CD40L signaling, production of immunoglobulin A is increased[43]. Thus, our findings presented here are consistent with the hypothesis that $V_{\alpha}19i$ T cells are involved in the homeostasis of gut immunity[2,7]. We have shown that $V_{\alpha}19i$ T cells help B cells produce

IL-10, which in nonpathogenic conditions may inhibit inflammation against gut flora required for $V_\alpha 19i$ T cell selection. Therefore, we propose a model of $V_\alpha 19i$ T cell–induced protection from EAE whereby $V_\alpha 19i$ T cells interact with B cells in lymphoid tissue through ICOS–B7RP-1 and to a lesser degree through other costimulatory pathways to induce IL-10 production, which in turn can inhibit the production of disease-promoting T_H1 cytokines such as IFN-γ and TNF. In conclusion, here we have identified a protective function for invariant $V_\alpha 19i$ T cells in autoimmune disease. In contrast to 'conventional' $V_\alpha 14i$ NKT cells, more T cells express the $V_\alpha 19i$ TCR human homolog $V_\alpha 7.2$-$J_\alpha 33$ than in mice and therefore these cells may prove to be useful therapeutic targets for the treatment of autoimmune disease.

METHODS

Mice and induction of EAE. C57BL/6 mice (CLEA Laboratory Animal), μMT mice (Jackson Laboratories), $V_\alpha 19i$Tg mice[5], $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice, *Cd1d1*$^{-/-}$ mice and *Mr1*$^{-/-}$ mice[7] were maintained in specific pathogen–free conditions in accordance with institutional guidelines (National Institute of Neuroscience, Tokyo, Japan.). *Mr1*$^{-/-}$ mice were backcrossed to C57BL/6 mice for ten generations[24]. Mice were injected subcutaneously with 100 μg MOG(35–55) and 1 mg heat-killed *Mycobacterium tuberculosis* H37RA (Difco) emulsified in complete Freund's adjuvant. Pertussis toxin (200 ng in PBS; List Biological Laboratories) was injected intraperitoneally on days 0 and 2 after immunization. EAE clinical symptoms were assigned scores daily as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, impaired righting reflex; 3, partial hindlimb paralysis; 4, total hindlimb paralysis.

Cell sorting and adoptive transfer. For depletion of NK cells, mice were injected intraperitoneally with 100 μg anti-asialo-GM_1 (ref. 44) 48 h before purification of $V_\alpha 19i$ T cells. Liver or spleen cells were isolated from mice by Percoll density-gradient centrifugation, and NKT cells, B cells and T cells were purified with the AutoMACS cell purification system (Miltenyi Biotech). NKT cells were isolated using phycoerythrin-conjugated anti-NK1.1 (PK136; BD Pharmingen) and anti-phycoerythrin microbeads (Miltenyi Biotech). The purity of isolated $NK1.1^+$ T cells, assessed by flow cytometry, was more than 90%. In some experiments, single-cell suspensions were incubated with fluorescein isothiocyanate–anti-CD3 (2C11; BD Pharmingen) and phycoerythrin–anti-NK1.1 (PK136, BD Pharmingen) for sorting by flow cytometry. B cells and T cells were isolated from the spleen with anti-CD19 microbeads or the 'pan T cell' kit (Miltenyi Biotech). For adoptive transfer studies, liver $CD3^+NK1.1^+$ $V_\alpha 19i$ T cells were sorted from naive $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice as described above, and 1×10^6 $V_\alpha 19i$ T cells were injected intraperitoneally into naive C57BL/6 recipient mice on the day of immunization with MOG(35–55). Control groups received identical numbers of $CD3^+NK1.1^-$ hepatic cells.

Cell proliferation and cytokine analysis. For *in vitro* stimulation of sorted $V_\alpha 19i$ T cells, $CD3^+NK1.1^+$ and $CD3^+NK1.1^-$ cells were suspended in RPMI 1640 medium (Sigma) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml of penicillin-streptomycin, 2 mM sodium pyruvate and 50 μM β-mercaptoethanol and were stimulated with immobilized anti-CD3 (5 μg/ml; BD Pharmingen). Cytokines were measured with inflammation cytometric bead assay kits (BD Biosciences) at 24, 48 and 72 h after stimulation with mouse T_H1-T_H2 cytokines. At 10 d after EAE induction without pertussis toxin, myelin-specific T cell responses were measured. Lymphocytes (1×10^6) were cultured with MOG(35–55) (1–100 μM for proliferation studies and 100 μM for cytokine analysis). Cytokines were measured with a cytometric bead assay kit (BD Biosciences) or an IL-17 enzyme-linked immunosorbent assay kit (BD Pharmingen) at 72 h after stimulation. Identical sets of wells were used for proliferation studies. After 72 h, cells were incubated with [^{3}H]thymidine (1 μCi/well) for the final 16 h of culture and incorporation of radioactivity was analyzed with a β-1205 counter (Pharmacia). Proliferation was determined from triplicate wells for each peptide concentration and is expressed as counts per minute.

Surface marker analysis, quantification of CNS leukocytes and histology. The surface phenotype of sorted $V_\alpha 19i$ T cells was analyzed by flow cytometry. Nonspecific staining was inhibited by incubation with anti-CD16/32 (BD Pharmingen). Cells were then stained with fluorescence-labeled antibodies specific for CD4, NK1.1, TCRβ, CD3, CD44, CD49d, CD19, CD8, CD14, CD28, CD278, CD86 or CD154 (BD Pharmingen) or CCR1 and CCR2 (Santa Cruz), followed by phycoerythrin-conjugated anti-goat immunoglobulin G (Santa Cruz), and were analyzed with a FACSCalibur (Becton Dickinson). Intracellular cytokines were analyzed by flow cytometry with the BD Cytofix/Cytoperm kit (BD Pharmingen). Staining of paraffin-embedded spinal cords with luxol fast blue and with haematoxylin and eosin was done by SRL. For quantification by flow cytometry, spinal cords were homogenized through 70-μm nylon mesh and by Percoll density-gradient centrifugation to form single-cell suspensions.

RNA extraction and real-time RT-PCR. The SV Total RNA isolation kit (Promega) was used for isolation of total RNA from sorted liver or splenic NKT cells, T cells or B cells according to the manufacturer's instructions. 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 real-time PCR. Gene expression values were normalized to expression of the hypoxanthine guanine phosphoribosyl transferase (*Hprt1*) 'housekeeping' gene. Primers from Bex Co are listed in **Supplementary Table 1** online.

Mixed-lymphocyte experiments. MOG(35–55)-specific spleen cells (2×10^6) isolated from wild-type nontransgenic mice 10 d after EAE induction were mixed with liver $V_\alpha 19i$ T cells (5×10^5) sorted from naive $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice, in the presence of 100 μg/ml of MOG(35–55) in 24-well plates or transwell plates (Corning). Where indicated, MOG(35–55)-specific spleen cells were isolated from *Mr1*$^{-/-}$ or μMT mice or were subjected to depletion with anti-CD19 microbeads (Miltenyi Biotech). Costimulatory molecules were blocked with 10 μg/ml of anti-B7RP-1 (HK5.3) or anti-CD40L (MR1) or with anti-CD80 and anti-CD86 (16-10A1 and GL1, respectively; all from BD Pharmingen)[35]. After 72 h, cytokines in the supernatant were analyzed by cytometric bead assay, enzyme-linked immunosorbent assay or intracellular flow cytometry. Proliferation of MOG(35–55)-specific lymph node cells was assessed 24 h after the addition of [^{3}H]thymidine (1 μCi/well) to 96-well plates.

Statistics. EAE clinical scores for groups of mice are presented as the mean group clinical score ± s.e.m., and statistical differences were analyzed by the Mann-Whitney U nonparametric ranking test. Cytokine secretion data were analyzed with the two-tailed Student's *t*-test or one-way analysis of variance with Tukey post-analysis for multiple group analysis.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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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 *J*α*281*$^{-/-}$ 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 *J*α*281*$^{-/-}$[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'-GTTGGATACAGGCCAGACTTT-GTTG-3'; HPRT reverse, 5 '-GAGGGTAGGCTGGCCTATAG-GCT-3'; RORγt forward, 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-

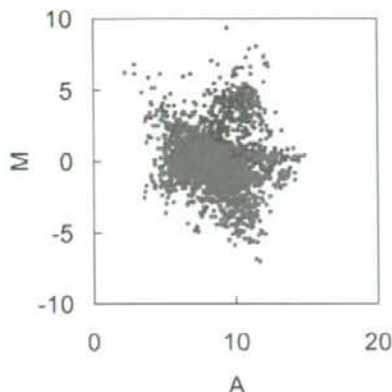

Figure 1. Altered composition of the intestinal microflora by oral administration of antibiotics KCV. A custom DNA microarray named 'FloraArray'[27] was used for evaluating the gut flora of mice. Briefly, genomic DNA was extracted from freshly collected fecal samples and fragmented by physical force.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

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

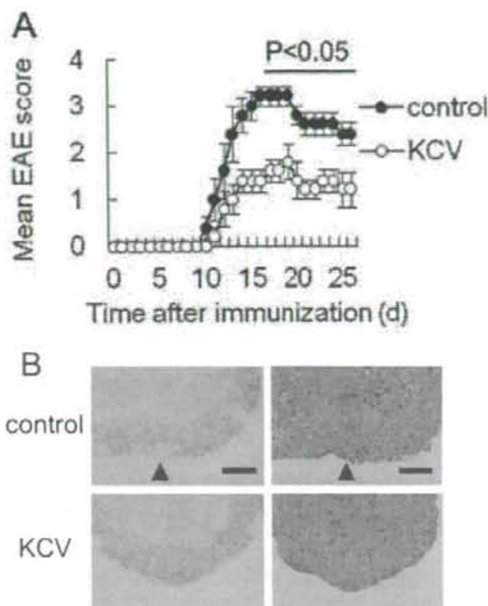

Figure 2. Suppression of EAE by oral KCV treatment. **A:** Clinical score of EAE. After immunized with MOG (35–55) mice were treated with KCV as described in Materials and Methods. Clinical EAE scores of KCV-treated mice (KCV) and of control mice (control) are shown. Data represent mean score ± SEM from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

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

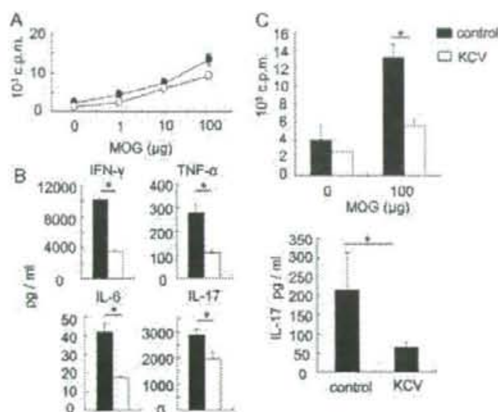

Figure 3. Reduced MOG-specific responses in dLN and MLN cells from KCV-treated mice. **A:** Effect of KCV on the lymphocyte proliferative responses. Draining lymph nodes (dLNs) were removed from control or KCV-treated mice 11 days after immunization with MOG (35–55) and the total lymphoid cells (1×10^6) 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).

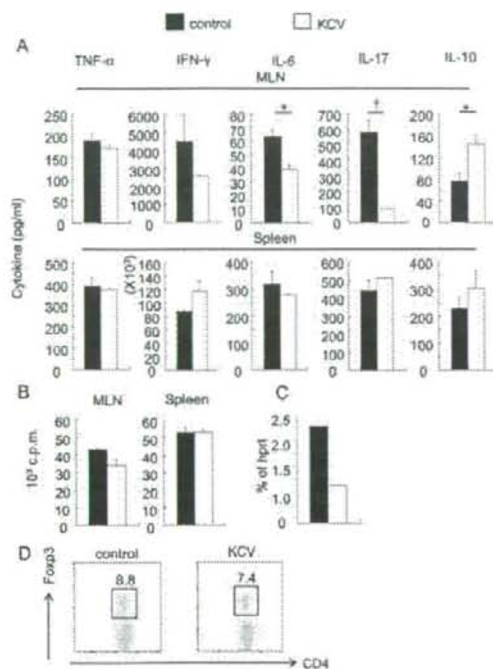

Figure 4. Decreased production of inflammatory cytokines from MLN cells after oral KCV treatment. **A:** Cytokine production from MLN T cells of naïve mice after KCV treatment. Mice were continuously given KCV-containing or control water for 7 days. Then MLN cells and splenocytes were isolated and stimulated 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

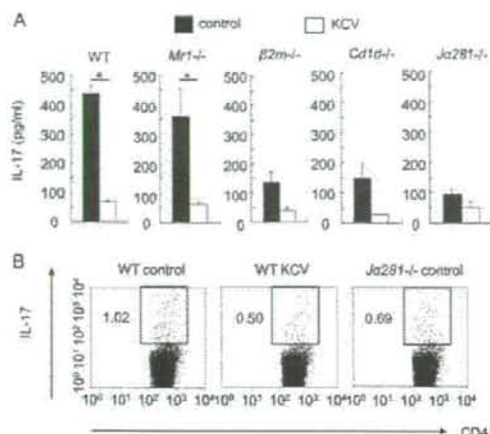

Figure 5. A role of Vα14 iNKT cells in the regulation of mesenteric Th17 cells. **A:** IL-17 production by the MLN T cells of mice lacking invariant iNKT or MAIT cells. After 1 week of KCV treatment, MLN cells were isolated from control or KCV-treated mice, including wild-type (WT), $Mr1^{-/-}$, $\beta 2m^{-/-}$, $Cd1^{-/-}$, or $J\alpha 281^{-/-}$ 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 $J\alpha 281^{-/-}$ 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*$^{-/-}$ ($\beta 2m^{-/-}$) 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 $\beta 2m^{-/-}$ 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 $\beta 2m^{-/-}$ 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 $J\alpha 281^{-/-}$ 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 $IL\text{-}17^+$ $CD4^+$ 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 $J\alpha 281^{-/-}$ mice (Figure 5B), we have confirmed that the number of $IL\text{-}17^+$ $CD4^+$ T cells corresponding to Th17 cells is reduced in the KCV-treated wild-type mice and in the iNKT cell-deficient $J\alpha 281^{-/-}$ mice. We also noticed that $IL\text{-}17^+$ iNKT cells are 15 times lower than $IL\text{-}17^+$ $CD4^+$ 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

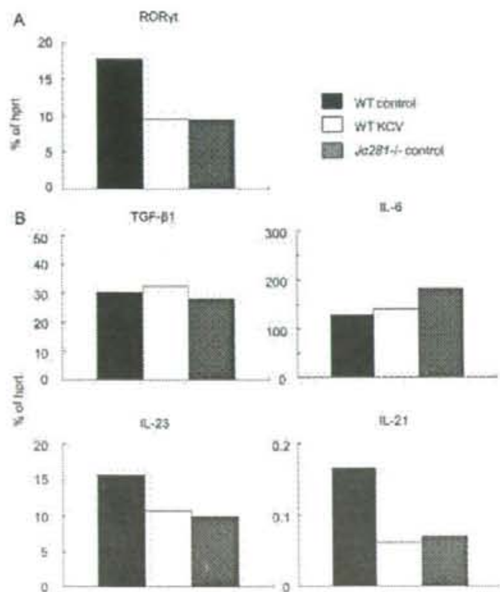

Figure 6. Reduced expression of Th17-promoting cytokines in the intestinal lamina propria lymphocytes from KCV-treated mice as well as iNKT-deficient mice. **A:** RORãt expression in the intestinal LP after treatment with oral KCV. Total RNA was isolated from pooled LPLs prepared from wild-type control B6 mice (WT control), KCV-treated wild-type B6 mice (WT KCV), and control $J\alpha 281^{-/-}$ mice ($J\alpha 281^{-/-}$ 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 $J\alpha 281^{-/-}$ 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 $J\alpha 281^{-/-}$ mice showed

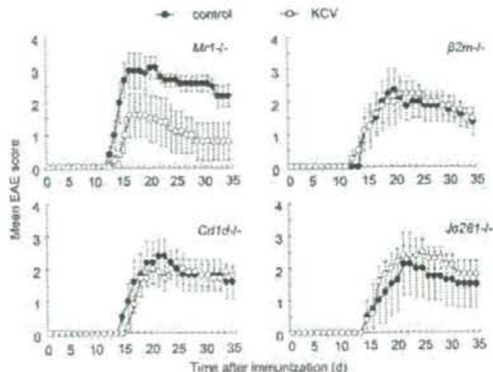

Figure 7. Suppressive effect of oral KCV treatment on EAE is abolished in Vα14 iNKT-deficient mice. $Mr1^{-/-}$, $\beta 2m^{-/-}$, $Cd1^{-/-}$, and $J\alpha 281^{-/-}$ mice were treated with KCV as described in Materials and Methods. After immunization of mice with MOG (35–55) clinical EAE scores of 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 $J\alpha 281^{-/-}$ 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 ($\beta 2m^{-/-}$, $Cd1^{-/-}$, $J\alpha 281^{-/-}$) 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 $\beta 2m^{-/-}$, $Cd1^{-/-}$, and $J\alpha 281^{-/-}$ 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.

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多発性硬化症と環境因子

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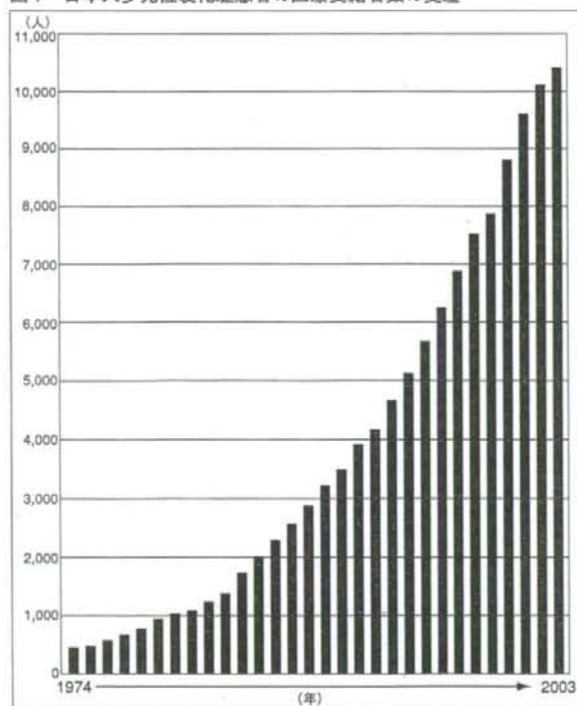
● はじめに

日本人の多発性硬化症（multiple sclerosis：MS）は、過去30年間で明らかに増加した。それは、神経内科医の増加やMRI検査の普及だけでは説明できないような増加傾向である（**図1**）。また、日本には視神経と脊髄に病変の限局する病型（アジア型MS）が多いことが強調されていたが、近年は大脳に多くの病変を有する病型（欧米型MS）の増加傾向が著しく、女性患者優位の傾向も明瞭になってきている。MSは遺伝と環境の両者で規定される疾患であるが、この30年間の欧米型MS増加の原因は遺伝因子（先天的因子）ではなく、環境因子（後天的因子）に求められなければならないことは明らかである。MSにおける環境因子の影響については、従来、移民の疫学調査の結果がしばしば引用されている。大まかに言うと、15歳以前に育った場所がMSの多発する地域（高緯度地域など）であれば、MSを発症しやすくなるという結論が導かれており、免疫系が発達する時期（幼小児期）に、どのような環境に曝されたかどうかがMSの発症リスクを規定することが推測されている。それでは現代日本におけるMSの増加は、どのような環境変化で説明できるのであろうか？

図1　日本人多発性硬化症患者の医療受給者数の変遷



● 衛生仮説

先進諸国では近年、免疫系の変調を背景に持つ疾患（アレルギー疾患、炎症性腸炎など）の増加傾向が顕著である。その原因は生活環境が過度に清潔になったためであると言う説があり、「衛生仮説（hygiene hypothesis）」と呼ばれている。免疫系は細菌や寄生虫感染から生体を守る必須の防御機能であるが、多くの感染症を経験することによって、バランスの取れた免疫系が形成される。先進諸国では幼小児期に、細菌、ウイルス、寄生虫などに感染する機会が極端に減った。そのために、免疫制御系（免疫反応を制御する細胞など）の発達に問題が生じ、本来免疫系が反応するべきではない抗原（花粉、自己抗原など）に対する免疫応答が亢進しているのではないか？これが「衛生仮説」である。

最近オーストラリアで行われた疫学調査は、幼児期に一緒に育てられた弟や妹の数が少ないほどMSを発病する可能性が高まる可能性を示唆している[1]。弟や妹が少ないと“ありふれた”ウイルス（麻疹など）に遭遇する機会が減り、そのために免疫系の発達に問題が生じ、MSの発症リスクが高まるという解釈が成り立つ。この考え方が正しいとすると、一人っ子が増えている現代社会で、アレルギー疾患や自己免疫病の増加傾向が見られるのは当然かもしれない。しかし、この論文で示された兄弟（姉妹）数のMS発症に与える影響は小さく、それだけで昨今のMSの増加を説明できるとは思えない。

● 腸内細菌叢と免疫の関係

一方、病原性のある感染体のほかに、病気には直接関係しない微生物（腸内細菌、常在細菌など）も免疫系の形成に大きな影響を与えることがわかってきた。たとえば、最近、腸内細菌がないと発達しないユニークな腸の免疫制御細胞が発見された[2,3]。また腸内細菌の少ないマウスで、NKT細胞（後述）の数が少ないという報告もある。腸内細菌叢が免疫制御系に少なからぬ影響を与えることが、さまざまな実験で示され、米国の免疫学会では特別シンポジウムが企画されるほどである。過去30年間で日本人の食生活は大きく変化したが、腸内細菌叢も変化し、それが免疫制御細胞の機能変調を生み、MSやアレルギーの発症増加につながったという可能性は十分にある。この仮説が正しければ、MSは生活習慣の改善によって予防できる「生活習慣病」ということになる。

● 欧米型MSとアジア型MS

欧米型MSはヨーロッパや北米などに多いが、アジア型MSは世界中に分布しており、地域差がない。このことから、欧米型MSの方が衛生環境や生活習慣に影響されやすいことが推測される。冒頭に述べたように、日本国内では欧米型MSの増加傾向が著しいが、アジア型MSについてはあまり変化がない。現代日本人で

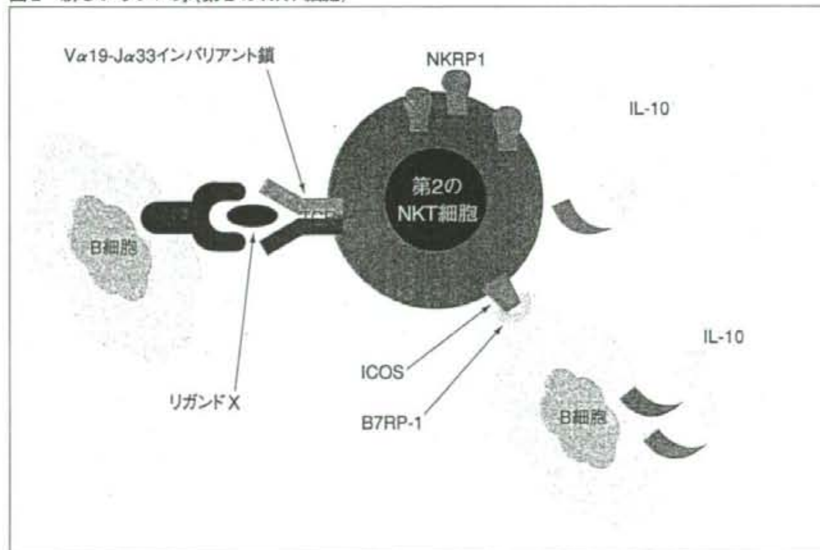
は、欧米型MSの発症を制御するようなメカニズムに問題が生じているのかもしれない。従来、MSの脳内炎症を惹起するリンパ球はインターフェロン(interferon：IFN)-γを産生するTh1細胞であるとされてきた。しかし、最近、インターロイキン(interleukin：IL)-17を産生するTh17も重要な炎症惹起細胞であることがわかった[4]。Th17細胞はTh1細胞よりも強い脳炎惹起能を有し、その誘導にはIL-6とtransforming growth factor(TGF)-βの組み合わせが重要である[5]。欧米型MSはおもにTh1細胞の介在する病態であるが、アジア型MSではTh17細胞の役割が推測されている[6]。現代の日本人では、Th1応答を制御するシステムに変調をきたしている傾向があり、それが欧米型MSの増加につながっているという可能性が考えられる。

● インバリアントVα19i T細胞

ー腸管粘膜固有相に集積する第2のNKT細胞ー

生活習慣と免疫系には強い関連があるにもかかわらず、生活習慣に隠された、何が、どのようにして、免疫制御系を変調に追い込むかについて、これまで手がかりはほとんどなかった。しかし、最近になって、腸内細菌叢と密接に関連を持つリンパ球が見つかった。このリンパ球は研究者によってMAIT細胞(mucosal associated invariant T cells)[2]、インバリアントVα19i T細胞などいろいろな名前で呼ばれるが、T細胞抗原受容体(マウスではVα19-Jα33インバリアント鎖)とNK細胞のマーカー分子(NKRP1など)をあわせて発現する点が、NKT細胞[7]と類似している(“第2のNKT細胞”)。しかし、NKT細胞がMHCクラスⅠb分子であるCD1d分子に依存性であるのに対し、Vα19i T細胞はMR1分子というクラスⅠb分子に依存している点が異なる(図2)。さらに大きな特徴は、このリンパ球が腸管の粘膜固有相に集積しており、腸内細菌のないマウスでは欠損するという点である[2]。この特異な分布様態から、この細胞の発生には腸内細菌が必要であり、腸管における免疫制御に関わる重要な細胞であることが推測されている[2]。しかし、われわれは、この細胞がMS患者の脳に浸潤することを明らかにし[8]、腸の免疫に限らず、MSの重要な免疫制御細胞として機能する可能性を提起してきた。ごく最近、この仮説をMSの動物モデルで実証することに成功した[3]。すなわち、この細胞の多いマウスではMSの動物モデルEAE(experimental autoimmune encephalomyelitis：実験的自己免疫性脳脊髄炎)が軽くなり、欠損するマウスでは重症化し、この細胞を注射するとEAEの症状は改善することを動物実験で証明した。さらに、この細胞は欧米型MSを惹起するTh1細胞を強く抑制するが、アジア型MSでの役割が推測されるTh17細胞に対する抑制作用はほとんどないことを発見した[3]。すなわち、同細胞は、欧米型MSの発症を予防する細胞であることが考えられる。このような細胞が腸管で見つかったことから、生活習慣、食生活と免疫制御細胞(Vα19i T細胞など)の関係について、詳しく解析を進める必要があることは明らかである。それはMSの予防や治療に有効な新しい方法の開発につながる可能性がある。

図2　新しいリンパ球(第2のNKT細胞)



このリンパ球は抗原受容体α鎖に固定鎖(Vα19-Jα33インバリアント鎖)を持ちMR1分子に結合したリガンドを認識する。またICOS分子を介してB細胞のB7RP-1分子と結合し、免疫抑制性サイトカインであるIL-10をB細胞と協同して産生する。これが自己免疫性炎症抑制のメカニズムである[3]。

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解　説

Mucosal associated invariant T（MAIT）cells—MR1拘束性の新しいNKT細胞*

山村　隆**　三宅幸子**

Key Words：MR1, NKT cell, mucosal associated invariant T（MAIT）cells, gut flora, experimental autoimmune encephalomyelitis（EAE）

はじめに

本稿では，抗原受容体(TCR)アルファ鎖にインバリアント鎖(invariant α-chain)を発現する新しいＴ細胞集団mucosal associated invariant T（MAIT）cells[1]について概説する．このＴ細胞はマウスではVα19-Jα33インバリアント鎖，ヒトではVα7.2-Jα33インバリアント鎖を発現するが，腸内細菌に依存し，腸管粘膜に集積するというユニークな性質をもつ．「MAIT」という名称はそのユニークな分布様態(粘膜指向性)に基づいている．しかし，名称についてはいまだ統一されておらず，インバリアント鎖を冠した命名(Vα19-Jα33Ｔ細胞)のほか，拘束分子(MR1)に関連した名称なども使われている．マウスVα19-Jα33Ｔ細胞はNK1.1陽性の分画に存在することから[2]，「第二のNKT細胞」と呼ばれることもある．

これまで研究されてきたNKT細胞は主にCD1d分子に拘束されるが，「MAIT細胞」はMHCクラスIb分子であるMR1分子[3]に拘束される．MR1もCD1dもともに，種や系統を超えて高度に保存されており，それが対応するTCRの均一性に対応している．なお，「MAIT」という名称は覚えやすいが，この細胞の性格を必ずしも正確に表したものではない．実際，このＴ細胞は自己免疫疾患である多発性硬化症(MS)の脳病変で検出できるし[4]，自己免疫病の動物モデル実験的自己免疫性脳脊髄炎(experimental autoimmune encephalomyelitis；EAE)では，臓器特異的炎症に対して抑制的に働く[5]．したがって，粘膜免疫に限らず，自己免疫性臓器炎症の制御細胞としても，重要な役割を果たすものと考えられる．

発見の経緯

ヒトCD4$^-$CD8$^-$Ｔ細胞のTCR配列を調べると，特定のTCRアルファCDR3配列が繰り返して検出されることが，初期の研究で注目を集めた[6]．その中でVα24-Jα18インバリアント鎖はマウスのCD1d拘束性NKT細胞の発現するインバリアント鎖と高度なホモロジーを有し，ヒトNKT細胞のTCR配列であることが明らかになった[7]．一方Lantzらは，Vα24-Jα18以外のインバリアント鎖に着目し，1999年にVα7.2-Jα33インバリアント鎖を発現するヒトＴ細胞が，非ペプチド抗原を認識するユニークな細胞集団であることを明らかにした[8]．この研究で彼らは，このインバリアント鎖を発現するＴ細胞(以下，Vα7.2-Jα33Ｔ細胞と略)が主にCD4$^-$CD8$^-$もしくはCD8αα$^+$であり，transporters associated with antigen processing(TAP)分子を欠損する患者において残存することを示した．ちなみにTAP欠損患者では，ペプチド反応性の通常Ｔ細胞は欠損する．さらに，マウスでもVα7.2-Jα33に類似したインバリアント鎖(Vα19-Jα33)を発現するCD4$^-$CD8$^-$Ｔ細胞が存在することを示した．これらのＴ細胞はTAP

* Mucosal associated invariant T（MAIT）cells—MR1-restricted NKT cells.

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ウシ	ASYLCVV	MDGNYQ	WIWGSGTKL
マウス	ASYLCAV	RDGNYQ	LIWGSGTKL
ヒト	ASYLCVV	KDGNYQ	WIWGSGTKL

図1 MR1拘束性T細胞アルファ鎖のホモロジー
ウシ，マウスおよびヒトのMR1拘束性T細胞は高度に保存されたTCRアルファ鎖を発現する．ボックスで囲まれた部分はCDR3に相当する． (文献[8]より引用)

に依存しないので非ペプチド抗原を認識することが予測されたが，CD1d欠損マウスに存在するのでCD1d拘束性NKT細胞ではない．しかし，β_2 microglobulin(β_2m)欠損マウスでは欠損するので，CD1d以外のMHC class I様分子に拘束されたT細胞集団であることが示唆された．

一方Shimamuraらは，CD1dノックアウト(CD1d KO)マウスにNK1.1$^+$ T細胞が若干残存することから，CD1dに拘束されないNKT細胞の存在することを推測していた．彼らは，CD1d KOマウスのNK1.1$^+$ T細胞を分離し，そのT細胞ハイブリドーマを樹立し，TCR配列を決定した．その結果，その多くがVα19-Jα33インバリアント鎖を発現することがわかった[2]．この報告はLantzらの報告したT細胞が，CD1dに拘束されない「第二のNKT細胞」である可能性を示唆した．

その翌年，Lantzらは，ヒトVα7.2-Jα33 T細胞が腸管へのホーミングに重要なα4β7インテグリンを発現し，腸管の粘膜固有相(lamina propria)や肺の粘膜で選択的に検出されることを示した．粘膜組織への選択的な分布は，マウスVα19-Jα33 T細胞においても確認された．この特異な体内分布から，彼らは同細胞が粘膜における過剰な免疫応答を制御する制御性細胞と考え，「mucosal-associated invariant T cells(MAIT細胞)」と呼ぶことを提唱した．また，骨髄キメラの実験からVα19-Jα33 T細胞はB細胞による選択を受けることも示された．

MAIT細胞のTCRアルファ鎖CDR3領域は，ヒト，マウス，ウシにおいて高度に保存されていることもわかった(図1)[8]．ベータ鎖のCDR3は多様であるが，限られたVβセグメント(マウスではVβ8.2など，ヒトではVβ11など)を優先的に使用する点は，CD1d拘束性NKT細胞と類似している．Lantzらは，非古典的MHC class I分子の中で，種間の相同性がもっとも高いMR1分子に注目し，同分子の欠損マウスを調べたところ，MAIT細胞を完全に欠損していることがわかった．一連の結果から，MAIT細胞はMR1分子で拘束される新しい細胞集団であると考えられる．なお，Shimamuraらの報告[2]，われわれの解析結果[5]，さらに最近のGilfillanらの報告は[9]，Vα19-Jα33 T細胞の多くがNK細胞マーカーNK1.1を発現することを示している．MAIT細胞を「新しいNKT細胞」と位置づけることも可能である(表1)．

Vα7.2-Jα33 T細胞と多発性硬化症

多発性硬化症(multiple sclerosis；MS)はT細胞やB細胞の介在する中枢神経系の自己免疫疾患で，患者末梢血では，NKT細胞[10]，NK細胞[11]，CD25陽性制御性T細胞[12]などの制御性細胞が数や機能において変化している．われわれは，NKT

表1 CD1d拘束性NKT細胞とMR1拘束性NKT細胞(MAIT)の比較

	CD1d拘束性NKT	MR1拘束性NKT
名称	NKT cell, iNKT cell Vα14i NKT(マウス)/Vα24i NKT(ヒト)	MAIT細胞，Vα19i T cell(マウス) Vα19-Jα33 T cell(マウス)
TCRアルファ鎖	Vα14-Jα18(マウス)/Vα24-Jα18(ヒト)	Vα19-Jα33(マウス)/Vα7.2-Jα33(ヒト)
TCRベータ鎖	Vβ8, Vβ2, Vβ7(マウス)/Vβ11(ヒト)	Vβ8, Vβ6(マウス)/Vβ2, Vβ13(ヒト)
Positive selectionに関与する細胞	胸腺CD4$^+$CD8$^+$細胞	B細胞
TAP依存性	なし	なし
β_2-microglobulin依存性	あり	あり
腸内細菌依存性	なし	あり
臓器分布	肝臓，脾臓，骨髄，胸腺	腸管粘膜，肺粘膜，肝臓
対応抗原	α-galactosylceramide, iGb3など	α-mannosylceramide(?)
産生サイトカイン	IL-2, IL-4, IL-5, IL-10, IL-13, GM-CSF, IFN-γ	IL-4, IL-5, IL-10, TNF-α, IFN-γ

細胞の研究を進めるうちに，Vα7.2-Jα33インバリアント鎖の存在に興味をもち，MSの脳病変や脳脊髄液サンプルにおけるVα7.2-Jα33インバリアント鎖の発現を検証した．解析にはRT-PCR-SSCPによるクロノタイプ検出法[13]を用いた．その結果，Vα7.2-Jα33鎖のメッセージは，MSの病変や髄液のサンプル，および炎症性末梢神経炎の生検サンプルの多くで検出されることがわかった[4]．この結果は，Vα7.2-Jα33 T細胞の役割が，腸管免疫の制御に限られるものではなく，広く自己免疫性炎症の制御に及ぶものであることを推測させた．

Vα19-Jα33 T細胞は自己免疫性炎症の制御細胞である

MSの脳病変に浸潤するVα7.2-Jα33 T細胞は，炎症抑制的に働いているのであろうか？ あるいは炎症を悪化させているのであろうか？ ヒト材料を使った研究では結論を出すのは難しい．そこでわれわれは，Vα19-Jα33 TCRを過剰発現するトランスジェニックマウス(以下，Vα19 Tgと略)[14]と，同マウスをCD1d KOマウスに交配したVα19 Tg x CD1d KOを導入した．CD1d KOマウスにおける肝臓NK1.1$^+$ T細胞の割合は2％程度であり，野生型マウスのそれに比べて6分の1程度に減少している．一方，Vα19 Tg x CD1d KOの肝臓NK1.1$^+$ T細胞は野生型マウスのそれとほぼ同数で，ほぼ純粋なVα19-Jα33 T細胞であると考えられる．

研究にはMSの動物モデルEAEを用いた[5]．EAEの誘導はB6マウスに脳炎惹起性ペプチドである

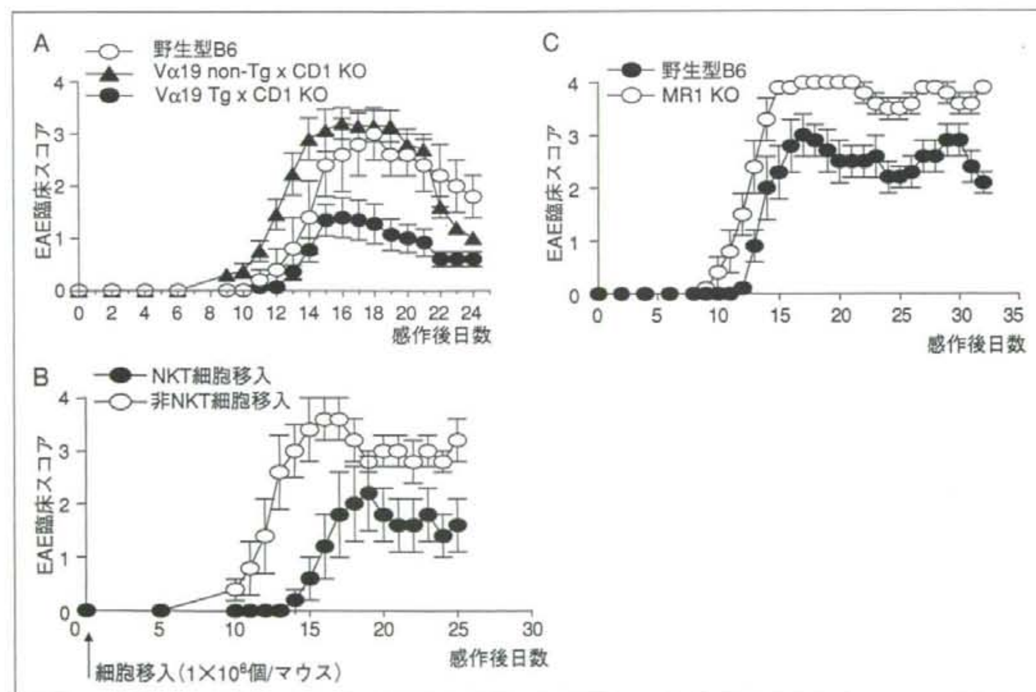

図2 Vα19-Jα33 T細胞のEAEに対する影響

A：Vα19 TgにおけるEAEの軽症化．対照(野生型B6, およびCD1d KOのリッターメート)に比較し，MR1拘束性NKT細胞を過剰に発現するVα19 Tg x CD1d KOマウスでは，EAEの臨床経過が軽症化した．この結果から，Vα19-Jα33 T細胞はCD1d拘束性NKT細胞の存在しない環境においても，十分な免疫制御能を発揮することがわかる．データは臨床スコア平均値±SEMで示した．

B：Vα19-Jα33 T細胞の移入によるEAEの軽症化．野生型B6マウスにEAEを誘導し，その直後にVα19 Tg x CD1d KOマウスの肝臓から分離したVα19-Jα33 T細胞，またはNK1.1陰性細胞を100万個移入した．Vα19-Jα33 T細胞移入群において，有意なEAEの抑制がみられた．

C：MR1 KOマウスにおけるEAEの重症化．野生型B6マウスおよびMR1 KOマウスにEAEを誘導した．MR1 KOにおけるEAEの重症化と炎症性サイトカイン産生亢進が確認された．