Table 1 $V_\alpha 19i$ T cells in EAE

Group	Mice with EAE	Group score	EAE score	Day of onset
Wild-type	10 of 10	3.3 ± 0.3	3.3 ± 0.3	13.6 ± 0.7
Cd1d1$^{-/-}$	18 of 18	3.4 ± 0.2	3.4 ± 0.2	11.7 ± 0.5
$V_\alpha 19i$Tg*Cd1d1*$^{-/-}$	13 of 22	1.3 ± 0.3***	2.2 ± 0.2**	14.3 ± 0.6**
Wild-type	7 of 7	3.6 ± 0.2	3.6 ± 0.2	13.6 ± 0.5
Cd1d1$^{+/+}$	11 of 11	3.3 ± 0.4	3.3 ± 0.4	14.8 ± 0.7
$V_\alpha 19i$Tg*Cd1d1*$^{+/+}$	9 of 13	1.3 ± 0.3**	1.9 ± 0.4*	18.6 ± 1.2**
NK1.1$^-$ AdTx	10 of 10	3.6 ± 0.3	3.6 ± 0.3	11.6 ± 0.5
$V_\alpha 19i$ AdTx	8 of 10	2.2 ± 0.4*	2.8 ± 0.3	15.8 ± 0.6***
Mr1$^{+/+}$	10 of 10	3.0 ± 0.2	3.0 ± 0.2	13.9 ± 0.5
Mr1$^{-/-}$	8 of 8	4.0 ± 0.0**	4.0 ± 0.0*	11.5 ± 0.5***

Clinical outcome of mice immunized with MOG(35–55) to induce EAE. Data represent number of mice with EAE (of total mice in group); mean group EAE score (± s.e.m.); mean EAE score excluding mice without evidence of EAE (± s.e.m.); and mean day of onset (± s.e.m.). In one experiment, mice received adoptive transfer (AdTx) of $V_\alpha 19i$ T cells or NK1.1$^-$ cells as a control. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared with control groups (Mann-Whitney U nonparametric test).

asialo-GM1 (anti-asialo-GM1). We then sorted NK1.1$^+$ cells from the liver. When activated by plate-bound anti-CD3, NK1.1$^+$ T cells from *Cd1d1*$^{+/+}$ mice secreted more interferon-γ (IFN-γ), tumor necrosis factor (TNF) and interleukin 4 (IL-4) than did those from *Cd1d1*$^{-/-}$ mice, confirming that CD1d-restricted T cells are a chief source of cytokines (**Fig. 1c**). However, NK1.1$^+$ T cells from $V_\alpha 19i$Tg mice secreted more T_H1 cytokines (IFN-γ and TNF) and T_H2 cytokines (IL-4 and IL-10) than did NK1.1$^+$ T cells from nontransgenic littermates (**Fig. 1c**). During subsequent experiments, we used $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice as a source of $V_\alpha 19i$ T cells.

$V_\alpha 19i$ T cells in EAE

To determine if an abundance of $V_\alpha 19i$ T cells could modulate autoimmune disease, we analyzed the development and progression of EAE in $V_\alpha 19i$Tg mice. We induced EAE by immunizing mice with a peptide of amino acids 35–55 of myelin oligodendrocyte glycoprotein (MOG(35–55)). The presence of the $V_\alpha 19i$ transgene suppressed the development and progression of EAE, regardless of whether CD1d-restricted NKT cells were present (**Fig. 2a,b** and **Table 1**). The onset of EAE was delayed in $V_\alpha 19i$Tg mice, and the incidence and severity of clinical EAE was reduced.

Histological examination of the lumbar (L3) region of the spinal cord 15 d after EAE induction showed less monocyte infiltration and demyelination (assessed by luxol fast blue staining) in $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice than in *Cd1d1*$^{-/-}$ mice (**Fig. 2c**). In agreement with the histology, spinal cords of *Cd1d1*$^{-/-}$ mice contained three times more infiltrating cells than did those from $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice (0.09×10^6 and 0.03×10^6 cells respectively, pooled from three mice). Flow cytometry showed fewer CD4$^+$ T cells infiltrating the CNS at an active stage of EAE (day 15) in $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice (6%) than in nontransgenic littermates (21%; **Fig. 2d**). Moreover, 11% and 15% of CNS-infiltrating CD3$^+$ T cells expressed NK1.1$^+$ in *Cd1d1*$^{-/-}$ and $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice, respectively, and NK1.1$^+$ T cells comprised between 1% and 2% of total CNS-infiltrating cells (**Fig. 2d**). Also, few B cells trafficked into the CNS during EAE (3% and 2% in *Cd1d1*$^{-/-}$ and $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$, respectively, **Fig. 2d**). To determine potential mechanisms of reduced CNS infiltration, we analyzed the expression of chemokine receptors and adhesion molecules necessary for T cell migration into the CNS. TCRβ$^+$CD4$^+$ T cells isolated from the CNS, lymph nodes and spleens of $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ and *Cd1d1*$^{-/-}$ mice on day 18 after EAE induction had similar surface expression of CCR1 and CCR2 (data not shown). However, $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice had fewer CD44$^+$ and CD49d$^+$ TCRβ$^+$ splenocytes than did *Cd1d1*$^{-/-}$ mice (**Supplementary Fig. 1** online).

Next we examined recall responses of MOG(35–55)-primed T cells by *ex vivo* rechallenge with MOG(35–55) on day 10 after disease induction. Compared with nontransgenic cells, lymph node cells from MOG(35–55)-primed $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice produced less pro-inflammatory cytokines (IFN-γ, TNF, IL-2 and IL-17) and more immunosuppressive IL-10 ($P < 0.05$; **Fig. 3a**). IL-4 and IL-5 were below the limits of analysis detection (less than 5 pg/ml).

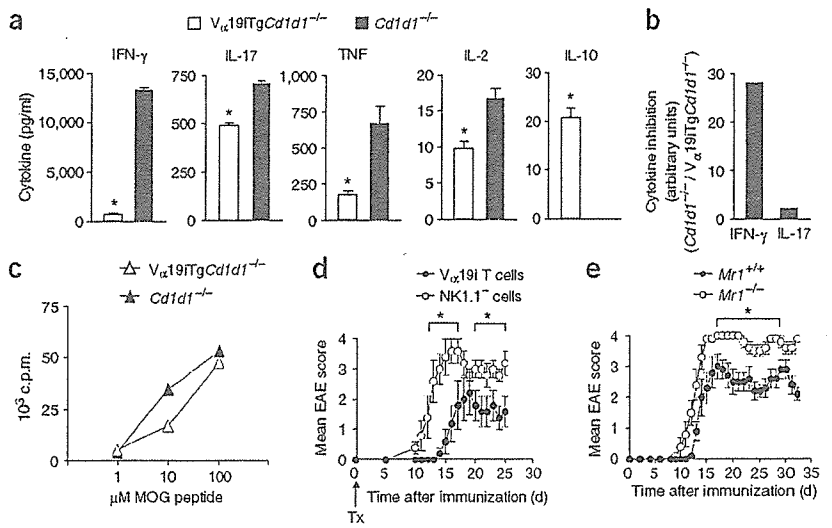

Figure 3 Inhibition of EAE is associated with decreased T_H1 cytokine production. (**a**) Cytometric bead assay of cytokines in the supernatants of MOG-specific lymph node cells (1×10^6) isolated from mice on day 10 after EAE induction and rechallenged with 100 μM MOG(35–55) *in vitro*, measured 72 h after rechallenge. Data represent the mean ± s.e.m. of duplicate samples from three separate experiments. *, $P < 0.05$ (two-tailed Student's *t*-test). (**b**) Inhibition of IFN-γ or IL-17 in $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice versus *Cd1d1*$^{-/-}$ mice from a, presented as 'fold inhibition' of cytokine, calculated as the cytokine concentration from *Cd1d1*$^{-/-}$ mice divided by the cytokine concentration from $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice. (**c**) T cell proliferation of cell preparations identical to those in a from lymph nodes (mouse genotypes, key) rechallenged for 72 h with varying doses of MOG(35–55), assessed by [^{3}H]thymidine incorporation. Data represent the mean of triplicate samples from three separate experiments. (**d**) Clinical EAE scores of wild-type nontransgenic mice ($n = 10$) that received 1×10^6 sorted $V_\alpha 19i$ T cells or an equal number of NK1.1$^-$ TCRβ$^+$ liver cells from $V_\alpha 19i$Tg*Cd1d1*$^{-/-}$ mice on the day of immunization with MOG(35–55). Tx indicates the day of adoptive transfer of cells. (**e**) Clinical EAE scores of *Mr1*$^{-/-}$ and *Mr1*$^{+/+}$ mice ($n = 8$–10) immunized with MOG(35–55). Data are representative of triplicate samples from three separate experiments.

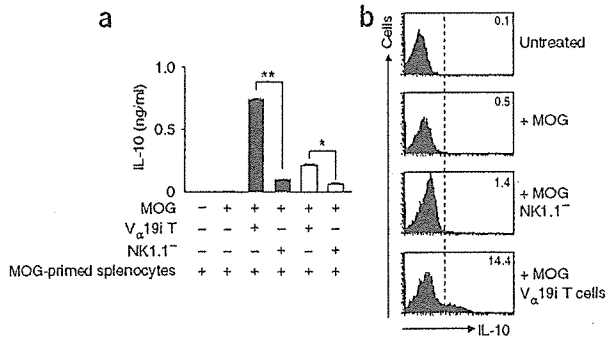

Figure 4 Interactions of V_α19i T cells and splenocytes induce IL-10. (a) Cytometric bead assay of IL-10 in the supernatants of liver V_α19i T cells from naive V_α19iTg*Cd1d1*$^{-/-}$ mice, cultured for 72 h with MOG(35–55)-specific splenocytes and MOG(35–55) (filled bars). In some cases, V_α19i T cells were separated from splenocytes by transwell inserts (open bars). Controls received NK1.1$^-$ liver cells from V_α19iTg*Cd1d1*$^{-/-}$ 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.

IFN-γ secretion was more susceptible to the inhibitory effects of V_α19i T cells than was IL-17 (**Fig. 3b**). Splenocytes acted like lymph node cells (data not shown).

Overexpression of the V_α19i 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_α19iTg*Cd1d1*$^{-/-}$ mice, despite the inhibition of T_H1 cytokine production (**Fig. 3c**). Therefore, it is unlikely that the degree of EAE suppression seen in V_α19iTg*Cd1d1*$^{-/-}$ 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_α19i T cells isolated from V_α19iTg*Cd1d1*$^{-/-}$ mice into nontransgenic mice on the day of EAE induction. Mice that received TCRβ$^+$ 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_α19i$^-$ NK1.1$^-$ T cells.

Next we sought to determine if V_α19i T cell deficiency could also influence clinical EAE. As no V_α19i-specific TCR antibody is available to deplete mice of V_α19i T cells *in vivo*, we used *Mr1*$^{-/-}$ mice, which lack V_α19i T cells[7]. As wild-type nontransgenic mice have about four times more V_α14i NKT cells than V_α19i 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_α19i T cells have a regulatory function in a T_H1-mediated autoimmune disease.

V_α19i T cells induce B cell IL-10 production

MOG(35–55)-primed V_α19iTg 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_α19i 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_α19iTg*Cd1d1*$^{-/-}$ 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_α19iTg*Cd1d1*$^{-/-}$ mice (**Fig. 4a**). NK1.1$^+$ T cells from V_α19iTg*Cd1d1*$^{-/-}$ 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_α19iTg*Cd1d1*$^{-/-}$ mice (**Fig. 4b**). However, in the presence of transwell inserts, IL-10 production was inhibited, indicating that V_α19i 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_α19i 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$^+$,

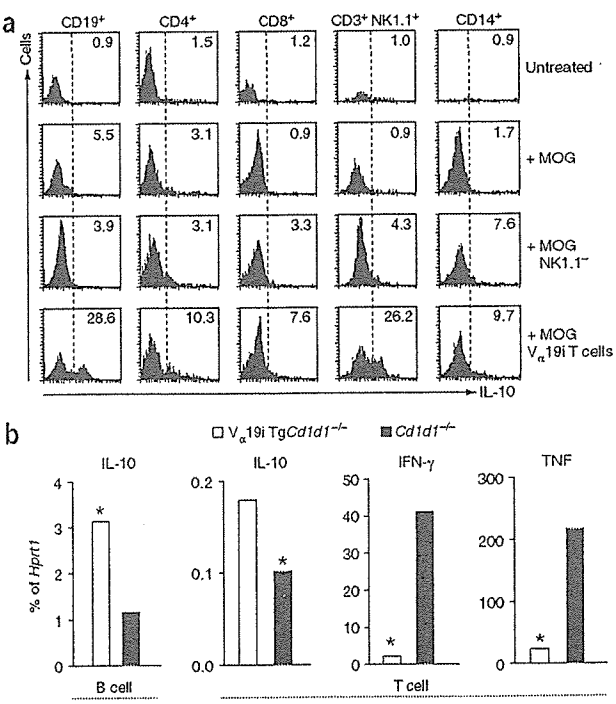

Figure 5 V_α19i T cells induce B cells to secrete IL-10. (a) Intracellular flow cytometry of IL-10 production by liver V_α19i T cells from naive V_α19iTg*Cd1d1*$^{-/-}$ mice, cultured for 72 h with MOG(35–55)-specific splenocytes and MOG(35–55). Areas to the right of dashed lines indicate positive cellular 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).

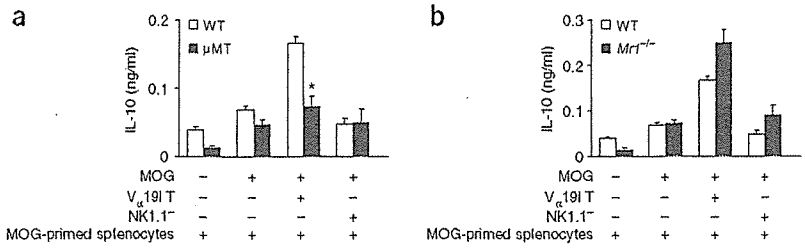

Figure 6 V_α19i T cell–induced IL-10 production is partially B cell dependent but completely MR1 independent. Cytometric bead assay of IL-10 in the supernatants of liver V_α19i T cells from naive V_α19iTg*Cd1d1*$^{-/-}$ mice, cultured for 72 h with MOG(35–55) plus MOG(35–55)-specific splenocytes from 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).

$CD3^+NK1.1^+$ 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

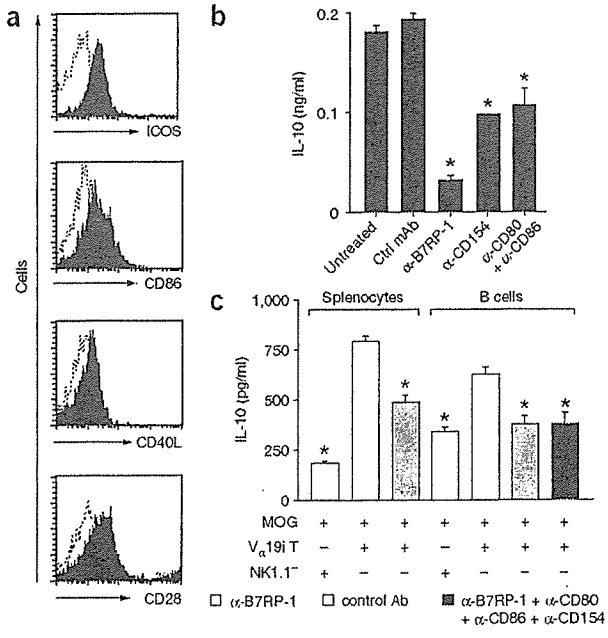

Figure 7 ICOS–B7RP-1 costimulation contributes to V_α19i T cell–induced B cell IL-10 production. (**a**) Flow cytometry of costimulatory molecule expression on the surface of liver V_α19i T cells (filled histograms) and naive splenic T cells from C57BL/6 mice (dotted lines). Data are representative of 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_α19i 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_α19i T cells and splenocytes and fully inhibited IL-10 production in cocultures of V_α19i 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_α19i T cell–induced IL-10 production. Furthermore, the ICOS–B7RP-1 pathway is vital for V_α19i 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_α19i T cell–induced IL-10 production from whole splenocytes (**Fig. 7b**).

DISCUSSION

Although T cells expressing the invariant V_α19-J_α33 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_α14i NKT cells, which influence autoimmunity, have similar properties, we speculated that MR1-resricted T cells would also be capable of modifying autoimmunity. However, V_α19i T cells are distinct from V_α14i 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_α7.2i T cells, the human homolog of V_α19i 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_α19i T cells can suppress autoimmune inflammation. In addition, we have shown that V_α19i 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_α19iTg mice produced more cytokines than did NK1.1$^+$ T cells from V_α19iTg*Cd1d1*$^{-/-}$ mice, indicating a possible interaction between CD1d- and MR1-restricted lymphocytes.

We undertook several approaches to determine whether V_α19i T cells regulate EAE pathogenesis. Overexpression of V_α19i 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_α19i 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_α19iTg mice. Furthermore, we adoptively transferred liver V_α19i T cells from naive V_α19iTg*Cd1d1*$^{-/-}$ mice into wild-type nontransgenic mice with EAE, which express natural TCR diversity. In those experiments, V_α19i T cells effectively inhibited EAE, suggesting that V_α19i T cells have a regulatory function during EAE. However, a potential limitation of our model is the difficulty of obtaining pure V_α19i T cell preparations because of the lack of a V_α19 TCR–specific antibody. Therefore, experiments using sorted CD3$^+$ NK1.1$^+$ cells from V_α19iTg*Cd1d1*$^{-/-}$ mice may also contain small numbers of non–V_α14i TCR NK1.1$^+$ T cells of other TCR specificities. To ascertain whether normal numbers of V_α19i 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_α19i T cells resulted in a more severe clinical disease than that of wild-type nontransgenic mice.

V_α19i 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_α19i T cells was independent of V_α14i NKT cells by using V_α19iTg mice on a CD1d-deficient background. Notably, we found reduced adhesion molecule expression on effector T cells from V_α19iTg*Cd1d1*$^{-/-}$ mice, which correlated with reduced T cell infiltration of the CNS. However, we did note low numbers of V_α19i T cells (CD3$^+$NK1.1$^+$ from V_α19iTg*Cd1d1*$^{-/-}$mice) and B cells in the CNS of mice with EAE, suggesting that V_α19i 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_α19iTg*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_α19i T cell–mediated immune regulation. B cells express MR1 (ref. 34), and V_α19i 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_α19i T cell selection, is not essential for V_α19i 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_α19i T cells was unknown before; we have demonstrated here that V_α19i 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_α19i 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_α19i T cells[7]. V_α19i T cells may also control gut production of immunoglobulin A from B cells, suggesting involvement of V_α19i 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_α19i T cells are involved in the homeostasis of gut immunity[2,7]. We have shown that V_α19i T cells help B cells produce

IL-10, which in nonpathogenic conditions may inhibit inflammation against gut flora required for $V_\alpha19i$ T cell selection. Therefore, we propose a model of $V_\alpha19i$ T cell–induced protection from EAE whereby $V_\alpha19i$ 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 $V_\alpha19i$ T cells in autoimmune disease. In contrast to 'conventional' $V_\alpha14i$ NKT cells, more T cells express the $V_\alpha19i$ TCR human homolog $V_\alpha7.2$-$J_\alpha33$ 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), $V_\alpha19iTg$ mice[5], $V_\alpha19iTgCd1d1^{-/-}$ 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 $V_\alpha19i$ 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 $CD3^+NK1.1^+$ $V_\alpha19i$ T cells were sorted from naive $V_\alpha19iTgCd1d1^{-/-}$ mice as described above, and 1×10^6 $V_\alpha19i$ 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 $V_\alpha19i$ 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 $V_\alpha19i$ 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 $V_\alpha19i$ T cells (5×10^5) sorted from naive $V_\alpha19iTgCd1d1^{-/-}$ 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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1. Kronenberg, M. Toward an understanding of NKT cell biology: progress and paradox. *Annu. Rev. Immunol.* **23**, 877–900 (2005).
2. Treiner, E. *et al.* Mucosal-associated invariant T (MAIT) cells: an evolutionarily conserved T cell subset. *Microbes Infect.* **7**, 552–559 (2005).
3. Kawano, T. *et al.* CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science* **278**, 1626–1629 (1997).

4. Zhou, D. *et al.* Lysosomal glycosphingolipid recognition by NKT cells. *Science* **306**, 1786–1789 (2004).
5. Okamoto, N. *et al.* Synthetic α-mannosyl ceramide as a potent stimulant for an NKT cell repertoire bearing the invariant $V_{\alpha}19$-$J_{\alpha}26$ TCR α chain. *Chem. Biol.* **12**, 677–683 (2005).
6. Chen, Y.H., Chiu, N.M., Mandal, M., Wang, N. & Wang, C.R. Impaired $NK1^+$ T cell development and early IL-4 production in CD1-deficient mice. *Immunity* **6**, 459–467 (1997).
7. Treiner, E. *et al.* Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* **422**, 164–169 (2003).
8. Spada, F.M., Koezuka, Y. & Porcelli, S.A. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J. Exp. Med.* **188**, 1529–1534 (1998).
9. Tilloy, F. *et al.* An invariant T cell receptor α chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted α/β T cell subpopulation in mammals. *J. Exp. Med.* **189**, 1907–1921 (1999).
10. Godfrey, D.I., MacDonald, H.R., Kronenberg, M., Smyth, M.J. & Van Kaer, L. NKT cells: what's in a name? *Nat. Rev. Immunol.* **4**, 231–237 (2004).
11. Lehuen, A. *et al.* Overexpression of natural killer T cells protects Vα14-Jα281 transgenic nonobese diabetic mice against diabetes. *J. Exp. Med.* **188**, 1831–1839 (1998).
12. Mars, L.T. *et al.* Vα14-Jα281 NKT cells naturally regulate experimental autoimmune encephalomyelitis in nonobese diabetic mice. *J. Immunol.* **168**, 6007–6011 (2002).
13. Wagner, M.J., Hussain, S., Mehan, M., Verdi, J.M. & Delovtch, T.L. A defect in lineage fate decision during fetal thymic invariant NKT cell development may regulate susceptibility to type 1 diabetes. *J. Immunol.* **174**, 6764–6771 (2005).
14. Pál, E. *et al.* Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of Vα14 NK T cells. *J. Immunol.* **166**, 662–668 (2001).
15. Sharif, S. *et al.* Activation of natural killer T cells by α-galactosylceramide treatment prevents the onset and recurrence of autoimmune type 1 diabetes. *Nat. Med.* **7**, 1057–1062 (2001).
16. Hong, S. *et al.* The natural killer T-cell ligand α-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat. Med.* **7**, 1052–1056 (2001).
17. Miyamoto, K., Miyake, S. & Yamamura, T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing T_H2 bias of natural killer T cells. *Nature* **413**, 531–534 (2001).
18. Chiba, A. *et al.* Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of α-galactosylceramide. *Arthritis Rheum.* **50**, 305–313 (2004).
19. Miyake, S. & Yamamura, T. Therapeutic potential of glycolipid ligands for natural killer (NK) T cells in the suppression of autoimmune diseases. *Curr. Drug Targets Immune Endocr. Metabol. Disord.* **5**, 315–322 (2005).
20. Chiba, A., Kaieda, S., Oki, S., Yamamura, T. & Miyake, S. The involvement of Vα14 natural killer T cells in the pathogenesis of arthritis in murine models. *Arthritis Rheum.* **52**, 1941–1948 (2005).
21. Kim, H.Y. *et al.* NKT cells promote antibody-induced joint inflammation by suppressing transforming growth factor β1 production. *J. Exp. Med.* **201**, 41–47 (2005).
22. Porcelli, S., Yockey, C.E., Brenner, M.B. & Balk, S.P. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood $CD4^-8^-$ α/β T cells demonstrates preferential use of several V_{β} genes and an invariant TCR α chain. *J. Exp. Med.* **178**, 1–16 (1993).
23. Shimamura, M. & Huang, Y.Y. Presence of a novel subset of NKT cells bearing an invariant Vα19.1-Jα26 TCR α chain. *FEBS Lett.* **516**, 97–100 (2002).
24. Kawachi, I., Maldonado, J., Strader, C. & Gilfillan, S. MR1-restricted Vα19i mucosal-associated invariant T cells are innate T cells in the gut lamina propria that provide a rapid and diverse cytokine response. *J. Immunol.* **176**, 1618–1627 (2006).
25. Illés, Z., Shimamura, M., Newcombe, J., Oka, N. & Yamamura, T. Accumulation of Vα7.2-Jα33 invariant T cells in human autoimmune inflammatory lesions in the nervous system. *Int. Immunol.* **16**, 223–230 (2004).
26. Illés, Z. *et al.* Differential expression of NK T cell Vα24JαQ invariant TCR chain in the lesions of multiple sclerosis and chronic inflammatory demyelinating polyneuropathy. *J. Immunol.* **164**, 4375–4381 (2000).
27. Croxford, J.L., Feldmann, M., Chernajovsky, Y. & Baker, D. Different therapeutic outcomes in experimental allergic encephalomyelitis dependent upon the mode of delivery of IL-10: a comparison of the effects of protein, adenoviral or retroviral IL-10 delivery into the central nervous system. *J. Immunol.* **166**, 4124–4130 (2001).
28. Croxford, J.L. *et al.* Cytokine gene therapy in experimental allergic encephalomyelitis by injection of plasmid DNA-cationic liposome complex into the central nervous system. *J. Immunol.* **160**, 5181–5187 (1998).
29. Cua, D.J., Groux, H., Hinton, D.R., Stohlman, S.A. & Coffman, R.L. Transgenic interleukin 10 prevents induction of experimental autoimmune encephalomyelitis. *J. Exp. Med.* **189**, 1005–1010 (1999).
30. Bettelli, E., Nicholson, L.B. & Kuchroo, V.K. IL-10, a key effector regulatory cytokine in experimental autoimmune encephalomyelitis. *J. Autoimmun.* **20**, 265–267 (2003).
31. Langrish, C.L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201**, 233–240 (2005).
32. Fillatreau, S., Sweenie, C.H., McGeachy, M.J., Gray, D. & Anderton, S.M. B cells regulate autoimmunity by provision of IL-10. *Nat. Immunol.* **3**, 944–950 (2002).
33. Mauri, C., Gray, D., Mushtaq, N. & Londei, M. Prevention of arthritis by interleukin 10-producing B cells. *J. Exp. Med.* **197**, 489–501 (2003).
34. Riegert, P., Wanner, V. & Bahram, S. Genomics, isoforms, expression, and phylogeny of the MHC class I-related MR1 gene. *J. Immunol.* **161**, 4066–4077 (1998).
35. Hayakawa, Y. *et al.* Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *J. Immunol.* **166**, 6012–6018 (2001).
36. Ikarashi, Y. *et al.* Dendritic cell maturation overrules H-2D-mediated natural killer T (NKT) cell inhibition: critical role for B7 in CD1d-dependent NKT cell interferon γ production. *J. Exp. Med.* **194**, 1179–1186 (2001).
37. Kitamura, H. *et al.* The natural killer T (NKT) cell ligand α-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* **189**, 1121–1128 (1999).
38. Kaneda, H. *et al.* ICOS costimulates invariant NKT cell activation. *Biochem. Biophys. Res. Commun.* **327**, 201–207 (2005).
39. Hutloff, A. *et al.* ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* **397**, 263–266 (1999).
40. McAdam, A.J. *et al.* ICOS is critical for CD40-mediated antibody class switching. *Nature* **409**, 102–105 (2001).
41. Song, F. *et al.* Expression of the neutrophil chemokine KC in the colon of mice with enterocolitis and by intestinal epithelial cell lines: effects of flora and proinflammatory cytokines. *J. Immunol.* **162**, 2275–2280 (1999).
42. Kaneko, M., Akiyama, Y., Takimoto, H. & Kumazawa, Y. Mechanism of up-regulation of immunoglobulin A production in the intestine of mice unresponsive to lipopolysaccharide. *Immunology* **116**, 64–70 (2005).
43. Cognasse, F. *et al.* Differential downstream effects of CD40 ligation mediated by membrane or soluble CD40L and agonistic Ab: a study on purified human B cells. *Int. J. Immunopathol. Pharmacol.* **18**, 65–74 (2005).
44. Muhlen, K.A. *et al.* NK cells, but not NKT cells, are involved in *Pseudomonas aeruginosa* exotoxin A-induced hepatotoxicity in mice. *J. Immunol.* **172**, 3034–3041 (2004).

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

Toshimasa Aranami, Sachiko Miyake, and Takashi Yamamura[2]

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

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

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

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

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

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

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

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

Materials and Methods

Subjects

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

Reagents

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

Cell preparation and NK cell purification

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

Flow cytometry

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

Stimulation of purified NK cells with proinflammatory cytokines

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

RT-PCR

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

Statistical analysis of remission rate

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

Results

CD11c on NK cells is up-regulated in MS remission

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

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

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

Absence of NK2 bias in $CD11c^{high}$ MS

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

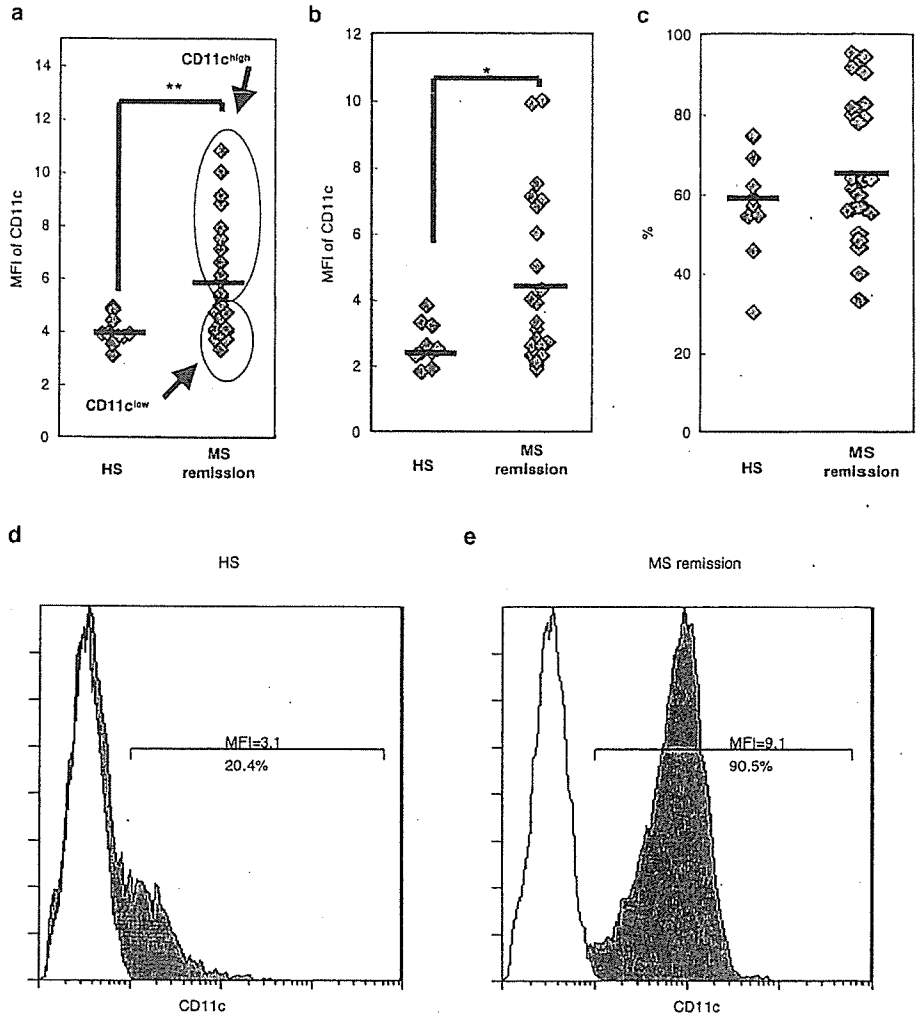

FIGURE 1. CD11c on NK cells is up-regulated in MS in remission. *a*, PBMC from HS (n = 10) and MS patients in remission (n = 25) were stained with anti-CD11c-PE, -CD3-ECD, and -CD56-PC5 mAb, and CD11c expression was measured on the $CD11c^+$ fraction gated within whole NK cells ($CD11c^+CD3^-CD56^+$ cells) as mean fluorescence intensity (MFI). Each dot represents the data from individual patients. $CD11c^{high}$ and $CD11c^{low}$ groups of patients are encircled as described in the text. *b*, In parallel, CD11c expression (MFI) was measured for the whole NK cells ($CD3^-CD56^+$ cells), which yielded a similar result. *c*, The proportions of $CD11c^+$ cells among whole NK cells are plotted. No significant difference was noted between HS and MS remission. *d* and *e*, Representative histogram patterns of CD11c on NK cells (closed histogram) from a single healthy subject (HS) (*d*) and a patient corresponding to $CD11c^{high}$ MS (*e*). Open histograms represent isotype control staining. Values represent proportions of $CD11c^+$ fraction (%) and MFI for $CD11c^+$ cells. Mann-Whitney U test was used for statistical analysis. Horizontal bars indicate the mean values. *, $p < 0.05$; **, $p < 0.01$.

remission (3). Although the mechanism for NK2 bias in MS remains to be further studied, up-regulation of GATA-3 has recently been reported in the induction of NK2 cells in mice (17). To explore the possible difference in the functions of $CD11c^{high}$ and $CD11c^{low}$ NK cells, we isolated NK cells from $CD11c^{high}$ or $CD11c^{low}$ group of patients and measured the mRNA levels of representative cytokines IFN-γ and IL-5 as well as corresponding transcription factors T-bet and GATA-3. As shown in Fig. 3, mRNA expression of both IL-5 and GATA-3 was significantly higher in $CD11c^{low}$ MS compared with HS or $CD11c^{high}$ MS, indicating that NK2 bias thought to be characteristic of MS remission is restricted to $CD11c^{low}$ MS. In contrast, there were no differences in mRNA expression of IFN-γ and T-bet among these three groups. Because NK cells from $CD11c^{high}$ patients expressed HLA-DR most brightly, we speculate that NK2 bias associated with $CD11c^{low}$ MS would attenuate when NK cells are further activated or differentiated.

NK cell stimulatory proinflammatory cytokines induce up-regulation of CD11c

We next attempted to explore the mechanism(s) for up-regulation of CD11c on NK cells in $CD11c^{high}$ MS. Because both NK cells and $CD4^+$ T cells overexpressed HLA-DR in $CD11c^{high}$, is it probable that immune signals influencing both innate and acquired immunity are operative. So we hypothesized that cytokine signals that have been implicated in the pathogenesis of MS may play a role. We cultured NK cells from HS in the presence or absence of cytokine(s) for 3 days, and evaluated the CD11c expression (MFI). We focused our attention to IL-12, IL-15, and IL-18, which are known to stimulate NK cells with or without help of other cytokines. Notably, they are reportedly elevated in the serum or blood lymphocytes of MS patients as compared with HS (11–14, 18, 19), and prior studies suggest that they may play an important role in autoimmune diseases (20–24). As shown in Fig. 4, although IL-12 and IL-18 showed only a marginal effect on purified NK cells, IL-15 consistently induced 2- to 3-fold up-regulation of CD11c compared with control culture without addition of cytokines. As IL-12 and IL-18 were reported to synergistically work in various settings (25, 26), we then examined whether combinations of these cytokines may induce CD11c. Combination of IL-15 and IL-12 or of IL-15 and IL-18 did not augment the CD11c expression to the level higher than that could be induced by IL-15 alone. However, the combination of IL-12 and IL-18 did up-regulate CD11c on NK cells, which was comparable to the effect of IL-15 alone (Table I). Additionally, we tested the effects of several cytokines involved in differentiation of DC (TNF-α, GM-CSF, IL-4) (27), or known to up-regulate CD11c in granulocytes (IL-8) as controls (28) in the same assay. These cytokines showed no significant effect (Table I).

$CD11c^{high}$ MS relapsed earlier

Given the significant difference in activation status and cytokine phenotype of NK cells as well as HLA-DR expression by $CD4^+$ T cells, it was particularly interesting to know whether $CD11c^{low}$ and $CD11c^{high}$ MS may follow a different clinical course. A new cohort of

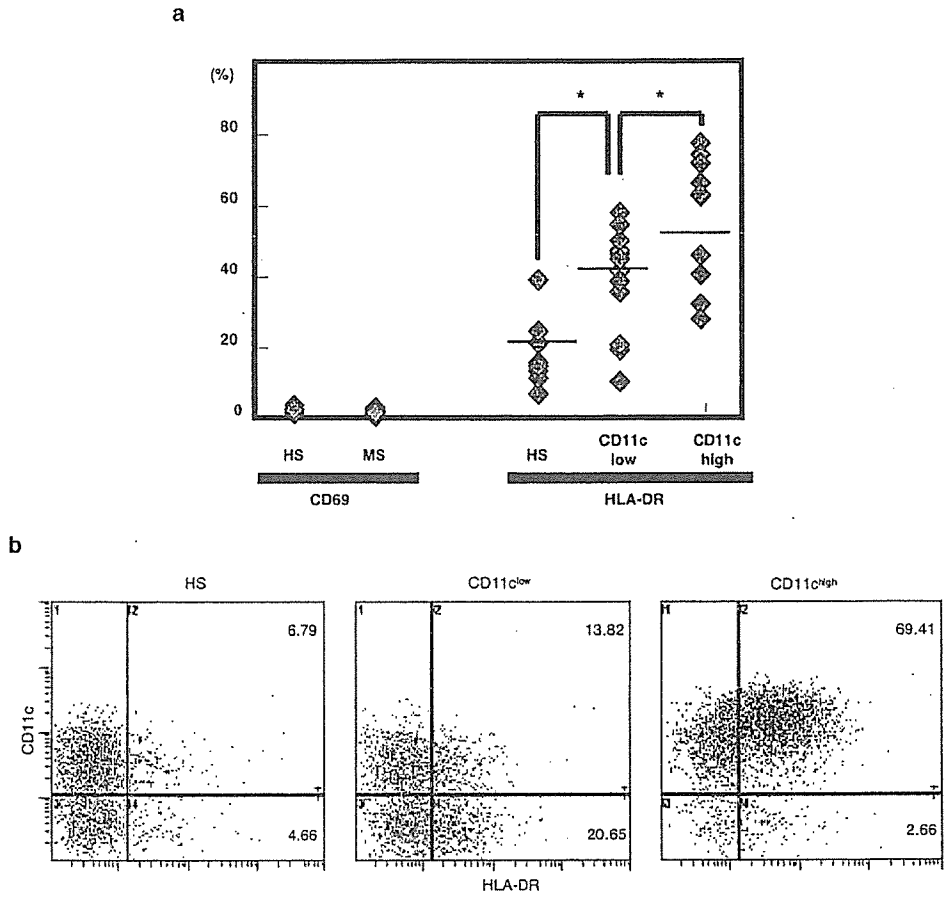

FIGURE 2. Proportions of HLA-DR$^+$ NK cells increase in CD11c^{high} MS. *a*, CD69 and HLA-DR expression on NK cells (CD3$^-$ CD56$^+$ cells). Data are expressed as proportions (percent) of CD69$^+$ cells (7 HS and 16 MS patients in remission) or HLA-DR$^+$ cells (10 HS and 25 MS patients) within whole NK cells. The Student *t* test was used for statistical analysis. Horizontal bars indicate the mean values. *, $p < 0.05$. *b*, Representative expression patterns of HLA-DR vs CD11c on NK cells from a healthy subject (*left*), CD11c^{low} MS (*middle*), and CD11c^{high} MS (*right*).

13 CD11c^{low} and 10 CD11c^{high} MS patients listed in Table II were followed for up to 120 days. In this preliminary exploration, we set the first episode of relapse after blood sampling as an end point. When the neurologist prescribed corticosteroids without knowing any information on the NK cell phenotype, the patient was considered as the dropout at that time point. Remission rate was calculated as Kaplan-Meier survival rate, and statistical difference between CD11c^{low} and CD11c^{high} MS was evaluated with the log-rank test (Fig. 5*a*). At entry, there was no significant difference in the age and disease duration between CD11c^{low} and CD11c^{high} MS (Table II). On analyzing the collected data after completing the study, we found that 8 patients developed a single relapse during the observation period and that the proportion of patients who have had relapse during the follow-up period was greatly higher in CD11c^{high} MS (6 of 10, 60%) than in CD11c^{low} MS (2 of 13, 15.3%). Furthermore, the log-rank test revealed that CD11c^{high} MS relapsed significantly earlier than CD11c^{low} MS ($p = 0.003$), suggesting a possible role of CD11c as a temporal marker for predicting relapse within months after examination. We also explored whether the difference between CD11c^{high} and CD11c^{low} could be influenced by age or sex. When we selected a group of patients younger than 38.5 years old (the mean age of all the patients), a significantly earlier relapse in CD11c^{high} than CD11c^{low} MS was confirmed in this group of patients ($p = 0.0067$, Fig. 5*b*). In the rest of the patients (<38.5 years old), the difference was less clear and not significant ($p = 0.095$). In female patients, CD11c^{high} MS relapsed significantly earlier than CD11c^{low} MS ($p = 0.035$, Fig. 5*c*), whereas this tendency was not statistically significant in male patients ($p = 0.083$). By examining the patients' medical records, we also found that the duration from the last relapse tended to be shorter in CD11c^{high} than CD11c^{low} MS (14.7 ± 12 mo in CD11c^{high} vs 26.7 ± 24.3 mo in CD11c^{low}) and that the mean number of relapses per year was higher in CD11c^{high} MS (0.9 ± 0.6 in CD11c^{high} vs 0.5 ± 0.5 in CD11c^{low}). These are consistent with the postulate that CD11c^{high} MS might be immunologically more active than CD11c^{low} MS (Table II).

Alteration of CD11c expression in the course of MS

We previously described that NK cells may lose NK2 phenotype during relapse (3). It is interesting to know whether the CD11c phenotype also changes in the course of MS. During the follow-up period of 120 days, 8 patients developed a relapse. We were able to take blood samples at relapse before treatment with corticosteroid and then compared the relapse samples with the samples obtained during remission at initiation of the study. As shown in Fig. 6, we saw an obvious tendency that the levels of CD11c expression would decline during relapse ($p < 0.05$). HLA-DR expression on NK cells was also reduced in some patients during relapse, but the difference between remission and relapse samples was not statistically significant.

Expression pattern of CD95 vs CD11c on NK cells in MS

In a previous study, we showed that MS patients could be divided into CD95^{high} and CD95^{low} according to the frequency of CD95$^+$ cells among NK cells (4). Additionally, we examined whether expression of CD11c and CD95 may independently reflect the status of MS. We found no significant correlation between CD95 (%) and CD11c (MFI) on NK cells in MS ($r = 0.29$, $p = 0.16$ with Spearman's correlation coefficient by rank test), indicating that expression of CD95 and CD11c on NK cells may be regulated independently. By setting the upper limits of CD95$^+$ (%) and CD11c MFI as (the average + 2 × SD) of HS (CD95: 44.6%, CD11c: 5.04),

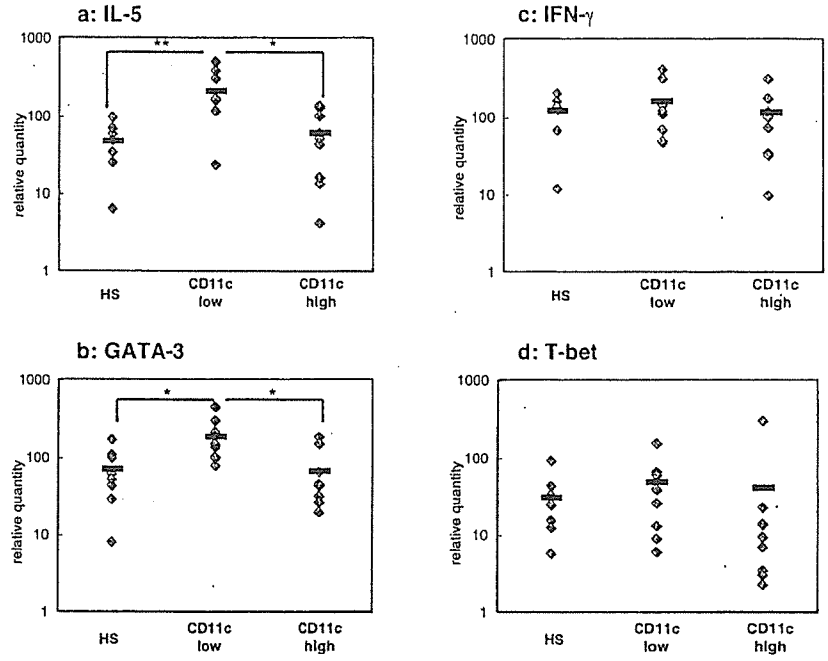

FIGURE 3. IL-5 and GATA-3 mRNA are increased in CD11c^{low} but not in CD11c^{high} MS. Total RNAs were extracted from purified NK cells of HS (n = 8), CD11c^{low} (n = 9), or CD11c^{high} MS (n = 8). mRNA expression of IL-5 (*a*), GATA-3 (*b*), IFN-γ (*c*), and T-bet (*d*) was evaluated by quantitative PCR. The data are normalized to endogenous β-actin expressions in the same samples. ANOVA was used for statistical analysis. Horizontal bars indicate the mean values. *, $p < 0.05$; **, $p < 0.01$.

we then examined whether there is a correlation between CD11c CD95 phenotype and clinical conditions (Fig. 7). Naturally, all the healthy subjects were plotted in the *left lower quadrant* (CD95^{low}CD11c^{low}). In contrast, MS patients were plotted in all the four quadrants with differential proportions of patients who have no relapse during 120 days: CD95^{low}CD11c^{low}; 3/3 (100%), CD95^{low}CD11c^{high}; 1/2 (50%), CD95^{high}CD11c^{low}; 8/10 (80%), CD95^{high}CD11c^{high}; 2/7 (28.6%). Although the data for CD95^{low} subjects (*lower left* and *lower right*) need to be omitted due to the limited sample size, we found that the difference between CD95^{high}CD11c^{low} and CD95^{high}CD11c^{high} in remission rate was significant with log-rank test (p = 0.028). Provided that CD95^{high} patients possessed an increased frequency of memory autoreactive T cells (4), this result is consistent with the idea that when comparable numbers of autoimmune T cells are present in the peripheral circulation, remission of MS is more stable in patients with CD11c^{low} NK cells.

Discussion

Blood examination of systemic autoimmune diseases such as systemic lupus erythematosus usually exhibits measurable abnormalities such as elevation of autoantibodies, which is useful for evaluating activity of disease. In contrast, patients with MS do not accompany such systemic abnormalities in laboratory tests except

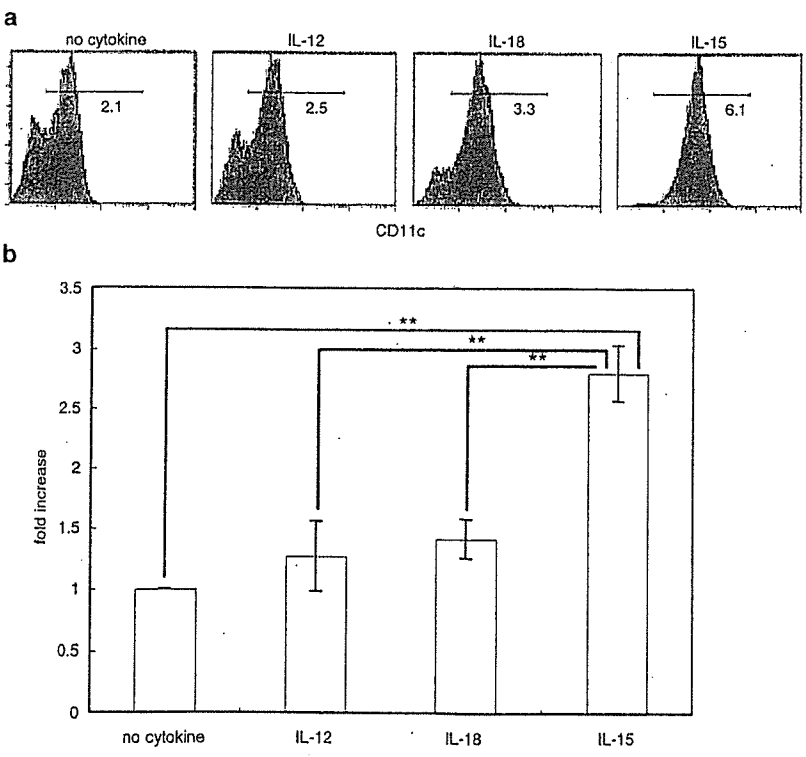

FIGURE 4. CD11c expression on NK cells is upregulated with addition of IL-15. *a*, Purified NK cells were cultured in the absence or presence of IL-12, IL-18, or IL-15. Three days later, the cells were stained with anti-CD11c-PE, -CD3-ECD, and -CD56-PC5 mAb. CD11c expression on NK cells (CD3$^-$CD56$^+$ cells) is demonstrated as single histogram. Values indicate CD11c MFI of CD11c$^+$ fractions. A representative of three independent experiments is shown. *b*, Data are expressed as mean fold increase of CD11c MFI (the MFI in the presence of cytokine/the MFI in the absence of cytokine) + SD from three independent experiments. ANOVA was used for statistical analysis. **, $p < 0.01$.

Table I. *Effect of several cytokines on CD11c expression on NK cells*

	No Cytokine	IL-12	IL-18	IL-15	IL-12 + IL-18	IL-4	TNF	GM-CSF	IL-23	IL-8
Expt. 1	1.00[a]	1.19	1.57	**2.90**	ND	ND	ND	ND	ND	ND
Expt. 2	1.00	1.04	1.43	**2.96**	**2.86**	ND	ND	ND	ND	ND
Expt. 3	1.00	1.59	1.25	**2.53**	**3.44**	ND	ND	ND	ND	ND
Expt. 4	1.00	ND	ND	**2.62**	ND	1.19	1.10	0.95	1.14	ND
Expt. 5	1.00	ND	ND	**2.81**	ND	1.24	ND	1.05	1.05	1.00
Mean	1.00	1.27	1.42	**2.77**	**3.15**	1.21	1.10	1.00	1.10	1.00
SD	0.00	0.29	0.16	0.19	0.41	0.03		0.07	0.07	

[a] Purified NK cells were stimulated with cytokines. Data are expressed as fold increase of CD11c MFI (the MFI in the presence of the indicated cytokines/the MFI in the absence of cytokines) in the presence of indicated cytokines. More than a 2-fold increase is highlighted (bold).

in unusual cases. It is currently recognized that autoreactive T cells might be activated and expanded to various degrees in the peripheral blood and peripheral lymphoid organs of MS even during remission (1–4). In fact, our previous work suggests that a higher number of memory autoreactive T cells is linked with unstable disease course (4). If we are able to accurately evaluate the immune status of each patient with a relatively simple test, it should be most helpful in treatment and management of MS. In this line, it is currently of particular importance to identify measurable indicators which would serve as clinically appropriate biomarkers in MS (2).

This study has clarified for the first time to our knowledge that CD11c expression on peripheral NK cells is significantly up-regulated in a major proportion of patients with MS in remission. To obtain insights into the mechanism and the biological meaning of the NK cell expression of CD11c in autoimmune disease MS, we have attempted to clarify the difference between $CD11c^{high}$ and $CD11c^{low}$ patients regarding phenotypes of NK cells, cytokine profile, and temporal clinical activity. We also explored which inflammatory cytokines might induce CD11c on NK cells. According to the NK cell expression of CD11c, we have classified the patients with MS in remission into $CD11c^{high}$ and $CD11c^{low}$. Most notably, NK2 phenotype characterized by predominant IL-5 production was seen in $CD11c^{low}$ patients, but not in $CD11c^{high}$. Consistently, the $CD11c^{high}$ patients were found to be clinically more active than $CD11c^{low}$ as judged by the remission rate during the 120 days after examination. These results indicate that up-regulation of CD11c on NK cells would reflect the temporal disease activity and therefore could be used to identify patients who are likely to exacerbate within months. It has been reported that $CD11c^{+}$ NK cells in mice could serve as APCs (6, 7). However, we could not reveal Ag presenting capacity of human $CD11c^{+}$ NK cells (data not shown).

Regarding the mechanism of CD11c induction on NK cells, we have found that in $CD11c^{high}$ patients, HLA-DR is concomitantly up-regulated with CD11c on NK cells (Fig. 2), which suggests that up-regulation of CD11c may represent an activation-induced change. After exploring the culture condition that may induce CD11c on NK cells, we have found that the addition of IL-15 or combination of IL-12 and IL-18 would increase the expression levels of CD11c on NK cells from healthy individuals. Because increased levels of these proinflammatory cytokines are detected in the blood samples of MS (11–13, 18, 19, 23), it is possible that in

Table II. *Information on the patients whose clinical courses were followed for up to 120 days*

Identification No.	Group	Age (years)	Sex	Disease Period (Years)	Total Number of Relapses	Duration from the Last Relapse (mo)	Mean Numbers of Relapse/Year
1	Low	17	F[a]	9.6	2	24	0.2
2	Low	52	M	12.2	9	3	0.7
3	Low	31	F	6.2	13	7	2.1
4	Low	32	F	3.9	1	34	0.3
5	Low	42	F	2.2	1	8	0.5
6	Low	35	M	20	3	88	0.2
7	Low	37	M	8.5	3	50	0.4
8	Low	35	F	2.4	1	38	0.4
9	Low	26	F	4.8	2	10	0.4
10	Low	26	F	1.5	1	8	0.7
11	Low	41	M	5.5	1	24	0.2
12	Low	64	F	4.5	2	8	0.4
13	Low	42	F	6.3	1	45	0.2
Mean + SD		36.9 + 12.0		6.7 + 5.0	3.1 + 3.7	26.7 + 24.3	0.5 + 0.5
14	High	39	M	4.4	2	22	0.5
15	High	31	F	9.2	11	14	1.2
16	High	46	F	7.4	>20[b]	2	ND
17	High	53	F	2.1	4	5	1.9
18	High	59	F	4.9	2	19	0.4
19	High	27	M	9.3	4	9	0.4
20	High	36	F	2.7	1	19	0.4
21	High	34	F	3.8	2	43	0.5
22	High	60	F	3.4	6	10	1.8
23	High	21	F	1.8	2	4	1.1
		40.6 + 13.4		4.9 + 2.8	3.8 + 3.1	14.7 + 12.0	0.9 + 0.6

[a] F, Female; M, male.
[b] This value is eliminated from calculation of the mean.

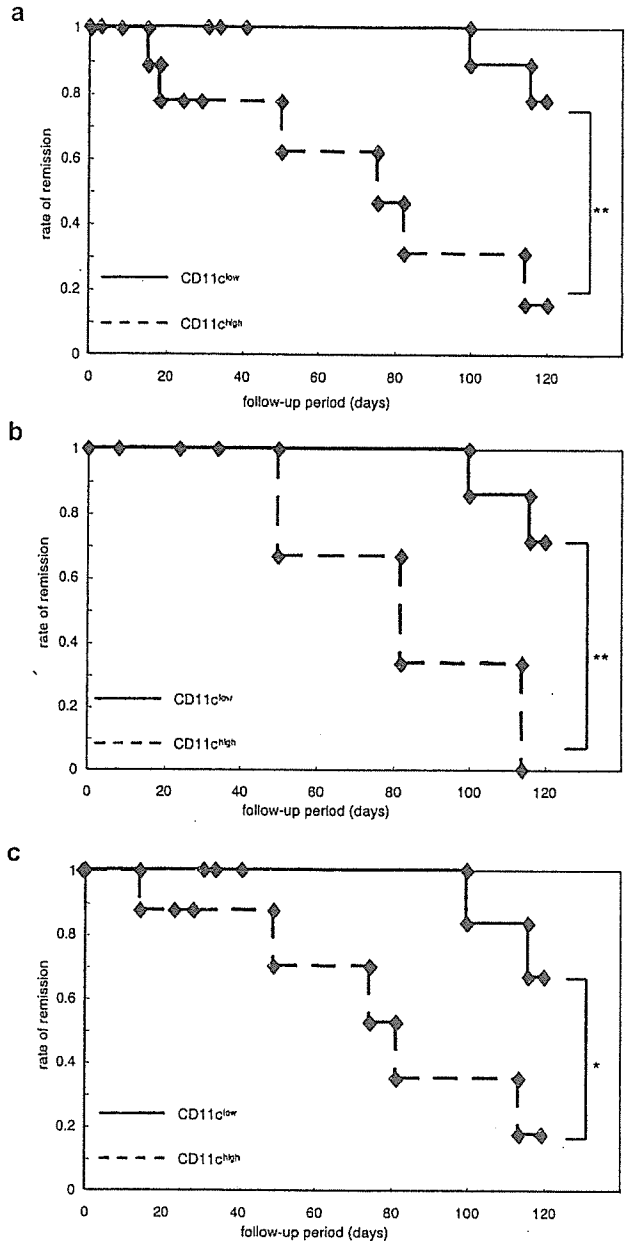

FIGURE 5. Rate of remission is lower in $CD11c^{high}$ MS. The first episode of relapse after blood sampling was set as an end point and clinical course of each patient was followed for up to 120 days. The remission rate was calculated in all (*a*), the younger (*b*), or female (*c*) patients as Kaplan-Meier survival rate, and statistical difference between $CD11c^{low}$ and $CD11c^{high}$ MS was evaluated with log-rank test at day 120. *, $p < 0.05$; **, $p < 0.01$.

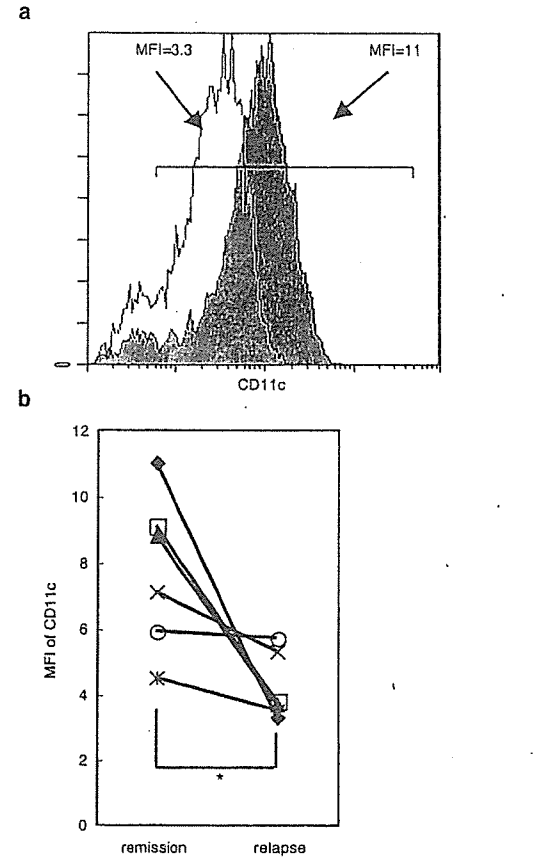

FIGURE 6. Down-regulation of CD11c expression during relapse. *a*, Representative CD11c histograms from the same patient in remission (closed) and relapse (open). Values indicate CD11c MFI of $CD11c^+$ fractions. *b*, Comparison of NK cells from remission and relapse from the same patients ($n = 6$). The data obtained from the same patients are connected with lines. Wilcoxon signed-ranks test was used for statistical analysis. *, $p < 0.05$.

vitro CD11c induction on NK cells may recapitulate the phenotypic alteration of NK cells in $CD11c^{high}$ patients. Interestingly, IL-18 is not only a cytokine able to facilitate IFN-γ production by NK cells in cooperation with IL-12 (25, 26) but is crucial in inducing pathogenic autoimmune responses (21). Furthermore, autoimmune encephalitogenic T cells can induce more serious disease upon adoptive transfer when they are preactivated in the presence of IL-12 and IL-18 (20). Taken together, these results allow us to speculate that the proinflammatory cytokines may be involved in the up-regulation of CD11c on NK cells. Although the relationship between serum cytokine concentration and levels of CD11c expression on NK cells should be estimated in future studies, a previous work (11, 29, 30) showing that a probable link between IL-15 and temporal disease activity, indicates that NK cell expression of CD11c is likely to correlate with the levels of cytokines.

In the Th cell differentiation, specific transcription factors have been identified that play a crucial role in inducing Th1 or Th2 cells. Namely, Th1 differentiation characterized by IFN-γ induction requires a transcription factor T-bet, whereas GATA-3 and c-*maf* act to promote Th2 cytokine production (31–33). Human NK cells cultured in the presence of IL-12 or IL-4 differentiate into NK1 or NK2 populations, reminiscent of Th1 and Th2 cells (5). Whereas NK1 cells produce IL-10 and IFN-γ, NK2 cells would serve as immune regulators by producing IL-5 and IL-13. Notably, up-regulation of GATA-3 has been reported in mouse NK2 cells (17), raising a possibility that Th cells and NK cells might share the same transcription factor for inducing the key cytokine. We have previously reported that IL-5 expression is one of the characteristics of NK cells in the remission state of MS (3). However, it was not excluded that overexpression of IL-5 could be restricted to a proportion of the patients. Here, we have addressed whether NK cells from $CD11c^{high}$ and $CD11c^{low}$ may differ with regard to expression levels of IFN-γ and IL-5 and of their transcription factors T-bet and GATA-3. By measuring the mRNAs, we found that expression levels of IL-5 and GATA-3 are elevated in $CD11c^{low}$ MS but not in $CD11c^{high}$ (Fig. 3). Furthermore, we showed that

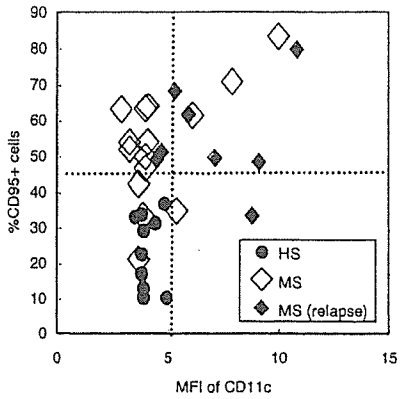

FIGURE 7. Expression pattern of CD95 vs CD11c on NK cells from MS. PBMC from MS or HS were stained with CD95-FITC, CD11c-PE, CD3-ECD, and CD56-PC5. After determining the proportion of $CD95^+$ cells among NK cells and CD11c expression (MFI) of CD11c, we plotted each patient according to the obtained values. Dotted lines represent the upper limits of $CD95^+$ cell (percent) and CD11c MFI for HS as (the average + two times SD) of HS. ●, HS; ◇, MS; ◆, MS patients who relapsed during the 120 days follow-up period.

neither IFN-γ nor T-bet was increased in $CD11c^{high}$ MS. This suggests that NK cells from $CD11c^{low}$ are NK2-biased but those from $CD11c^{high}$ are not, although MS in remission as a whole is NK2-biased as compared with control subjects. More recently, we have observed that stimulation with IL-15 or IL-12 plus IL-18 would decrease IL-5 and GATA-3 mRNA in purified NK cells with reciprocal up-regulation of CD11c (data not shown). This further supports a model that proinflammatory cytokines may play a crucial role in the absence of NK2 bias in $CD11c^{high}$ MS.

To clarify the clinical differences between $CD11c^{high}$ and $CD11c^{low}$, we followed up the clinical course of the patients after blood sampling. Although there was no significant difference in clinical parameters at examination of NK cells, we have found that $CD11c^{high}$ MS showed a significantly earlier relapse than $CD11c^{low}$ MS. This is consistent with our assumption that the absence of NK2 bias in $CD11c^{high}$ MS should imply that regulatory NK cell functions are defective in this group of patients. When we reanalyzed the data regarding various clinical parameters, we found that an earlier relapse in $CD11c^{high}$ than $CD11c^{low}$ MS is more remarkable in the younger group (<38.5 years old) or in female patients. Furthermore, the duration from the last relapse tended to be shorter and the mean number of relapses per year higher in $CD11c^{high}$ MS, supporting that $CD11c^{high}$ MS is more active than $CD11c^{low}$ MS.

When we analyzed expression of CD95 and CD11c on NK cells simultaneously, we found that MS patients in remission could be divided into four subgroups (Fig. 7). When we compared clinical course after examination of NK cell phenotypes, we found that $CD95^{high}CD11c^{high}$ MS relapsed significantly earlier than $CD95^{high}CD11c^{low}$ MS ($p = 0.028$ with log-rank test). This result indicates that $CD95^{high}CD11c^{high}$ MS may be most unstable subgroup of MS, among the patients whose clinical state could be judged as being in clinical remission.

In this study, we have demonstrated that MS patients differentially express CD11c on peripheral blood NK cells and a higher expression of CD11c on NK cells may reflect the temporal disease activity as well as functional alteration of regulatory NK cells. Our results have a clinical implication because of a lack of appropriate biomarker to monitor the immunological status in MS at present. To verify the reliability of this marker, longitudinal examination of CD11c expression on NK cells in the same patients should be performed in the future study.

Disclosures

The authors have no financial conflict of interest.

References

1. Sospedra, M., and R. Martin. 2005. Immunology of multiple sclerosis. *Annu. Rev. Immunol.* 23: 683–747.
2. Bielekova, B., and R. Martin. 2004. Development of biomarkers in multiple sclerosis. *Brain* 127: 1463–1478.
3. Takahashi, K., S. Miyake, T. Kondo, K. Terao, M. Hatakenaka, S. Hashimoto, and T. Yamamura. 2001. Natural killer type 2 bias in remission of multiple sclerosis. *J. Clin. Invest.* 107: R23–R29.
4. Takahashi, K., T. Aranami, M. Endoh, S. Miyake, and T. Yamamura. 2004. The regulatory role of natural killer cells in multiple sclerosis. *Brain* 127: 1917–1927.
5. Peritt, D., S. Robertson, G. Gri, L. Showe, M. Aste-Amezaga, and G. Trinchieri. 1998. Differentiation of human NK cells into NK1 and NK2 subsets. *J. Immunol.* 161: 5821–5824.
6. Homann, D., A. Jahreis, T. Wolfe, A. Hughes, B. Coon, M. J. van Stipdonk, K. R. Prilliman, S. P. Schoenberger, and M. G. von Herrath. 2002. CD40L blockade prevents autoimmune diabetes by induction of bitypic NK/DC regulatory cells. *Immunity* 16: 403–415.
7. Pillarisetty, V. G., S. C. Katz, J. I. Bleier, A. B. Shah, and R. P. Dematteo. 2005. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-γ via autocrine IL-12. *J. Immunol.* 174: 2612–2618.
8. Bilsland, C. A., M. S. Diamond, and T. A. Springer. 1994. The leukocyte integrin p150,95 (CD11c/CD18) as a receptor for iC3b: activation by a heterologous β subunit and localization of a ligand recognition site to the I domain. *J. Immunol.* 152: 4582–4589.
9. Stacker, S. A., and T. A. Springer. 1991. Leukocyte integrin P150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. *J. Immunol.* 146: 648–655.
10. Morelli, A. E., A. T. Larregina, W. J. Shufesky, A. F. Zahorchak, A. J. Logar, G. D. Papworth, Z. Wang, S. C. Watkins, L. D. Falo, Jr., and A. W. Thomson. 2003. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood* 101: 611–620.
11. Blanco-Jerez, C., J. F. Plaza, J. Masjuan, L. M. Orensanz, and J. C. Alvarez-Cermeno. 2002. Increased levels of IL-15 mRNA in relapsing–remitting multiple sclerosis attacks. *J. Neuroimmunol.* 128: 90–94.
12. Huang, W. X., P. Huang, and J. Hillert. 2004. Increased expression of caspase-1 and interleukin-18 in peripheral blood mononuclear cells in patients with multiple sclerosis. *Mult. Scler.* 10: 482–487.
13. Balashov, K. E., D. R. Smith, S. J. Khoury, D. A. Hafler, and H. L. Weiner. 1997. Increased interleukin 12 production in progressive multiple sclerosis: induction by activated $CD4^+$ T cells via CD40 ligand. *Proc. Natl. Acad. Sci. USA* 94: 599–603.
14. Karni, A., D. N. Koldzic, P. Bharanidharan, S. J. Khoury, and H. L. Weiner. 2002. IL-18 is linked to raised IFN-γ in multiple sclerosis and is induced by activated $CD4^+$ T cells via CD40-CD40 ligand interactions. *J. Neuroimmunol.* 125: 134–140.
15. McDonald, W. I., A. Compston, G. Edan, D. Goodkin, H. P. Hartung, F. D. Lublin, H. F. McFarland, D. W. Paty, C. H. Polman, S. C. Reingold, et al. 2001. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* 50: 121–127.
16. Lima, M., J. Almeida, M. dos Anjos Teixeira, M. L. Queiros, B. Justica, and A. Orfao. 2002. The "ex vivo" patterns of CD2/CD7, CD57/CD11c, CD38/CD11b, CD45RA/CD45RO, and CD11a/HLA-DR expression identify acute/early and chronic/late NK-cell activation states. *Blood Cells Mol. Dis.* 28: 181–190.
17. Katsumoto, T., M. Kimura, M. Yamashita, H. Hosokawa, K. Hashimoto, A. Hasegawa, M. Omori, T. Miyamoto, M. Taniguchi, and T. Nakayama. 2004. STAT6-dependent differentiation and production of IL-5 and IL-13 in murine NK2 cells. *J. Immunol.* 173: 4967–4975.
18. Nicoletti, F., R. Di Marco, K. Mangano, F. Patti, E. Reggio, A. Nicoletti, K. Bendtzen, and A. Reggio. 2001. Increased serum levels of interleukin-18 in patients with multiple sclerosis. *Neurology* 57: 342–344.
19. Fassbender, K., A. Ragoschke, S. Rossol, A. Schwartz, O. Mielke, A. Paulig, and M. Hennerici. 1998. Increased release of interleukin-12p40 in MS: association with intracerebral inflammation. *Neurology* 51: 753–758.
20. Ito, A., A. Matejuk, C. Hopke, H. Drought, J. Dwyer, A. Zamora, S. Subramanian, A. A. Vandenbark, and H. Offner. 2003. Transfer of severe experimental autoimmune encephalomyelitis by IL-12- and IL-18-potentiated T cells is estrogen sensitive. *J. Immunol.* 170: 4802–4809.
21. Shi, F. D., K. Takeda, S. Akira, N. Sarvetnick, and H. G. Ljunggren. 2000. IL-18 directs autoreactive T cells and promotes autodestruction in the central nervous system via induction of IFN-γ by NK cells. *J. Immunol.* 165: 3099–3104.
22. Takeda, K., H. Tsutsui, T. Yoshimoto, O. Adachi, N. Yoshida, T. Kishimoto, H. Okamura, K. Nakanishi, and S. Akira. 1998. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* 8: 383–390.

23. Makhlouf, K., H. L. Weiner, and S. J. Khoury. 2001. Increased percentage of IL-12$^+$ monocytes in the blood correlates with the presence of active MRI lesions in MS. *J. Neuroimmunol.* 119: 145–149.
24. Waldmann, T. A. 2004. Targeting the interleukin-15/interleukin-15 receptor system in inflammatory autoimmune diseases. *Arthritis Res. Ther.* 6: 174–177.
25. Fehniger, T. A., M. H. Shah, M. J. Turner, J. B. VanDeusen, S. P. Whitman, M. A. Cooper, K. Suzuki, M. Wechser, F. Goodsaid, and M. A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* 162: 4511–4520.
26. Okamura, H., S. Kashiwamura, H. Tsutsui, T. Yoshimoto, and K. Nakanishi. 1998. Regulation of interferon-γ production by IL-12 and IL-18. *Curr. Opin. Immunol.* 10: 259–264.
27. Young, J. W., P. Szabolcs, and M. A. Moore. 1995. Identification of dendritic cell colony-forming units among normal human CD34$^+$ bone marrow progenitors that are expanded by c-*kit*-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α. *J. Exp. Med.* 182: 1111–1119.
28. Detmers, P. A., S. K. Lo, E. Olsen-Egbert, A. Walz, M. Baggiolini, and Z. A. Cohn. 1990. Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. *J. Exp. Med.* 171: 1155–1162.
29. Kivisakk, P., D. Matusevicius, B. He, M. Soderstrom, S. Fredrikson, and H. Link. 1998. IL-15 mRNA expression is up-regulated in blood and cerebrospinal fluid mononuclear cells in multiple sclerosis (MS). *Clin. Exp. Immunol.* 111: 193–197.
30. Pashenkov, M., M. Mustafa, P. Kivisakk, and H. Link. 1999. Levels of interleukin-15-expressing blood mononuclear cells are elevated in multiple sclerosis. *Scand. J. Immunol.* 50: 302–308.
31. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell.* 100: 655–669.
32. Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science* 295: 338–342.
33. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89: 587–596.

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T cell gene expression profiling identifies distinct subgroups of Japanese multiple sclerosis patients

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Abstract

To clarify the molecular background underlying the heterogeneity of multiple sclerosis (MS), we characterized the gene expression profile of peripheral blood $CD3^+$ T cells isolated from MS and healthy control (CN) subjects by using a cDNA microarray. Among 1258 cDNAs on the array, 286 genes were expressed differentially between 72 untreated Japanese MS patients and 22 age- and sex-matched CN subjects. When this set was used as a discriminator for hierarchical clustering analysis, it identified four distinct subgroups of MS patients and five gene clusters differentially expressed among the subgroups. One of these gene clusters was overexpressed in MS versus CN, and particularly enhanced in the clinically most active subgroup of MS. After 46 of the MS patients were treated with interferon-beta (IFNβ-1b) for two years, IFNβ responders were clustered in two of the four MS subgroups. Furthermore, the IFNβ responders differed from nonresponders in the kinetics of IFN-responsive genes at 3 and 6 months after starting IFNβ treatment. These results suggest that T-cell gene expression profiling is valuable to identify distinct subgroups of MS associated with differential disease activity and therapeutic response to IFNβ.

Keywords: Gene expression profile; Hierarchical clustering analysis; IFNβ responder; Microarray; Multiple sclerosis; T cells

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) white matter mediated by an autoimmune process whose development is triggered by a complex interplay of both genetic and environmental factors (Compston and Coles, 2002). Intravenous administration of interferon-gamma (IFNγ) to MS patients in a previous clinical trial provoked acute relapses accompanied by activation of the systemic immune response, indicating a central role of proinflammatory T helper type 1 (Th1) lymphocytes in the immunopathogenesis of MS (Panitch et al., 1987). In contrast, treatment with interferon-beta (IFNβ) produced a

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beneficial effect on MS patients with a reduction of the relapse rate by approximately 30% (The IFNB Multiple Sclerosis Study Group, 1993; Jacobs et al., 1996; Saida et al., 2005). Recent studies indicated that an early initiation of IFNβ delays the conversion to clinically definite MS in the patients who experienced a first demyelinating event (Jacobs et al., 2000).

MS exhibits a great range of phenotypic variability. It is classified into relapsing–remitting MS (RRMS), secondary progressive MS (SPMS), or primary progressive MS (PPMS) with respect to the disease course, conventional MS (CMS) or opticospinal MS (OSMS) in terms of the lesion distribution (Saida et al., 2005), and IFNβ responder or nonresponder based on the therapeutic response to IFNβ (Waubant et al., 2003). MS brain lesions show a remarkable heterogeneity in the degree of inflammation, complement activation, antibody deposition, demyelination and remyelination, oligodendrocyte apoptosis, and axonal degeneration (Lucchinetti et al., 2000). These observations suggest that MS is a kind of neurological syndrome caused by different immunopathological mechanisms leading to the final common pathway that provokes inflammatory demyelination. Therefore, it is not surprising to find that individual MS patients show highly variable responses to IFNβ treatment. Currently, very little is known about the molecular background underlying clinical and pathological heterogeneity of MS.

DNA microarray technology is a novel approach that allows us to systematically monitor the expression of a large number of genes in disease-affected tissues (Staudt, 2001). This approach has discovered therapeutically relevant targets and prognostic markers for cancers (Alizadeh et al., 2000; van de Vijver et al., 2000), and has given new insights into the complexity of molecular interactions promoting the autoimmune process in MS (Steinman and Zamvil, 2003). Importantly, the comprehensive gene expression profiling of MS brain tissues and peripheral blood lymphocytes identified a battery of genes deregulated in MS, whose role has not been previously predicted in its pathogenesis (Lock et al., 2002; Graumann et al., 2003; Tajouri et al., 2003; Stürzbecher et al., 2003; Achiron et al., 2004). However, most of previous studies have focused on gene expression in heterogeneous populations of unfractionated lymphocytes and brain cells. Recently, by using microarray we showed that IFNβ treatment elevates the expression of 7 IFN-responsive genes in highly purified peripheral blood $CD3^+$ T cells of 13 Japanese RRMS patients (Koike et al., 2003). More recently, we found that the majority of differentially expressed genes in $CD3^+$ T cells between 72 untreated MS patients and 22 healthy control (CN) subjects were categorized into apoptosis signaling-related genes (Satoh et al., 2005).

To extend our previous studies, we conducted hierarchical clustering analysis of differentially expressed genes between MS and CN in peripheral blood $CD3^+$ T cells. Here we report that T-cell gene expression profiling classifies a heterogeneous population of Japanese MS into four subgroups that differ in the disease activity and therapeutic response to IFNβ, suggesting that this analysis could be applied for designing tailor-made treatment of MS.

2. Subjects and methods

2.1. The study population

The Research Group for IFNβ treatment of Japanese MS, sponsored by the Ministry of Health, Labour and Welfare of Japan, conducted the present study. It enrolled 72 clinically active Japanese MS patients, including 65 RRMS and 7 SPMS cases composed of 55 women and 17 men with the mean age of 36.1±10.3 years, and 22 healthy control (CN) subjects composed of 16 women and 6 men with the mean age of 38.6±12.3 years. The members of this research group (SK, KN, KY, KO, TK, TF and TY), all of who are certified neurologists, diagnosed individual cases according to the established criteria (McDonald et al., 2001), and followed up the patients for at least two years after entry. The patients showed the mean Expanded Disability Status Scale (EDSS) score of 2.8±2.0 upon entry. No patients had a history of treatment with interferons, glatiramer acetate or mitoxantrone before enrollment, or received corticosteroids and other immunosuppressants during at least one month before blood sampling. MS patients were divided into two groups according to their own determination upon entry: one treated with IFNβ and the other without IFNβ. The IFNβ-treated group included 46 patients who started to receive an administration of 8 million units of IFNβ-1b (Betaferon, Schering, Osaka, Japan) for two years given subcutaneously on alternate days, while the IFNβ-untreated group included 26 patients who were followed up without IFNβ treatment for successive two years. From the IFNβ-treated group, blood samples were taken at three time points: before starting IFNβ treatment (designated Pre) and at 3 and 6 months after starting the treatment. In the IFNβ-untreated group, they were collected twice: at enrollment and at 6 months after the enrollment. In case of acute relapse, the patients in both groups were given intravenous methylprednisolone pulse (IVMP) following the standard protocol, although none received glatiramer acetate, mitoxantrone, or other immunosuppressants. The samples obtained during clinically obvious relapses or episodes of infection were omitted. Written informed consent was obtained from all the subjects. The present study was approved by the Ethics Committee of National Center of Neurology and Psychiatry (NCNP).

2.2. IFN β responder/nonresponder score

To evaluate the therapeutic response to IFNβ, we monitored the following six parameters during four years spanning two years before and after initiation of IFNβ

treatment; the number of clinical relapse, the day of IVMP treatment, the day of hospitalization, EDSS score, the number of lesions on T2-weighted MRI, and the patient's satisfaction on the treatment (Table 1). When compared before and after IFNβ treatment, these parameters have given three ranks and scores; good (+1), intermediate (0), and poor (−1). The total score was calculated for each patient, ranging from the maximum value of +6 to the minimum value of −6. The patients with the total score equal to or greater than +3 were considered as being the responder (R), the score from 0 to +2 as one with the undetermined response (UD), and the score equal to or smaller than −1 as the nonresponder (NR) (Table 1).

2.3. cDNA microarray analysis

The present study utilized a custom microarray containing duplicate spots of 1258 cDNA immobilized on a poly-L-lysine-coated slide glass. They were composed of well annotated genes of various functional classes, including cytokines/growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, signal transducers, cell cycle regulators and housekeeping genes (Hitachi Life Science, Kawagoe, Saitama, Japan; http://www.hitachi.co.jp/LS). Peripheral blood mononuclear cells (PBMC) were isolated from 30 ml of heparinized blood by centrifugation on a Ficoll density gradient. They were labeled with anti-CD3 antibody-coated magnetic microbeads (#130-050-101, Miltenyi Biotec, Auburn, CA), and $CD3^+$ T cells were separated by AutoMACS (Miltenyi Biotec). The remaining cells after the positive selection of $CD3^+$ T cells were harvested as $CD3^-$ non-T cell fraction as described previously (Koike et al., 2003; Satoh et al., 2005). Total RNA was isolated from the cells by using RNeasy Mini Kit (Qiagen, Valencia, CA). Five micrograms of purified RNA was in vitro amplified, and the antisense RNA (aRNA) of MS patients and CN subjects was labeled with a fluorescent dye Cy5, while pooled aRNA of three independent healthy volunteers who were not included in the present study was labeled with Cy3 for a universal reference to standardize the gene expression levels throughout the experiments. The arrays were hybridized at 62 °C for 10 h in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cDNA, and they were then scanned by the ScanArray 5000 scanner (GSI Lumonics, Boston, MA). The data were analyzed by using the QuantArray software (GSI Lumonics). The average of fluorescence intensities (FI) of duplicate spots was obtained after global normalization between Cy3 and Cy5 signals. The gene expression level (GEL) was calculated according to the formula: GEL=FI (Cy5) of the sample/FI (Cy3) of the universal reference.

2.4. Hierarchical clustering analysis, principal component analysis, and statistical analysis

The genes whose expression was significantly different between MS and CN groups were identified by using *pierre* of the "R" statistical software system (www.cran.r-project.org) based on a Bayesian framework for analysis of microarray expression data (Baldi and Long, 2001). The error rate of this test smaller than 0.25 following the Bonferroni correction was considered as significant. Hierarchical clustering analysis and principal component analysis (PCA) were performed on a set of 286 genes differentially expressed between MS and CN groups, which were selected

Table 1
IFNβ responder/nonresponder score

Category	The parameters	Rank and score of the therapeutic response		
		Poor	Intermediate	Good
#1	Number of relapse after 2 years/number of relapse before 2 years	≧1.5	1.5–0.5	≦0.5
	Score	(−1)	0	(+1)
#2	Number of IVMP treatment after 2 years/number of IVMP treatment before 2 years	≧1.5	1.5–0.5	≦0.5
	Score	(−1)	0	(+1)
#3	Day of hospitalization after 2 years/day of hospitalization before 2 years	≧1.5	1.5–0.5	≦0.5
	Score	(−1)	0	(+1)
#4	EDSS score before treatment-EDSS score in 2 years after treatment	≦ −0.5	0.5–(−0.5)	≧0.5
	Score	(−1)	0	(+1)
#5	Number of lesions on T2-weighted MRI in 2 years after treatment/number of lesions on T2-weighted MRI before treatment	≧1.2	1.2–0.8	≦0.8
	Score	(−1)	0	(+1)
#6	Patient's satisfaction	Unsatisfied	Neither satisfied nor unsatisfied	Satisfied
	Score	(−1)	0	(+1)

The total responder/nonresponder score of six categories ranges from the maximum value of +6 to the minimum value of −6. The patients with the score equal to +3 or greater than +3 were classified as responder (R), the score ranging from 0 to +2 as undetermined (UD), and the score equal to −1 or smaller than −1 as nonresponder (NR). Abbreviations: IVMP, intravenous methylprednisolone pulse.

as a discriminator for a standard × standard algorithm on GeneSpring 7.2 (Agilent Technologies, Palo Alto, CA). The differences in clinical parameters among MS subgroups were evaluated by multiple comparison test following the Bonferroni correction.

3. Results

3.1. Microarray analysis identified 286 genes differentially expressed in peripheral blood T cells between MS and control subjects

Among 1258 genes on the microarray, 286 genes were expressed differentially in peripheral blood $CD3^+$ T cells between 72 untreated MS patients and 22 CN subjects. Among them, 78 genes were upregulated, while 208 genes downregulated in MS versus CN (Supplementary Table 1 online for all datasets). We also conducted the microarray analysis of $CD3^-$ non-T cells, composed of B cells, monocytes/macrophages and NK cells, and found that 96 genes were differentially expressed in the non-T cell fraction between MS and CN (data not shown).

3.2. Hierarchical clustering analysis identified four distinct subgroups of MS and five gene classes

Hierarchical clustering analysis was performed on $CD3^+$ T-cell samples of 72 untreated MS patients and 22 CN subjects, by using the set of 286 differentially expressed genes described above as a discriminator. This unsupervised approach, which arranged the genes and samples with a similar expression pattern to make a cluster in the dendrogram, identified four distinct

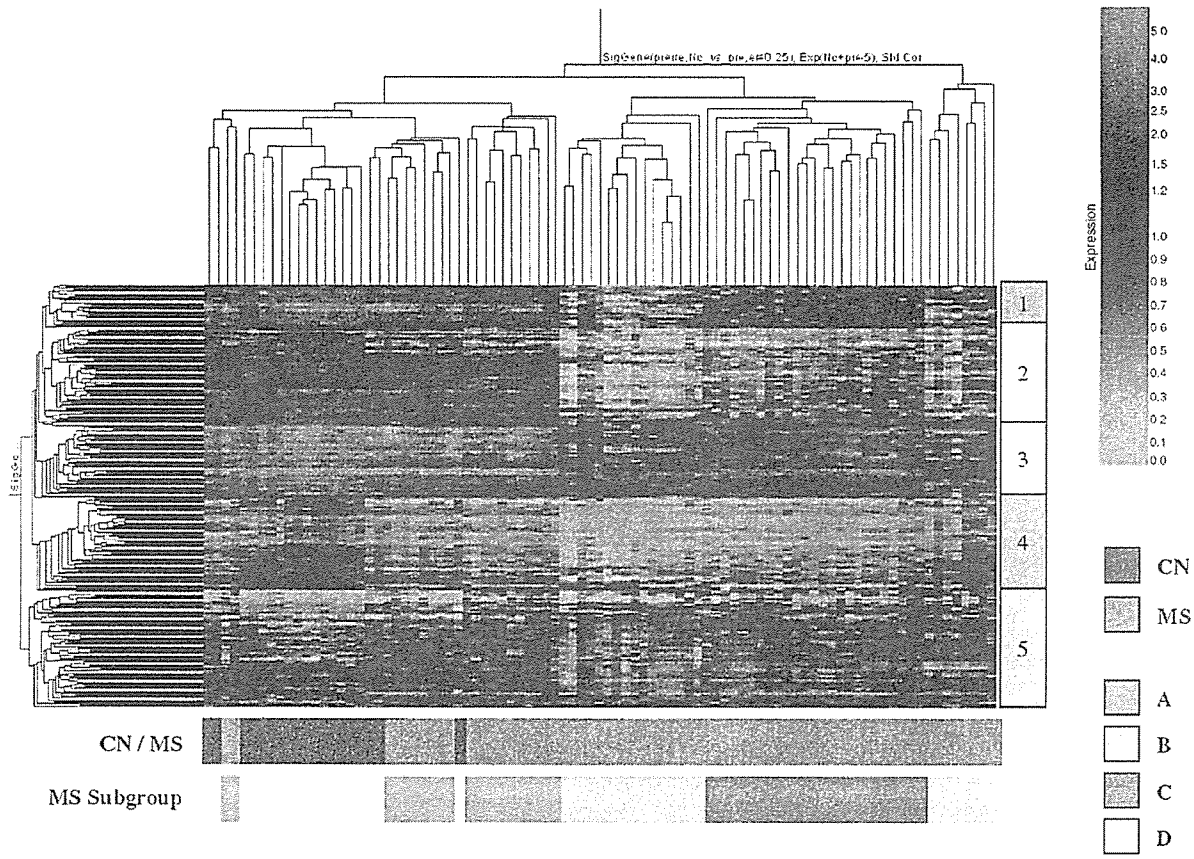

Fig. 1. Hierarchical clustering analysis of 286 genes differentially expressed between untreated MS patients and control subjects. The gene expression profile of peripheral blood $CD3^+$ T cells was studied in 72 untreated MS patients and 22 age- and sex-matched healthy control (CN) subjects, by using a 1258 cDNA microarray. Hierarchical clustering analysis was performed by selecting a set of 286 genes differentially expressed between MS and CN as a discriminator. The results are expressed in a matrix format, with each row representing the gene expression level (GEL) of a single gene in all the subjects and each column representing GEL of 286 genes in an individual subject. The matrix is shown by a pseudo-color, with red expressing upregulation, green expressing downregulation, and the color intensity representing the magnitude of the deviation from GEL 1.0 as shown on the upper right. Hierarchical clustering analysis separated MS (purple) from CN (dark blue), and classified the former into four subgroups named A (green), B (light blue), C (red) and D (yellow). The 286 genes were categorized into five classes numbered #1 (pink) to #5 (light blue).

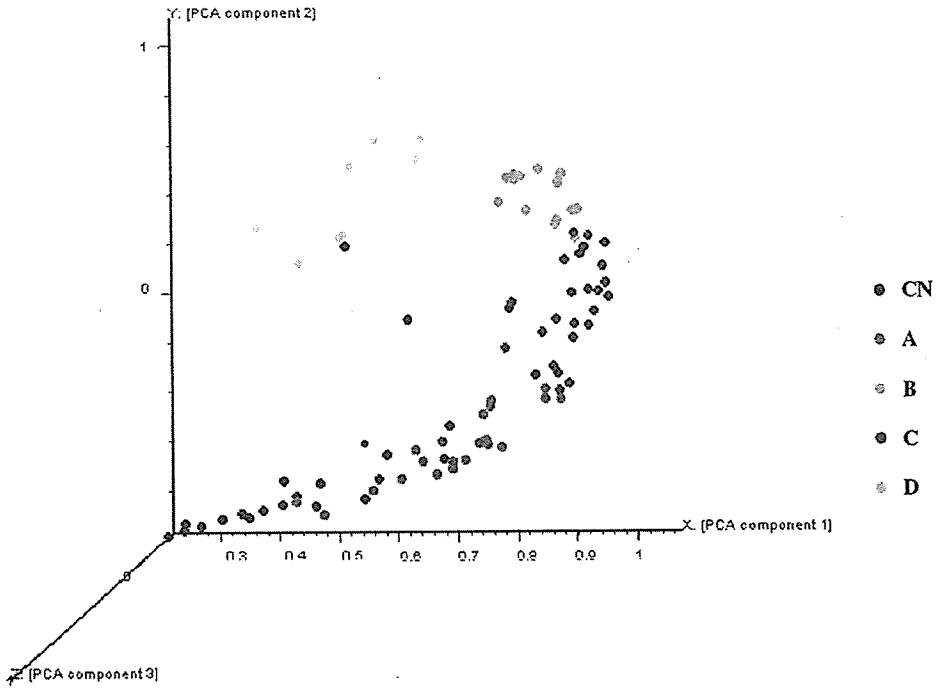

Fig. 2. Principal component analysis of 286 discriminator genes. Principal component analysis (PCA), which reduces all of the variance in the original dataset to three dimensions accounting for a significant fraction of the variance, verified a clear separation of the CN group (dark blue) and four MS subgroups named A (green), B (light blue), C (red) and D (yellow) identified by hierarchical clustering analysis.

subgroups of MS, clearly separated from the CN group (Fig. 1). We operationally designated each subgroup of MS as A, B, C and D, following the relative location in the dendrogram (Fig. 1). Principal component analysis (PCA) verified a clear discrimination of four MS subgroups and CN group (Fig. 2). Among 94 subjects examined, two MS patients and three CN subjects were considered as being unclassifiable (UC). In contrast, the clustering analysis of $CD3^-$ non-T cells did not clearly separate MS subgroups from CN (data not shown). Hierarchical clustering analysis categorized 281 of 286 differentially expressed genes into five distinct classes numbered #1 to #5 (Fig. 1 and Supplementary Table 1 online for all datasets). The remaining five, including TOP1, CHST4, SLC35A1, ST1B2, and TAF2H, were unable to be categorized into any classes. All the class #5 genes were upregulated in MS, whereas the genes of classes #1 to #4 were downregulated in MS, when compared with CN (Fig. 1). Upregulation of several class #5 genes in MS was validated by quantitative real-time RT-PCR analysis (data not shown).

3.3. Association of MS subgroups with gene clusters

Expression of the class #5 genes were elevated in all MS subgroups, whereas the classes #1 to #4 genes were downregulated in all of them, although the present study could not identify the marker genes specific for each MS subgroup. The subgroup A showed the gene expression pattern that is the most similar to CN. The similarity was supported by a partial overlap between A and CN in PCA (Fig. 2), and by the observations that one CN subject was incorporated in A, while two MS patients of A were included in CN (Fig. 1). Notably, the subgroup B showed the greatest upregulation of class #5 genes and the most prominent suppression of classes #1 to #4 genes (Fig. 1).

The class #5 genes ($n=78$) contain nine chemokines (11.5%), including CCL1, CCL3, CCL13, CCL18, CCL24, CXCL1, CXCL2, CXCL9, and CXCL14. In contrast, the classes #1 to #4 genes ($n=203$) contained only two chemokines (1.0%), such as CXCL5 and CXCL10. These observations suggest that the class #5 gene cluster is highly enriched in chemokine genes.

Fig. 3. Clinical characteristics of microarray-determined four MS subgroups. MS patients were classified into four distinct subgroups named A, B, C, and D by hierarchical clustering analysis. The bar indicates the data of individual patients. The number of relapse, the day of IVMP treatment, the day of hospitalization, and the number of lesions on T2-weighted MRI represent the data of 2 years before enrollment. Abbreviations: EDSS, Expanded Disability Status Scale; IVMP, intravenous methylprednisolone pulse; R/NR, responder/nonresponder.