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Invariant V_α19i T cells regulate autoimmune inflammation

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T cells expressing an invariant V_α19-J_α33 T cell receptor α-chain (V_α19i TCR) are restricted by the nonpolymorphic major histocompatibility complex class Ib molecule MR1. Whether V_α19i T cells are involved in autoimmunity is not understood. Here we demonstrate that T cells expressing the V_α19i TCR transgene inhibited the induction and progression of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Similarly, EAE was exacerbated in MR1-deficient mice, which lack V_α19i T cells. EAE suppression was accompanied by reduced production of inflammatory mediators and increased secretion of interleukin 10. Interleukin 10 production occurred at least in part through interactions between B cells and V_α19i T cells mediated by the ICOS costimulatory molecule. These results suggest an immunoregulatory function for V_α19i T cells.

Two distinct mouse T cell subsets express invariant TCRα chains: V_α14-J_α18 (V_α14i; ref. 1) and V_α19-J_α33 (V_α19i; ref. 2). Although conventional T cells recognize peptide antigens presented by polymorphic major histocompatibility complex class Ia molecules, V_α14i 'invariant' T cell populations recognize nonpeptide antigens[3,4] presented in the context of the nonpolymorphic major histocompatibility complex class Ib molecule CD1d. MR1 may be able to present glycolipids *in vitro* to V_α19i T cells[5], but the identity or type of endogenous ligand recognized by V_α19i T cells *in vivo* is unknown. However, antigen recognition is essential for the development of T cells expressing V_α14i and V_α19i TCR chains, as these subsets are absent from *Cd1d1*$^{-/-}$ and *Mr1*$^{-/-}$ mice, respectively[6,7]. Similar invariant T cell subsets are present in humans[8,9]. Many of these cells also express natural killer (NK) cell markers on their surface (such as mouse NK1.1). Consequently, CD1d-restricted invariant T cells have traditionally been referred to as 'NKT cells' (V_α14i NKT cells)[10].

Transgenic overexpression of the V_α14i TCR chain protects against the development of mouse models of type I diabetes[11] and multiple sclerosis[12], suggesting that V_α14i NKT cells may be involved in regulating autoimmunity. In addition, susceptibility to type I diabetes is linked to quantitative and functional deficiencies in V_α14i NKT cells[13]. Mechanistic studies suggest that V_α14i NKT cells may down regulate autoimmunity by increasing the production of T helper type 2 (T_H2) cytokines[14–19]. However, in other conditions, NKT cells may promote the exacerbation of autoimmune disease. V_α14i NKT cell–deficient mice show ameliorated arthritis compared with that of their wild-type counterparts[18,20,21].

The immune function of MR1-restricted invariant T cells remains less clear than that of CD1d-restricted lymphocytes. MR1-restricted invariant T cells were first identified among human peripheral blood CD4$^-$CD8$^-$ T cells as a clonally expanded population expressing an invariant V_α7.2-J_α33 TCR chain (V_α7.2i T cells)[22]. Subsequent studies identified clonally expanded T cells expressing the highly homologous invariant V_α19-J_α33 TCR chain in mice and cattle[9]. V_α19i T cell development has been found to depend on the nonpolymorphic major histocompatibility complex class Ib molecule MR1 and on the presence of B cells[7]. The V_α19i TCR is uniquely overexpressed in the gut lamina propria and V_α19i T cell development depends on the presence of commensal gut flora, indicating potential involvement of these cells in gut immunity[2,7]. As MR1 molecules are thought to be retained in the endoplasmic reticulum, intestinal flora might provide exogenous ligands for the V_α19i TCR, or a cellular 'stress' signal, that enables transit of MR1 from the endoplasmic reticulum to the cell surface[2,7].

Human V_α7.2i T cells[2] but not mouse gut V_α19i T cells express NKT cell markers[7]. In contrast, the V_α19i TCR is expressed by most T cell hybridomas derived from liver NK1.1$^+$ T cells from *Cd1d1*$^{-/-}$ mice[23]. Furthermore, 25–50% of V_α19i cells from V_α19i transgenic mice on a *Tcra*$^{-/-}$ background express NK1.1 (ref. 24). Those divergent results regarding NK1.1 expression remain unclear, but may be due to differences among mouse genetic backgrounds. Alternatively, as with CD1d-restricted T cells, a subpopulation of MR1-restricted T cells may lack NK1.1 expression. Based on their predominant distribution in the gut, MR1-restricted T cells are often referred to as 'mucosal-associated invariant T cells'[2,7]. To avoid confusion, we subsequently use the term 'V_α19i T cells' to describe V_α19i T cells expressing NK1.1.

The V_α7.2i TCR is over-represented in central nervous system (CNS) lesions from multiple sclerosis autopsy samples[25], whereas the V_α24i TCR is mostly absent[26]. Those findings led us to speculate that MR1-restricted T cells may 'preferentially' migrate to CNS lesions,

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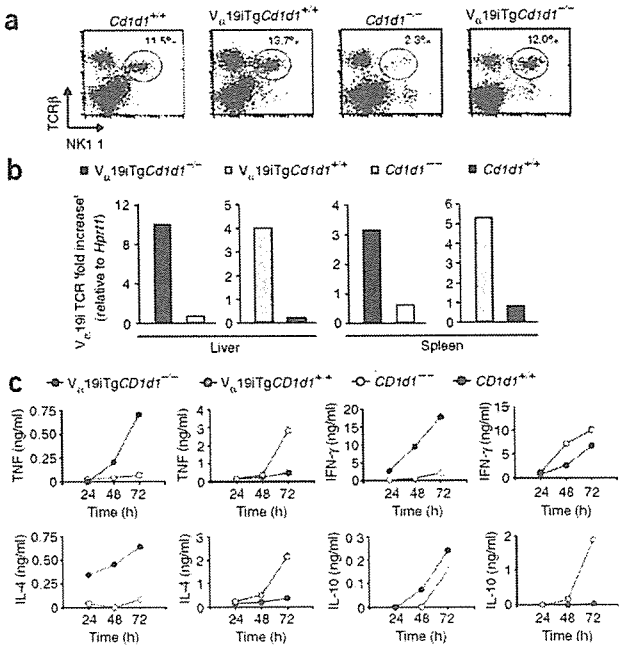

Figure 1 Characterization of NK1.1$^+$ T cells from V_α19iTg mice. (**a**) Flow cytometry of liver NK1.1$^+$ T cells 48 h after anti-asialo-GM1-mediated depletion of NK cells (mouse genotypes, above plots). Numbers above gated regions indicate the percentage of NK1.1$^+$TCRβ$^+$ cells. (**b**) Real-time RT-PCR of V_α19i TCR mRNA expression in liver or spleen NK1.1$^+$ T cells (mouse genotypes, key). Data are presented as 'fold increase' over expression of *Hprt1*. (**c**) Cytokines in the supernatants of sorted liver NK1.1$^+$ T cells (mouse genotypes, key) stimulated by immobilized anti-CD3 *in vitro*, measured at 24, 48 and 72 h after stimulation. Data are representative of two separate experiments (**a**,**b**) or the mean of two replicate values from two separate experiments (**c**).

where they regulate CNS inflammation. We designed this study to address the function of MR1-restricted T cells in experimental autoimmune encephalomyelitis (EAE)[14,17], a mouse model of multiple sclerosis. Here we report that over-representation of V_α19i T cells decreased the severity of EAE, whereas depletion of V_α19i T cells exacerbated EAE. Furthermore, V_α19i T cells exerted an influence on the phenotype and functions of autoimmune T cells in the draining lymph nodes and spleens of mice. In particular, over-representation of V_α19i T cells reduced the production of proinflammatory cytokines and increased the production of interleukin 10 (IL-10), which may account for V_α19i T cell–mediated suppression of autoimmune disease. Finally, interactions between V_α19i T cells and B cells mediated by the ICOS costimulatory molecule increased B cell IL-10 production and may therefore represent a mechanism by which V_α19i T cells regulate inflammation.

RESULTS

Characterization of transgenic V_α19i T cells

An antibody specific for the V_α19i TCR chain does not exist, and wild-type mice have very few MR1-restricted V_α19i T cells. Therefore, to circumvent those experimental hurdles and to evaluate the function of V_α19i T cells *in vivo*, we used V_α19i TCR–transgenic (V_α19iTg) mice[5], which were originally generated by injection into C57BL/6 mouse oocytes of a transgenic construct encoding a V_α19-J_α33 TCR construct driven by the endogenous *Tcra* promoter. We crossed the transgenic line with *Cd1d1*$^{+/+}$ and *Cd1d1*$^{-/-}$ C57BL/6 mice for seven to nine generations. First we compared numbers of liver NK1.1$^+$ T cells present in *Cd1d1*$^{+/+}$, *Cd1d1*$^{-/-}$, V_α19iTg*Cd1d1*$^{+/+}$ and V_α19iTg*Cd1d1*$^{-/-}$ mice (**Fig. 1a**). TCRβ$^+$NK1.1$^+$ T cells comprised 11.5% of total liver lymphocytes in *Cd1d1*$^{+/+}$ mice but only 2.3% of total liver lymphocytes in *Cd1d1*$^{-/-}$ mice. Therefore, most (about 80%) of NK1.1$^+$ T cells in *Cd1d1*$^{+/+}$ mice corresponded to CD1d-restricted V_α14i NKT cells, whereas about 20% were probably MR1 restricted[23]. Notably, V_α19iTg*Cd1d1*$^{-/-}$ mice had many NK1.1$^+$ T cells (12.0%), indicating that overexpression of the V_α19i TCR in *Cd1d1*$^{-/-}$ mice compensated for the reduction in NK1.1$^+$ T cells caused by CD1d deficiency. In contrast, the number of NK1.1$^+$ T cells was only slightly higher in V_α19iTg*Cd1d1*$^{+/+}$ mice, which had normal numbers of V_α14i NKT cells. To confirm that the NK1.1$^+$ T cell population in V_α19iTg mice was enriched in cells expressing the V_α19i TCR chain, we measured V_α19i mRNA transcripts in NK1.1$^+$ liver cells and splenocytes by real-time RT-PCR (**Fig. 1b**). V_α19i mRNA expression was much greater in liver and splenic NK1.1$^+$ T cell populations from V_α19iTg*Cd1d1*$^{+/+}$ or V_α19iTg*Cd1d1*$^{-/-}$ mice than in those from nontransgenic littermates (**Fig. 1b**). In V_α19i T cells, the V_α19i TCR chain 'preferentially' associates with TCRβ chains containing V_β8 or V_β6 segments[24]. Approximately 60–70% of liver NKT cells from V_α19iTg*Cd1d1*$^{-/-}$ or V_α19iTg*Tcra*$^{-/-}$ mice expressed either V_β8 or V_β6, compared with 30–40% of conventional T cells in the same mice (unpublished observations). These observations collectively demonstrate that NK1.1$^+$ T cell populations in V_α19iTg mice are highly enriched in cells expressing V_α19-J_α33 TCR chains and V_β6 or V_β8 TCR chains. Next we compared the ability of NK1.1$^+$ T cells from V_α19iTg and nontransgenic mice to produce immunosuppressive cytokines. To obtain V_α19i T cells, we depleted V_α19iTg*Cd1d1*$^{-/-}$ mice of NK cells by injecting antibody to

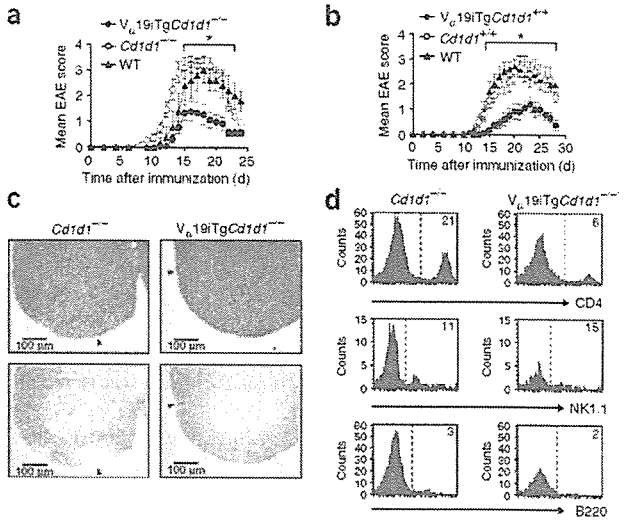

Figure 2 V_α19i T cells in EAE. (**a**,**b**) Clinical EAE scores of mice immunized with MOG(35–55). WT, wild-type. Data represent mean score ± s.e.m. from three independent experiments (n = 10–22 mice). (**c**) Monocyte infiltration and demyelination (arrowheads) of the lumbar spinal cord during EAE (day 15). (**d**) Quantification of spinal cord cellular infiltrates by flow cytometry. Areas to the right of dashed lines indicate positive cellular staining; numbers in histograms indicate percentage of CD4$^+$, NK1.1$^+$ (gated on CD3$^+$) or B220$^+$ cells. *, $P < 0.05$ (Mann-Whitney U-test). Data are representative of three separate experiments.

Table 1 V_α19i 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_α19iTg*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_α19iTg*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_α19i 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_α19i 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_α19iTg 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_α19iTg*Cd1d1*$^{-/-}$ mice as a source of V_α19i T cells.

V_α19i T cells in EAE

To determine if an abundance of V_α19i T cells could modulate autoimmune disease, we analyzed the development and progression of EAE in V_α19iTg 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_α19i 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_α19iTg 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_α19iTg*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_α19iTg*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_α19iTg*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_α19iTg*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_α19iTg*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_α19iTg*Cd1d1*$^{-/-}$ and *Cd1d1*$^{-/-}$ mice on day 18 after EAE induction had similar surface expression of CCR1 and CCR2 (data not shown). However, V_α19iTg*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_α19iTg*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).

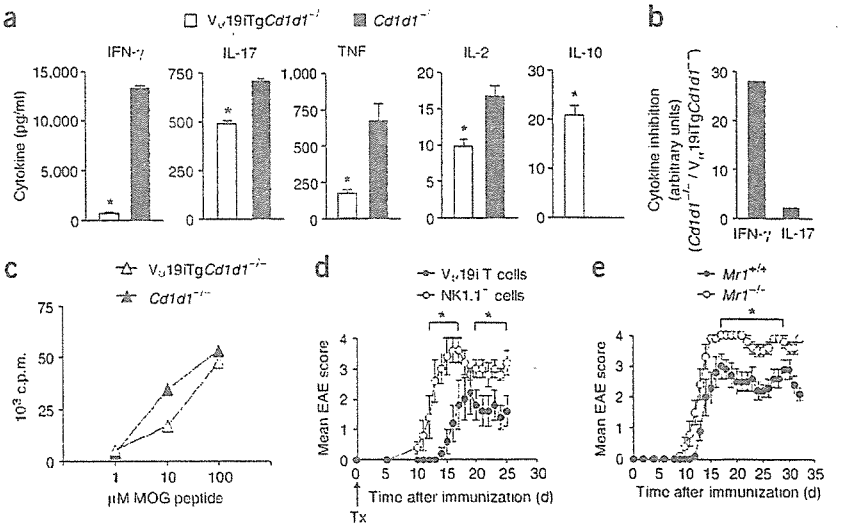

Figure 3 Inhibition of EAE is associated with decreased T_H1 cytokine production. (a) Cytometric bead assay of cytokines in the supernatants of MOG-specific lymph node cells (1×10^6) isolated from mice on day 10 after EAE induction and rechallenged with 100 μM MOG(35–55) *in vitro*, measured 72 h after 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_α19iTg*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_α19iTg*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_α19i T cells or an equal number of NK1.1$^-$ TCRβ$^+$ liver cells from V_α19iTg*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.

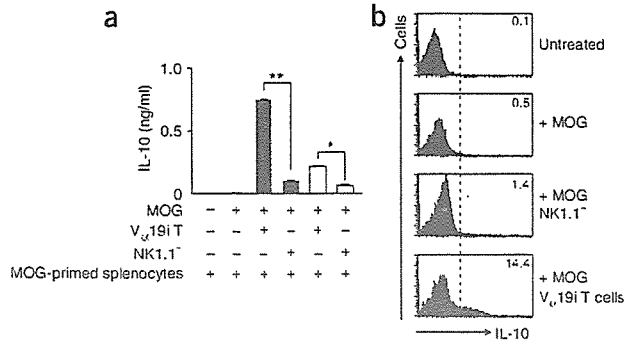

Figure 4 Interactions of V_α19i T cells and splenocytes induce IL-10. (a) Cytometric bead assay of IL-10 in the supernatants of liver V_α19i T cells from naive V_α19iTg*Cd1d1*$^{-/-}$ mice, cultured for 72 h with MOG(35–55)-specific splenocytes and MOG(35–55) (filled bars). In some cases, V_α19i T cells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

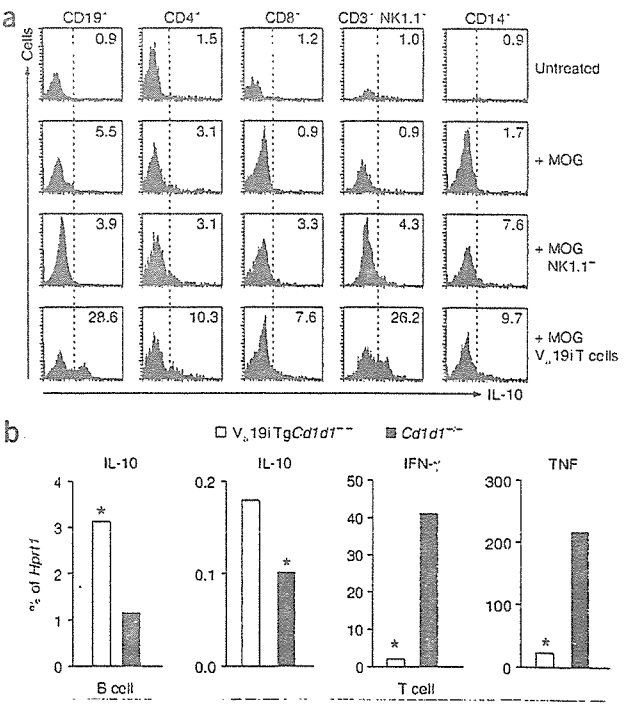

Figure 5 V_α19i T cells induce B cells to secrete IL-10. (a) Intracellular flow cytometry of IL-10 production by liver V_α19i T cells from naive V_α19iTg*Cd1d1*$^{-/-}$ mice, cultured for 72 h with MOG(35–55)-specific splenocytes and MOG(35–55). Areas to the right of dashed lines indicate positive cellular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

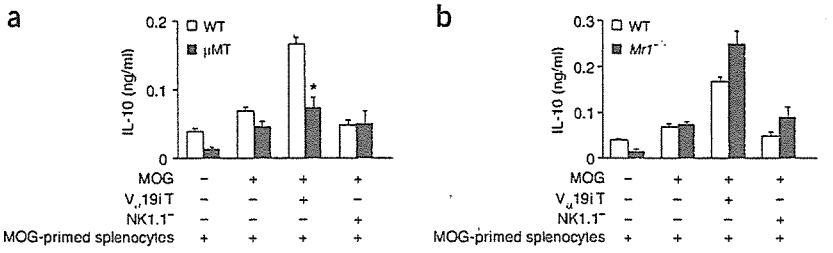

Figure 6 V_α19i T cell–induced IL-10 production is partially B cell dependent but completely MR1 independent. Cytometric bead assay of IL-10 in the supernatants of liver V_α19i T cells from naive V_α19iTg*Cd1d1*$^{-/-}$ mice, cultured for 72 h with MOG(35–55) plus MOG(35–55)-specific splenocytes from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

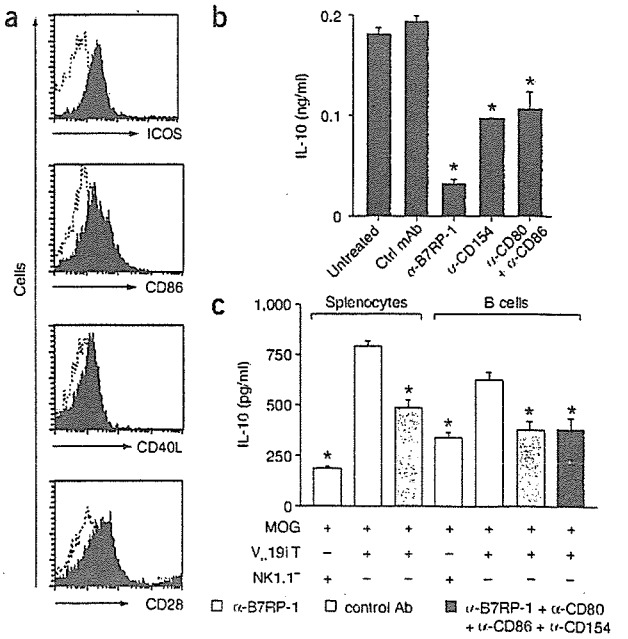

Figure 7 ICOS–B7RP-1 costimulation contributes to V_α19i T cell–induced B cell IL-10 production. (a) Flow cytometry of costimulatory molecule expression on the surface of liver V_α19i T cells (filled histograms) and naive splenic T cells from C57BL/6 mice (dotted lines). Data are representative of three 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 $V_\alpha19i$ 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 $V_\alpha19i$ T cells and splenocytes and fully inhibited IL-10 production in cocultures of $V_\alpha19i$ 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 $V_\alpha19i$ T cell–induced IL-10 production. Furthermore, the ICOS–B7RP-1 pathway is vital for $V_\alpha19i$ 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 $V_\alpha19i$ T cell–induced IL-10 production from whole splenocytes (Fig. 7b).

DISCUSSION

Although T cells expressing the invariant $V_\alpha19$-$J_\alpha33$ 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 $V_\alpha14i$ NKT cells, which influence autoimmunity, have similar properties, we speculated that MR1-resricted T cells would also be capable of modifying autoimmunity. However, $V_\alpha19i$ T cells are distinct from $V_\alpha14i$ CD1d-restricted T cells in their 'preferential' distribution in the gut mucosa and their dependence on the presence of B cells and gut flora.

$V_\alpha7.2i$ T cells, the human homolog of $V_\alpha19i$ 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 $V_\alpha19i$ T cells can suppress autoimmune inflammation. In addition, we have shown that $V_\alpha19i$ 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 $V_\alpha19iTg$ mice produced more cytokines than did $NK1.1^+$ T cells from $V_\alpha19iTgCd1d1^{-/-}$ mice, indicating a possible interaction between CD1d- and MR1-restricted lymphocytes.

We undertook several approaches to determine whether $V_\alpha19i$ T cells regulate EAE pathogenesis. Overexpression of $V_\alpha19i$ 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 $V_\alpha19i$ 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 $V_\alpha19iTg$ mice. Furthermore, we adoptively transferred liver $V_\alpha19i$ T cells from naive $V_\alpha19iTgCd1d1^{-/-}$ mice into wild-type nontransgenic mice with EAE, which express natural TCR diversity. In those experiments, $V_\alpha19i$ T cells effectively inhibited EAE, suggesting that $V_\alpha19i$ T cells have a regulatory function during EAE. However, a potential limitation of our model is the difficulty of obtaining pure $V_\alpha19i$ T cell preparations because of the lack of a $V_\alpha19$ TCR–specific antibody. Therefore, experiments using sorted $CD3^+$ $NK1.1^+$ cells from $V_\alpha19iTgCd1d1^{-/-}$ mice may also contain small numbers of non–$V_\alpha14i$ TCR $NK1.1^+$ T cells of other TCR specificities. To ascertain whether normal numbers of $V_\alpha19i$ T cells in wild-type nontransgenic mice could be involved during EAE, we induced EAE in $Mr1^{-/-}$ mice and found that the absence of $V_\alpha19i$ T cells resulted in a more severe clinical disease than that of wild-type nontransgenic mice.

$V_\alpha19i$ 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 $V_\alpha19i$ T cells was independent of $V_\alpha14i$ NKT cells by using $V_\alpha19iTg$ mice on a CD1d-deficient background. Notably, we found reduced adhesion molecule expression on effector T cells from $V_\alpha19iTgCd1d1^{-/-}$ mice, which correlated with reduced T cell infiltration of the CNS. However, we did note low numbers of $V_\alpha19i$ T cells ($CD3^+NK1.1^+$ from $V_\alpha19iTgCd1d1^{-/-}$mice) and B cells in the CNS of mice with EAE, suggesting that $V_\alpha19i$ 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 $V_\alpha19iTgCd1d1^{-/-}$ 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 $V_\alpha19i$ T cell–mediated immune regulation. B cells express MR1 (ref. 34), and $V_\alpha19i$ 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 $V_\alpha19i$ T cell selection, is not essential for $V_\alpha19i$ 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 $V_\alpha19i$ T cells was unknown before; we have demonstrated here that $V_\alpha19i$ 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 $V_\alpha19i$ 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 $V_\alpha19i$ T cells[7]. $V_\alpha19i$ T cells may also control gut production of immunoglobulin A from B cells, suggesting involvement of $V_\alpha19i$ 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 $V_\alpha19i$ T cells are involved in the homeostasis of gut immunity[2,7]. We have shown that $V_\alpha19i$ T cells help B cells produce

IL-10, which in nonpathogenic conditions may inhibit inflammation against gut flora required for V_α19i T cell selection. Therefore, we propose a model of V_α19i T cell–induced protection from EAE whereby V_α19i 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_α19i T cells in autoimmune disease. In contrast to 'conventional' V_α14i NKT cells, more T cells express the V_α19i TCR human homolog V_α7.2-J_α33 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_α19iTg mice[5], V_α19iTg*Cd1d1*$^{-/-}$ mice, *Cd1d1*$^{-/-}$ mice and *Mr1*$^{-/-}$ mice[7] were maintained in specific pathogen–free conditions in accordance with institutional guidelines (National Institute of Neuroscience, Tokyo, Japan.). *Mr1*$^{-/-}$ mice were backcrossed to C57BL/6 mice for ten generations[24]. Mice were injected subcutaneously with 100 μg MOG(35–55) and 1 mg heat-killed *Mycobacterium tuberculosis* H37RA (Difco) emulsified in complete Freund's adjuvant. Pertussis toxin (200 ng in PBS; List Biological Laboratories) was injected intraperitoneally on days 0 and 2 after immunization. EAE clinical symptoms were assigned scores daily as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, impaired righting reflex; 3, partial hindlimb paralysis; 4, total hindlimb paralysis.

Cell sorting and adoptive transfer. For depletion of NK cells, mice were injected intraperitoneally with 100 μg anti-asialo-GM_1 (ref. 44) 48 h before purification of V_α19i 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_α19i T cells were sorted from naive V_α19iTg*Cd1d1*$^{-/-}$ mice as described above, and 1×10^6 V_α19i 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_α19i 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_α19i 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_α19i T cells (5×10^5) sorted from naive V_α19iTg*Cd1d1*$^{-/-}$ mice, in the presence of 100 μg/ml of MOG(35–55) in 24-well plates or transwell plates (Corning). Where indicated, MOG(35–55)-specific spleen cells were isolated from *Mr1*$^{-/-}$ or μMT mice or were subjected to depletion with anti-CD19 microbeads (Miltenyi Biotech). Costimulatory molecules were blocked with 10 μg/ml of anti-B7RP-1 (HK5.3) or anti-CD40L (MR1) or with anti-CD80 and anti-CD86 (16-10A1 and GL1, respectively; all from BD Pharmingen)[35]. After 72 h, cytokines in the supernatant were analyzed by cytometric bead assay, enzyme-linked immunosorbent assay or intracellular flow cytometry. Proliferation of MOG(35–55)-specific lymph node cells was assessed 24 h after the addition of [^{3}H]thymidine (1 μCi/well) to 96-well plates.

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

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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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, ATTTGCGGTGGACGATGGAG; IFN-γ-sense, CAGGTCATTCAGATGTAGCG, and -antisense, GCTTTTCGAAGTCATCTCG; IL-5-sense, GCACACTGGAGAGTCAAACT, and -antisense, CACTCGGTGTTCATTACACC; GATA-3-sense, CTACGGAAACTCGGTCAGG, and -antisense, CTGGTACTTGAGGCACTCTT; T-bet-sense, GGAGGACACCGACTAATTTGGGA, 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

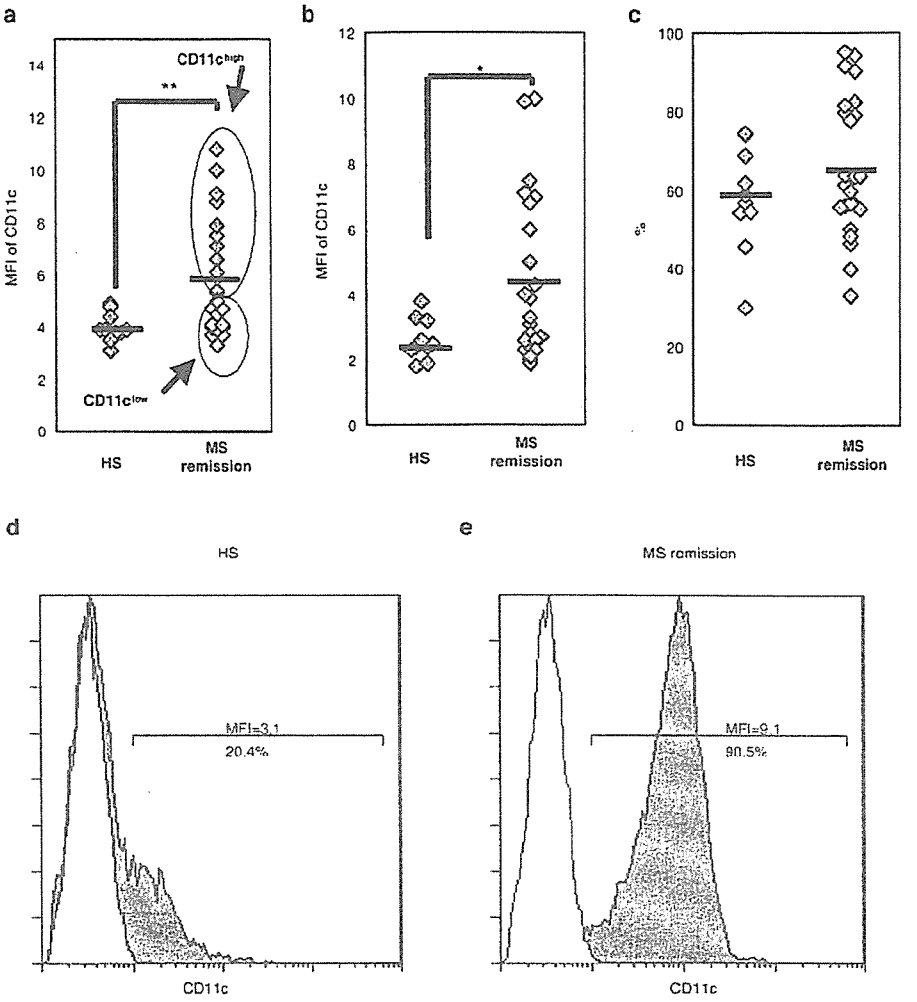

FIGURE 1. CD11c on NK cells is up-regulated in MS in remission. *a*, PBMC from HS (n = 10) and MS patients in remission (n = 25) were stained with anti-CD11c-PE, -CD3-ECD, and -CD56-PC5 mAb, and CD11c expression was measured on the $CD11c^+$ fraction gated within whole NK cells ($CD11c^+CD3^-CD56^+$ 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

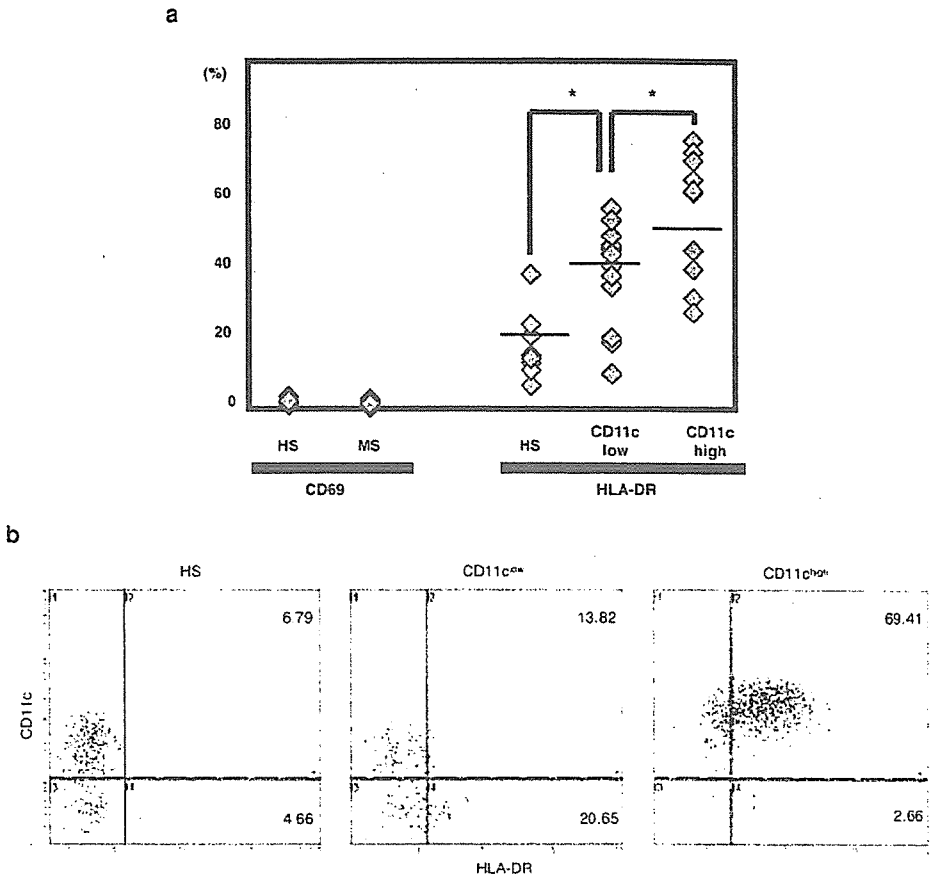

FIGURE 2. Proportions of HLA-DR$^-$ NK cells increase in CD11c^{high} MS. *a*, CD69 and HLA-DR expression on NK cells (CD3$^-$ CD56$^+$ cells). Data are expressed as proportions (percent) of CD69$^+$ cells (7 HS and 16 MS patients in remission) or HLA-DR$^+$ cells (10 HS and 25 MS patients) within whole 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),

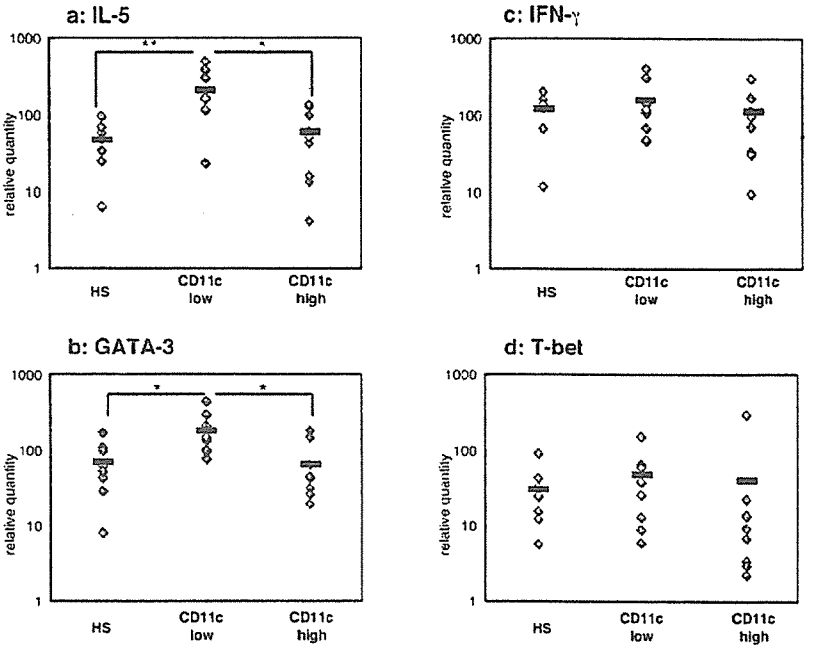

FIGURE 3. IL-5 and GATA-3 mRNA are increased in $CD11c^{low}$ but not in $CD11c^{high}$ MS. Total RNAs were extracted from purified NK cells of HS (n = 8), $CD11c^{low}$ (n = 9), or $CD11c^{high}$ MS (n = 8). mRNA expression of IL-5 (*a*), GATA-3 (*b*), IFN-γ (*c*), and T-bet (*d*) was evaluated by quantitative 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

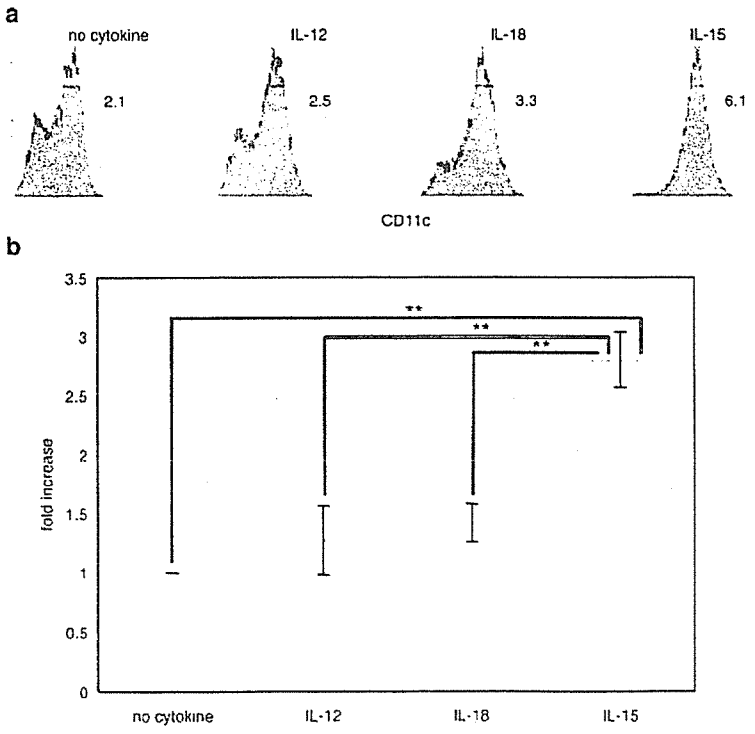

FIGURE 4. CD11c expression on NK cells is upregulated with addition of IL-15. *a*, Purified NK cells were cultured in the absence or presence of IL-12, IL-18, or IL-15. Three days later, the cells were stained with anti-CD11c-PE, -CD3-ECD, and -CD56-PC5 mAb. CD11c expression on NK cells ($CD3^-CD56^+$ 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.

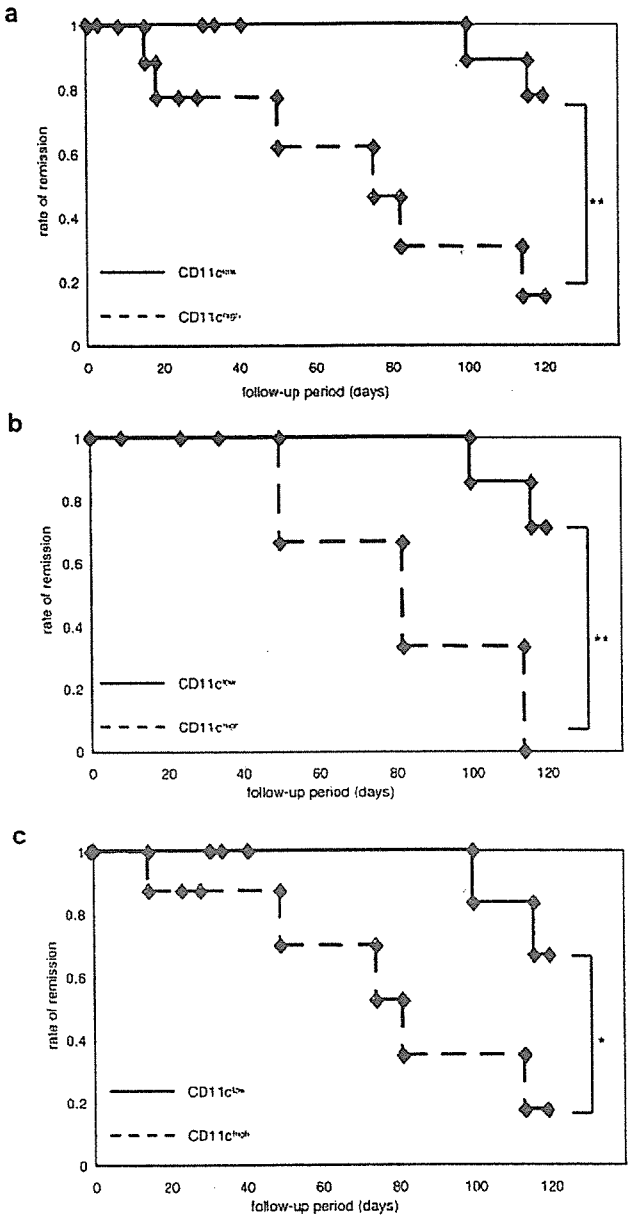

FIGURE 5. Rate of remission is lower in CD11c^{high} MS. The first episode of relapse after blood sampling was set as an end point and clinical course of each patient was followed for up to 120 days. The remission rate was calculated in all (*a*), the younger (*b*), or female (*c*) patients as Kaplan-Meier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

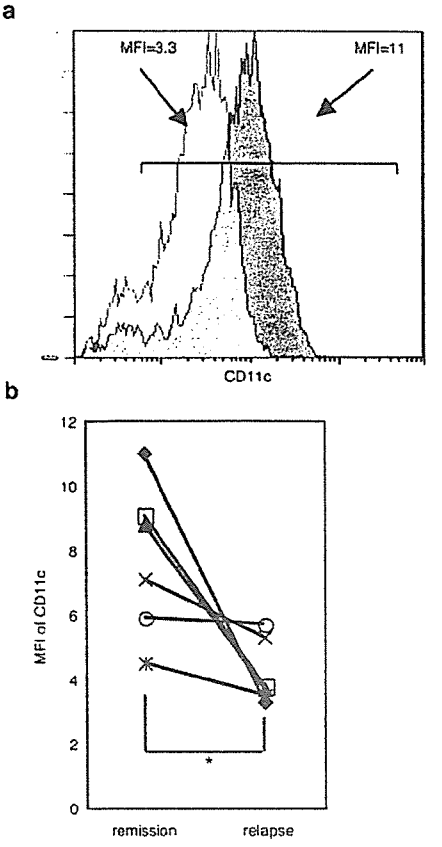

FIGURE 6. Down-regulation of CD11c expression during relapse. *a*, Representative CD11c histograms from the same patient in remission (closed) and relapse (open). Values indicate CD11c MFI of CD11c$^+$ fractions. *b*, Comparison of NK cells from remission and relapse from the same patients ($n = 6$). 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

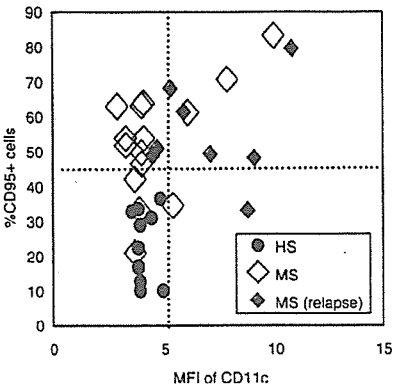

FIGURE 7. Expression pattern of CD95 vs CD11c on NK cells from MS. PBMC from MS or HS were stained with CD95-FITC, CD11c-PE, CD3-ECD, and CD56-PC5. After determining the proportion of $CD95^+$ cells among NK cells and CD11c expression (MFI) of CD11c, we plotted each patient according to the obtained 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.

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In vivo delivery of small interfering RNA targeting brain capillary endothelial cells

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Abstract

Brain capillary endothelial cells (BCECs) play an important role in blood–brain barrier (BBB) functions and pathophysiologic mechanisms in brain ischemia and inflammation. We try to suppress gene expression in BCECs by intravenous application of small interfering RNA (siRNA). After injection of large dose siRNA with hydrodynamic technique to mouse, suppression of endogenous protein and the BBB function of BCECs was investigated. The brain-to-blood transport function of organic anion transporter 3 (OAT3) that expressed in BCECs was evaluated by Brain Efflux Index method in mouse. The siRNA could be delivered to BCECs and efficiently inhibited endogenously expressed protein of BCECs. The suppression effect of siRNA to OAT3 is enough to reduce the brain-to-blood transport of OAT3 substrate, benzylpenicillin at BBB. The in vivo siRNA-silencing method with hydrodynamic technique may be useful for the study of BBB function and gene therapy targeting BCECs.

Keywords: Small interfering RNA; Blood–brain barrier; Organic anion transporter 3; Brain ischemia; Brain inflammation; Drug delivery system

In brain ischemia and inflammation, the brain capillary endothelial cells (BCECs) have no longer been regarded as an inert vascular lining that is injured and morphologically changed, but actively play many important roles of these pathophysiologic mechanisms. The inhibition of signaling molecule in BCECs of vascular endothelial growth factor (VEGF)-induced vasogenic edema can reduce an ischemic lesion [1]. The inflammatory cell adhesion molecules expressed in BCECs induced by ischemia, such as intercellular adhesion molecule (ICAM) and E-selectin, can be a target molecule [2,3] for the therapy of these diseases. Because leukocytes activation and adhesion to BCECs are believed to contribute to additional, secondary neuronal injury after reperfusion [4] and initiate immune-mediated encephalopathy such as multiple sclerosis [5]. Endothelial nitric oxide synthases expressed in BCECs are also a possible target molecule. In cerebral ischemia, nitric oxide is increased and works as a prooxidant via peroxynitrite [6]. Therefore, BCECs are an important platform in the cerebral ischemia and inflammation, and express many constitutively or transiently expressed molecules which might be a therapeutic target for these pathologies.

RNA interference is a powerful tool for post-transcriptional gene silencing. Recently, we showed an in vitro model whose function of the transporter protein expressed in BCECs is inhibited by siRNA [7]. Here, we try to introduce siRNA by hydrodynamic, intravenous injection method from mouse tail vein and investigate the siRNA effect on brain-to-blood transport function by inhibiting organic anion transporter 3 (OAT3) with Brain Efflux Index method.

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Materials and methods

Effect of siRNA on expression of recombinant OAT3 in culture cells. The mOAT3cDNA was subcloned from pGEM-HEN/Roct (OAT3) [8] into the *Renilla* luciferase expression vector, psiCHECK-1 (Promega).

Human embryonic kidney 293 (HEK293) cells were transfected with 80 ng of *Renilla* luciferase-fused OAT3 expression vector, 20 ng of *firefly* luciferase expression vector (pGL3; Promega), and 25 nM siRNA in each well of 24-well plates. *Renilla* luciferase activity was normalized with *firefly* luciferase activity. The luciferase activities were analyzed after 24 h after transfection using the Dual Luciferase System (Promega).

Effect of siRNA on uptake of OAT3 substrate in culture cells. The mOAT3 cDNA was subcloned into the pcDNA3 vector. HEK293 cells in 6-well plates were transfected by 0.5 μg of pcDNA3/OAT3 or vector alone with 100 nM siRNA using the Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were passaged into the 24-well plates, and after another 24 h the cells were washed with phosphate-buffered saline (PBS). The uptake study was initiated at 37 °C by applying 200 μl PBS containing 0.5 μCi [^{3}H]benzylpenicillin to estimate the volume of adherent water. After incubation for 2 min, the radioactivities of ^{3}H in the cells were measured. The uptake of [^{3}H]benzylpenicillin was expressed as the ratio to control siRNA (shuffle sequence).

Animals. Adult male of Institute of Cancer Research (ICR) mice, weighing 35–42 g and age 9–10 weeks, were purchased from Charles River Laboratories. All experiments were approved by the Animal Experiment Committee of Tokyo Medical and Dental University.

In vivo transduction of siRNA with hydrodynamic injection method. Hydrodynamic injection method has been performed according to a previously reported method in mice [9]. The 50 μg siRNA in a volume equivalent to 5–10% of the body weight was rapidly injected in 3–5 s into the mouse tail vein. For comparison, the same amount of siRNA in 0.2 ml PBS was injected slowly in more than 60 s into the mouse tail vein as a regular intravenous injection method.

Brain small vascular fractionation and Western blot analysis. Mice brains were harvested 24 h after application of 50 μg siRNA SOD1 with the hydrodynamic or regular injection method. The total brain homogenate [10] and the brain vascular fraction of small vessels were prepared using a modified method reported previously [11]. Briefly, brains were homogenized in Dulbecco's modified Eagle's medium (DMEM). The homogenates were dissociated further with 0.005% (wt/vol) dispase (grade 1; Roche Diagnostic) at 37 °C for 2 h. After centrifugation (800*g*, 5 min), the pellets were suspended with a dextran solution (M_w 70,000; 15% wt/vol; Sigma) and centrifuged (4 °C, 4500*g* for 10 min). The pellets were resuspended with 0.05 M PBS for 10 min. After centrifugation (800*g*, 5 min), the final pellets of small vessels were resuspended in lysis buffer (20 mM Tris–HCl, 0.1% SDS, and 1% Triton).

Fractionated mouse brain tissues and mouse brain capillary endothelial cell line [12] cells were homogenized in buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 4% Chaps, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease-inhibitor cocktail (Complete-Mini; Roche Diagnostic). The 2.5 μg samples were separated with 7.5% SDS–polyacrylamide mini-gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane. The membrane was probed with anti-glucose-transporter-1 antibodies (Alpha Diagnostic International) or anti-SOD1 antibodies (Stressgen Biotechnologies) and visualized by using an ECL Western blot system (Amersham–Pharmacia).

Assay for efflux function of OAT3 in vivo. Fifty micrograms of siRNA OAT3 or control siRNA was delivered to brain capillary endothelial cells with hydrodynamic injection via the tail vein. After 36 h, the in vivo brain efflux experiments were carried out using Brain Efflux Index (BEI) method as described previously [13]. Each mouse was anesthetized intramuscularly with a mixture of ketamine (125 mg/kg) and xylazine (1.22 mg/kg), then mounted on a stereotaxic frame (SRS-6; Narishige), to hold the head in position. Using a dental drill, a bore hole was made 3.8 mm lateral to the bregma. Then, extracellular fluid buffer (122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM Hepes, pH 7.4) containing 96 nCi [^{3}H]benzylpenicillin and 4.8 nCi [^{14}C]inulin was injected over a period 1 min using a 5.0-μl microsyringe (Hamilton Reno) fitted with a fine needle at a depth of 2.5 mm from the surface of the scalp, i.e., the secondary somatosensory cortex 2 (S2) region. The needle was left in this configuration for an additional 4 min to prevent reflux of the injected solution along the injection track, before being slowly retracted. After 40 min, the whole brain was subsequently removed and the left cerebrum was isolated. After weighing each of these, tissue samples were solubilized in 2 N NaOH at 60 °C for 1 h and then mixed with Hionic-fluor (Packard). The radioactivity in each sample was assayed in a liquid scintillation counter equipped with an appropriate crossover correction for ^{3}H and ^{14}C (LS-6500; Beckman).

The BEI was defined by Eq. (1) and the percentage of substrate remaining in the ipsilateral cerebrum was determined from Eq. (2).

$$\mathrm{BEI}(\%) = \frac{\text{test substrate undergoing efflux at the BBB}}{\text{test substrate injected into the brain}} \times 100 \quad (1)$$

$$100\text{-BEI}(\%) = \frac{(\text{amount of test substrate in the brain/amount of reference in the brain})}{(\text{concentration of test substrate injected/concentration of reference injected})} \times 100. \quad (2)$$

The percentage of [^{3}H]benzylpenicillin remaining in the brain is given by (100-BEI).

The data were used when the remaining amount of [^{14}C]inulin in the brain was more than 15% of the injected amount. No significant difference was observed in the remaining percentage of [^{14}C]inulin, which is a non-permeable marker, among all samples (#1, 39.7 ± 3.5%; #2, 27.5 ± 3.4%; #3, 31.4 ± 2.9%; #2 shuffle, 28.9 ± 2.2%) (ANOVA), showing that the hydrodynamic injection of siRNA did not damage the integrity of BBB.

Data analysis. All data represent means ± SEM. An unpaired, two-tailed Student's *t* test was used to determine the significance of differences between two group means. (The difference is certified when $P < 0.05$.)

Results

siRNA directed against the OAT3 and SOD1 genes

Sense sequences of the siRNA designed to OAT3 and SOD1 genes are described as follows. The siRNA of shuffle sequence of siRNA OAT3 #2 and siRNA against unrelated gene, GBV-B virus, were used as negative controls. Uppercase letters at 3′ end indicate deoxyribonucleotides.

siRNA OAT3 #1:	5′-ucuacaacagcaccagagaTT-3′
siRNA OAT3 #2:	5′-ccauuaucuugaauguggaTT-3′
siRNA OAT3 #3:	5′-aaacaaagcaggagccagaTT-3′
siRNA-shuffle sequence:	5′-agugguaaugucuauauccTT-3′
siRNA-unrelated control:	5′-agugguaaugucuauauccTT-3′
siRNA SOD1:	5′-gguggaaaugaagaaaguaTT-3′

Effect of siRNA on expression and function of recombinant OAT3 in culture cells

siRNA OAT3 #2 most effectively reduced the expression of OAT3 in HEK293 cells by 86.2% on luciferase activity compared with control siRNA with shuffle sequence of siRNA OAT3 #2 (Fig. 1). siRNA OAT3 #1 and #3 were moderately effective.

To investigate the inhibition effect of siRNA OAT3 to its efflux function in vitro, we measured uptake of OAT3 substrate, [^{3}H]benzylpenicillin. After expression of OAT3