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T_h2 bias of CD4+ NKT cells derived from multiple sclerosis in remission

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Abstract

Although CD1d-restricted NKT cells have been implicated as a participant in the regulatory mechanism of autoimmune diseases, it remains unclear how they would regulate human autoimmune diseases such as multiple sclerosis (MS). Furthermore, although the NKT cells comprise CD4+ and CD4– populations, prior studies have often represented them as simply a CD4– population. Given that CD4+ and CD4– NKT cells may represent functionally distinct populations, it appears crucial to examine the individual NKT subset in autoimmune diseases. Here we studied the frequency and cytokine phenotypes of the CD4+ and CD4– NKT cells in fresh peripheral blood mononuclear cells, and of α-galactosylceramide-stimulated short-term cell lines obtained during the remission or relapse phase of MS as compared with from healthy subjects (HS). Here we report that CD4+ NKT line cells expanded from MS in remission (MS-rem) would produce a larger amount of IL-4 than those from HS or from MS in relapse (MS-rel). They were significantly biased for T_h2 as judged by the IL-4/IFN-γ balance. However, there was no functional bias toward T_h1 or T_h2 in CD4– NKT line cells from MS-rem due to the defects in both IFN-γ and IL-4 production, compared with HS. Of note, although double-negative NKT cells in the periphery were greatly reduced, the reduction of CD4+ NKT cells was only marginal, if any, in MS-rem compared with HS. The T_h2 bias of CD4+ NKT line cells from MS-rem may support an immunoregulatory role for the CD4+ NKT cells *in vivo*.

Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease affecting the central nervous system (CNS), clinically characterized by alternating periods of relapse and remission (1,2). Whereas IFN-γ-producing T_h1 autoimmune T cells are considered to mediate the immunopathology of MS that results in the formation of multiple inflammatory demyelinating foci in the CNS, recovery or remission of the disease may be achieved by the contribution of regulatory cells controlling T_h1 cells. Although regulatory cells, down-modulating a T_h1 response, were shown to protect against autoimmune disease models in rodents (3–6), their role in MS was largely speculative. However, the contribution of regulatory cells has begun to be elucidated, as we have shown that NK cells may inhibit the activation of T_h1 autoimmune T cells by producing IL-5, thereby maintaining the remission phase of MS (7). Data from our laboratory and others also suggest that regulatory cells other than NK cells may be involved in MS in the context of complex cellular interaction. Of note, whereas pharmacological administration of IFN-γ in previous clinical trials significantly increased the frequency of relapses in MS (8,9), currently available therapeutics for MS tend to induce a T_h2 deviation (10–12). Hence, we reason that study of the regulatory cells producing T_h2 cytokine is particularly important in MS.

CD1d-restricted NKT cells (13,14) are regulatory cells, composed of CD4– and CD4+ populations, that have been intensively characterized in humans and rodents. They express a conserved canonical TCR α chain ($V_\alpha14J_\alpha281$ in mouse/$V_\alpha24J_\alpha Q$ in human) paired with a β chain using a selected V_β segment ($V_\beta8.2$ and 7 in mouse/$V_\beta11$ in human). Whereas conventional T cells respond to peptide–MHC ligand, the NKT cells with the canonical TCR sequence

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recognize a glycolipid antigen in association with the monomorphic CD1d molecule. Because they can explosively produce large amounts of IL-4 and IFN-γ after TCR ligation, they are thought to play regulatory roles in a wide range of immune responses. Studies of type I diabetes in the non-obese diabetic mouse have demonstrated that transfer or overexpression of NKT cells (15,16), as well as activation of NKT cells by the representative ligand α-galactosylceramide (α-GC) (17–19), leads to suppression of autoimmune diabetes. By contrast, elimination of NKT cells by knocking out CD1d expression led to augmentation of the disease (17). Furthermore, we have recently demonstrated in experimental autoimmune encephalomyelitis (EAE) that proper stimulation of NKT cells with glycolipid ligands could lead to suppression of the T_h1-mediated autoimmune disease by inducing a selective induction of IL-4 (20,21).

The frequency of NKT cells in the peripheral blood lymphocytes ranges from 0.1 to 0.3% in most healthy subjects. This low frequency in humans has raised some concern about the potential role of the cells in the regulatory network preventing human autoimmune diseases. However, a recent phase I clinical trial of α-GC (22) showed that i.v. injections of α-GC would induce an elevation of cytokines in the serum, which include IFN-γ, IL-4, IL-12 and granulocyte macrophage colony stimulating factor. This result supports that human NKT cells could exhibit immunoregulatory functions *in vivo*, if they are properly stimulated. In addition, it has been reported that NKT cells represent a predominant population in inflammatory lesions of human diseases (23,24), and that rodent NKT cells rapidly migrate to and accumulate in the inflammatory lesions like inflammatory cells (25). These results allow us to speculate that NKT cells might overcome the low frequency of the cells in the periphery by exploiting the unique ability to migrate to the inflammatory site.

In human autoimmune diseases, including systemic sclerosis, type I diabetes and MS, CD1d-restricted NKT cells were shown to be affected in number (23,26–29) or function (27,29). Of interest, $CD4^-CD8^-$ [double-negative (DN)] NKT cell clones generated from human type I diabetes were previously reported to exhibit a strong T_h1 bias as compared to those from control subjects (27). Provided that NKT cells regulate autoimmune diabetes by producing T_h2 cytokines, the T_h1 bias of NKT cells may be interpreted as a vicious change that would facilitate the development of diabetes. However, the functional bias of human NKT cells needs to be examined further in other disease conditions with a different methodology (27,30). Furthermore, it might be particularly important to examine $CD4^+$ NKT cells along with DN NKT cells, now that the $CD4^+$ (but not $CD4^-$) population was shown to be the predominant source of T_h2 cytokines produced by NKT cells in healthy individuals (31,32).

The primary purpose of this study was to enumerate the frequency and to characterize the production of T_h1/T_h2 cytokine by CD1d-restricted NKT cells in MS. To this end, we have examined the total, DN and $CD4^+$ NKT cell populations in the peripheral lymphocytes individually in each subject, and the IL-4 and IFN-γ production by NKT cell lines derived from the subjects. Here we report that $CD4^+$ NKT line cells expanded from MS in remission (MS-rem) produce a larger amount of IL-4 than those from healthy subjects (HS) or from MS in relapse (MS-rel) and that they were biased for T_h2 as judged by the IL-4/IFN-γ balance. Together with the accompanying observations, we suggest that the T_h2 bias of $CD4^+$ NKT line cells may support a regulatory role for $CD4^+$ NKT cells in MS.

Methods

Patients and controls

We examined 39 patients with relapsing–remitting MS (27 in remission and 12 in relapse) and 15 HS. None of the patients had been given corticosteroid or immunosuppressive agents for at least 3 months prior to examination. Relapses of MS were diagnosed by the presence of gadolinium-enhanced lesions in magnetic resonance imaging of the brain. The age and gender of the subjects were matched as follows: HS 36.3 ± 2.2 years old (nine females and six males), MS-rem 37.0 ± 2.5 years old (17 females and 10 males) and MS-rel 37.2 ± 4.4 years old (nine females and three males).

Antibodies and reagents

FITC-labeled anti-TCR $V_\alpha24$, phycoerythrin (PE)-labeled anti-$V_\beta11$, PE–Texas Red-labeled anti-human CD4 and PE–cyanin 5.1-labeled anti-human CD8 mAb were purchased from Immunotech (Marseille, France). We obtained anti-CD3/anti-CD28-coated microbeads (Dynabeads CD3/CD28 T Cell Expander) from Dynal (Oslo, Norway). α-GC was synthesized according to a previously described method (33). Human CD1d tetramer loaded with α-GC were prepared as described (31).

Flow cytometric analysis of NKT cells

Unless otherwise stated, we used anti-$V_\alpha24$/anti-$V_\beta11$ staining to estimate the frequency of CD1d-restricted NKT cells (28,29,34). Fresh peripheral blood mononuclear cells (PBMC) or α-GC-stimulated line cells were routinely stained with a combination of anti-$V_\alpha24$, -$V_\beta11$, -CD4 and -CD8 mAb, and were analyzed with an Epics XL (Coulter, Miami, FL) for counting the frequency of the total, DN and $CD4^+$ NKT cells. At least 100,000 cells were acquired from each sample for analysis. When we stained the cells with isotype-matched control antibody, the background count was null or only 1 cell per 100,000 (0.001%). Selected samples were also analyzed with CD1d tetramer (31) to assess the reproducibility of the results with a different method. For the tetramer staining, we incubated the cells first with 100 μg/ml of MOPC21 IgG1 blocking mAb, 0.5 μg/ml ovalbumin and 0.05% NaN_3 to block non-specific reaction, and then with α-GC-loaded or unloaded CD1d tetramer labeled with Alexa Fluor 488 as previously described (31). The background count with unloaded CD1d tetramer was 0.005 ± 0.003%. All the data were analyzed with EXPO 32 software (Coulter).

Expansion of NKT cells with α-GC and maintenance of the NKT-enriched line cells

We isolated the PBMC from peripheral blood of MS-rem, MS-rel or HS by density gradient centrifugation with Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). The cells were resuspended at 2×10^6/ml in

AIM-V medium (Life Technologies, Rockville, MD) supplemented with 10% FCS, human rIL-2 (40 U/ml; Shionogi, Osaka, Japan) and human rIL-7 (10 ng/ml; Peprotech EC, London, UK). They were seeded onto 24-well plates and stimulated with α-GC (100 ng/ml). From day 7 after initiation of the culture, we changed the medium every 3–5 days with the above culture medium further supplemented with human rIL-15 (10 ng/ml) (Peprotech EC).

Cell surface affinity matrix technology (cytokine secretion assay)

We used cell surface affinity matrix technology (IL-4/IFN-γ secretion assay; Miltenyi Biotec, Bergisch Gladbach, Germany) to identify cells secreting IL-4 or IFN-γ (35). In brief, α-GC-stimulated line cells on day 20 (1×10^6) were treated with phorbol myristate acetate (25 ng/ml) and ionomycin (1 μg/ml) for 4 h. Cytokine catch reagents (anti-IL-4 or anti-IFN-γ mAb conjugated to cell surface-specific mAb) were added and incubated at 37°C for 45 min. After washing, the cells were labeled with IL-4/IFN-γ detection mAb–PE, together with anti-Vα24–FITC for 10 min on ice. After washing, we measured the proportion of $V_\alpha24^+$ T cells producing IL-4 or IFN-γ by flow cytometry. We regarded the $V_\alpha24^+$ T cells as NKT cells in this setting, because virtually all $V_\alpha24^+$ cells co-expressed $V_\beta11$ on day 20 after culture.

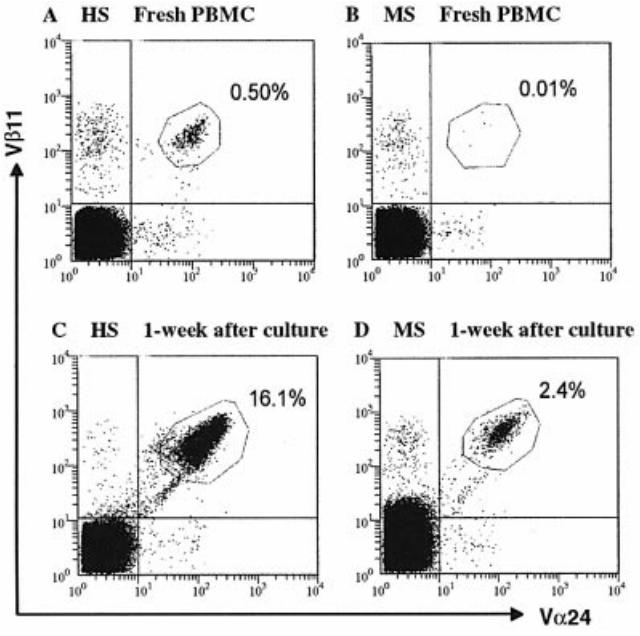

Fig. 1. Identification of $V_\alpha24^+V_\beta11^+$ NKT cells in PBMC and α-GC-stimulated culture. Representative flow fluorometric data for $V_\alpha24^+V_\beta11^+$ NKT cells are shown here. Freshly isolated PBMC (A and B) or the α-GC-stimulated cultures on day 7 (C and D) were stained and analyzed as described in Methods. The percentage value represents the frequency of the $V_\alpha24^+V_\beta11^+$ NKT cells among total lymphocytes. We routinely acquired at least 100,000 cells from each PBMC sample, among which ~70% were found to represent lymphocytes. Staining with irrelevant Ig isotype-matched antibodies showed that the background count was null or 1 dot per 100,000 counts in the NKT-gated area.

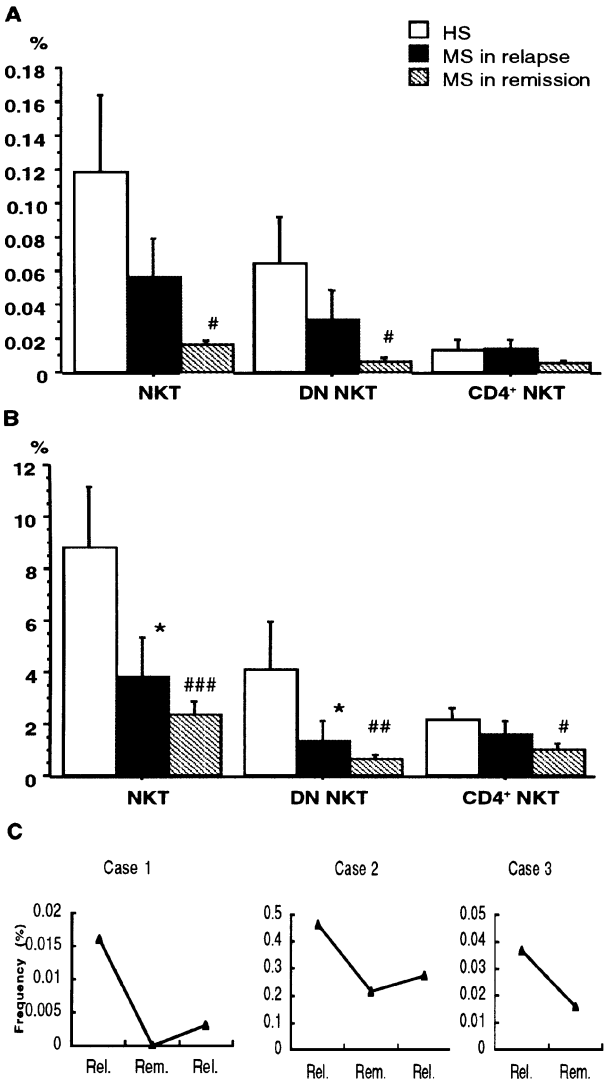

Fig. 2. The frequency of the total, DN and CD4+ NKT cells in MS and HS. (A) Freshly isolated PBMC. We obtained the PBMC samples from 15 HS, 12 MS-rel and 27 MS-rem. They were stained with the combination of anti-CD4–PE–Texas Red, anti-CD8–PE–cyanin 5.1, anti-$V_\alpha24$–FITC and anti-$V_\beta11$–PE mAb. Here we show the frequency (%) of the $V_\alpha24^+V_\beta11^+$ cells (NKT), $CD4^-CD8^-V_\alpha24^+V_\beta11^+$ cells (DN NKT) and $CD4^+CD8^-V_\alpha24^+V_\beta11^+$ cells ($CD4^+$ NKT) among total lymphocytes in each subject. There was a significant reduction in the total NKT and DN NKT in the lymphocytes from MS-rem compared with from HS ($^{\#}P < 0.01$). However, the difference between other pairs did not reach statistical significance. Error bars represent SEM. (B) α-GC-stimulated cultures. We stimulated the PBMC from HS ($n = 15$), MS-rel ($n = 12$) and MS-rem ($n = 27$) with α-GC, and cultured the cells in the presence of IL-2 and IL-7. On day 7 after stimulation, we evaluated the frequency of total NKT, DN NKT and CD4+ NKT cells in the NKT cell-enriched cultures. Indicated are the values showing a significant reduction as compared to those from HS: $^*P < 0.05$, $^{\#}P < 0.01$, $^{\#\#}P < 0.001$ or $^{\#\#\#}P < 0.0001$. (C) Follow-up of three patients regarding the NKT cell frequency. We were able to study both remission (Rem.) and relapse (Rel.) samples in these three patients (Case 1–3). They were followed over 1 year. The value shows the frequency of the total NKT cells among total lymphocytes.

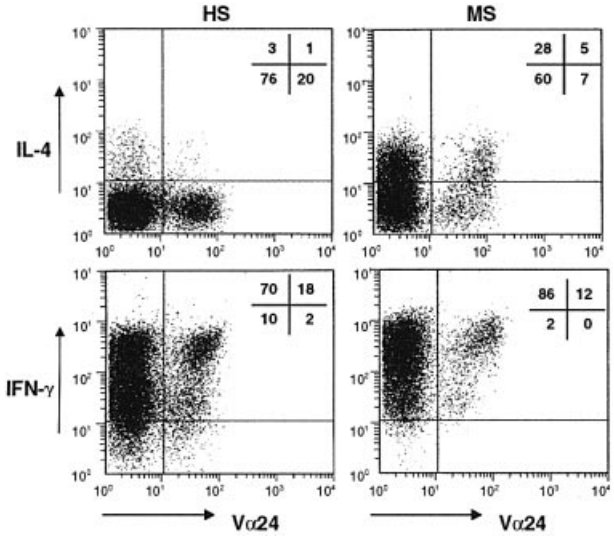

Fig. 3. Cytokine-secreting cells in α-GC-stimulated line cells on day 20. We used α-GC-stimulated line cells on day 20 for cytokine secretion assay with the cell-surface affinity matrix technique. One million of the line cells from HS or MS-rem were stimulated with phorbol myristate acetate (25 ng/ml) and ionomycin (1 μg/ml) for 4 h. They were reacted with cytokine catching reagents and then with IL-4/IFN-γ detection antibody together with anti-Vα24–FITC mAb. We conducted the analysis for HS (n = 10) and MS-rem (n = 11) (see Table 1). Here we show representative results from an HS and an MS-rem. Given values show the percentage of cells present in each quadrant among gated lymphocytes. Note that only one of 21 (0.5%) of NKT cells from the HS is IL-4+, whereas five of 12 (41.7%) from the MS-rem are judged as secreting IL-4.

Isolation of NKT cell subsets for cytokine production analysis

On days 20–30 after stimulation with α-GC, we isolated the total NKT cells ($V_\alpha24^+V_\beta11^+$) from the line cells using an Epics ALTRA cell sorter (Coulter). These cells (95–99% in purity) were further sorted into $CD4^+$ and $CD4^-$ populations. The purified NKT line cell populations were seeded onto 96-well flat-bottom plates (5×10^4/well) and were stimulated with 0.125 μl/well of Dynabeads CD3/CD28 T Cell Expander in the presence of human rIL-2 (40 U/ml). Two days later, we assayed the concentrations of IL-4 and IFN-γ in the supernatants by a sandwich ELISA (BD PharMingen, San Diego, CA).

Cell proliferation assay

We determined the proliferative responses of the sorted NKT cell populations to α-GC in selected experiments. In brief, we isolated $CD14^+$ monocytes [antigen-presenting cells (APC)] from autologous PBMC using a magnetic cell sorter (Miltenyi Biotec) and irradiated the cells with X-rays (50 Gy). The APC were seeded onto 96-well flat-bottom plates (4×10^4 cells/well) and incubated overnight with graded doses of α-GC (0–100 ng/ml). After removing the free α-GC by washing with PBS, we added the sorted NKT line cells ($1–4 \times 10^4$ cells/well) onto the APC and cultured them for 3 days. We measured the cell uptake of [^{3}H]thymidine (ICN, Irvine, CA) during the last 16 h of culture with a Beta-1205 counter (Pharmacia, Uppsala, Sweden).

Statistical analysis

The Mann–Whitney U-test was used for analyses of cell frequencies and of the cytokine data. For comparison of the anti-$V_\alpha24$/anti-$V_\beta11$ and α-GC-loaded CD1d tetramer staining, we evaluated the data with Spearson's correlation coefficient and regression analysis.

Results

DN NKT, but not CD4+ NKT, cell numbers are significantly reduced during remission of MS

Using the RT-PCR SSCP technique, we previously demonstrated a reduced mRNA expression of the invariant $V_\alpha24J_\alpha Q$ TCR for the CD1d-restricted NKT cells in the peripheral blood of individuals with MS, particularly during remission (23). Here we conducted a more quantitative assessment of the NKT cells with four-color analysis for expression of $V_\alpha24$, $V_\beta11$, CD4 and CD8. In accordance with the data obtained in other laboratories (32,34,36), the mean frequency of total NKT cells ($V_\alpha24^+V_\beta11^+$) was 0.118% in peripheral lymphocytes isolated from HS (Fig. 1A). Consistent with our previous report (23), there was a significant reduction of total NKT cells in the patients with MS as compared to HS (NKT in Fig. 2A) and the reduction was more pronounced in remission than during relapse [–86% ($P < 0.01$) versus –53% ($P = 0.196$)]. In addition, follow-up of three patients (Fig. 2C) confirmed the occurrence of a reduction of NKT cells in remission rather than during relapse in the same patients. Next we analyzed the frequencies of DN or $CD4^+$ $V_\alpha24^+V_\beta11^+$ cells. We observed that the DN subset was remarkably reduced in MS [–89%; HS versus MS-rem ($P < 0.01$)] (Fig. 2A). In contrast, the frequency of $CD4^+$NKT cells did not differ significantly between HS and MS ($P = 0.18$ for MS-rem versus HS) (Fig. 2A). The $V_\alpha24^+V_\beta11^+$ cells comprised a small number of $CD4^-CD8^+$ T cells expressing CD8αα homodimers. Of interest, this subset was significantly reduced in MS, as seen with the DN subset (data not shown).

NKT cells from MS-rem would normally expand in response to α-GC

The reduction of NKT cells might possibly arise from a dysfunction of this subset in response to their natural ligands. So we examined the responsiveness of peripheral NKT cells to their glycolipid ligand α-GC. After examining the frequency of NKT cells in fresh PBMC samples from MS or HS, we stimulated the PBMC with α-GC in the presence of IL-2 and IL-7. As expected, the percentage of NKT cells among lymphocyte populations had remarkably increased after 1 week of culture (Fig. 1C and D). Although NKT cells derived from patients with collagen diseases showed a reduced responsiveness to α-GC (29), NKT cells from the MS patients and those from HS did not clearly differ in the proliferative response to α-GC. This was reflected in the observation that the hierarchy of the NKT cell frequencies among groups of subjects before culture (Fig. 2A) has not apparently changed after culture (Fig. 2B). The frequencies of total NKT cells as well as of DN NKT cells after 1 week of culture were significantly reduced in MS-rem compared with HS [–89% for total NKT ($P < 0.0001$) and –84% for DN NKT

Table 1.

	NKT frequency[a]	IL-4 production[b]		IFN-γ production[b]	
		NKT	Conventional T	NKT	Conventional T
HS (*n*=10)	27.9±6.6	13.3±3.8	9.4±3.0	92.9±2.6	90.5±3.0
MS-rem (*n*=11)	6.9±1.6	20.0±5.4	17.0±4.7	95.0±1.8	92.1±3.0

[a]The percentage of total NKT cells in the line cells on day 20 after stimulation with α-GC.
[b]Using cell surface affinity matrix analysis, we identified the NKT or conventional T cells that produced IL-4 and IFN-γ after phorbol myristate acetate/ionomycin stimulation. The given values for NKT represent the mean±SEM of the percentage of the NKT cells secreting IL-4 or IFN-γ among total NKT cells, whereas as the values for conventional T cells represent the corresponding percentage values for conventional T cells.

($P < 0.001$)] (Fig. 2B). The frequency of CD4+ NKT cells was also reduced ($P < 0.01$), but the extent of reduction was less pronounced (–53%). As such, the consistent reduction of DN NKT cells in MS-rem and the relative persistence of CD4+ NKT cells was confirmed in both fresh PBMC and cultured cells on day 7.

T cells as well as NKT cells from MS-rem would become polarized towards T_h2 when NKT cells are stimulated with α-GC

Using the cytokine secretion assay, we found that the α-GC-stimulated NKT cells spontaneously produced IFN-γ in the first week of culture (data not shown). However, while maintained in the presence of IL-2, IL-7 and IL-15, the number of IFN-γ-secreting cells gradually reduced and was negligible in the third week. Because the cells spontaneously producing IFN-γ appeared to be inappropriate for functional assessment, we decided to conduct cytokine assays by using the α-GC-stimulated cell lines maintained in culture for 20–30 days. First, we used the cytokine secretion assay to evaluate the cytokine profile of conventional T cells as well as NKT cells in the α-GC-stimulated cell lines generated from MS-rem and from HS. Using the line cells on day 20, we examined the percentage of the cells secreting IFN-γ or IL-4 among total NKT cells and conventional T cells (Fig. 3 and Table 1). We first confirmed that the frequency of NKT cells is also reduced in the line cells from MS-rem used for analysis compared with those from HS (Table 1). HS-derived and MS-rem-derived lines did not differ significantly regarding the frequency of IFN-γ-secreting cells. In fact, >90% of the NKT cells and conventional T cells were IFN-γ+ in both HS- and MS-derived lines. However, the frequency of IL-4-secreting cells in NKT cells was increased in the lines from MS-rem compared with those from HS (+50.4%; Table 1). Although the difference was not statistically significant, a remarkable increase of IL-4-secreting NKT cells was seen in a few cases such as the one shown in Fig. 3. This result may indicate that NKT cells show a trend for T_h2 bias in MS-rem compared with HS. More interestingly, the frequency of IL-4-secreting cells in the conventional T cells was also elevated in the MS-rem lines compared with the HS lines (+80.9%). These results do not only indicate that NKT cells may be biased for T_h2 in MS-rem, but also demonstrate that despite the reduced frequency, they might induce a T_h2 bias of conventional T cells if properly stimulated.

Cytokine profiles of NKT cells from HS reveal a predominant production of IL-4 by CD4+ NKT cells

The cytokine secretion assay is a reliable method to evaluate the frequency of the cells secreting cytokine of interest, but it is not suitable for determining the amount of the cytokine produced by a cell population of interest. To examine the amount of cytokines produced by CD4+ or CD4– NKT line cells, we sorted $V_\alpha24^+V_\beta11^+$ cells from the line cells on days 20–30, and further separated them into CD4– and CD4+ populations. (The CD4– line cells contained 3–20% of CD8+ cells.) The sorted populations (total NKT, CD4– NKT and CD4+ NKT) showed significant proliferative responses to α-GC presented by APC (data not shown). However, we stimulated each population with microbeads coated with anti-CD3/anti-CD28 for further analysis, because anti-CD3 and anti-CD28 co-stimulation has been widely used for functional assessment of T cell populations, including NKT cells (36), and because we need not prepare APC for each experiment. Using this assay, we showed that CD4+ NKT line cells from HS would produce a significantly larger amount of IL-4 than DN NKT line cells from HS (Fig. 4E) [CD4+ versus DN: 140.8 ± 27.9 versus 59.5 ± 13.1 pg/ml ($P < 0.05$)], whereas the two NKT subsets from HS produced equivalent amounts of IFN-γ (Fig. 4F) (CD4+ versus CD4–: 411.2 ± 135.2 versus 577.4 ± 195.5 pg/ml). This result was reminiscent of previous studies, indicating that human CD4+ NKT line cells are able to produce a larger amount of IL-4 than CD4– line cells (37,38).

Predominant production of IL-4 by CD4+ NKT cells is more remarkable in MS in remission

In parallel, we examined the cytokine production by NKT line cells expanded from patients with MS (Fig. 4A–D). While CD4+ NKT line cells were superior to CD4– NKT cells in the ability to produce IL-4 in HS, this tendency was more remarkable in those from MS-rem (Fig. 4A). Namely, the CD4+ NKT line cells derived from MS-rem produced ~10 times more IL-4 than did CD4– NKT line cells from MS-rem [CD4+ versus CD4–; 321.9 ± 82.7 versus 27.1 ± 8.8 pg/ml ($P < 0.025$)]. In contrast, the ability to produce IFN-γ did not significantly differ between CD4– and CD4+ NKT line cells expanded from MS-rem (Fig. 4B). To evaluate the relative dominance of CD4+ NKT line cells in cytokine production, we calculated the CD4+/CD4– ratio (the cytokine amount produced by CD4+ NKT/that produced by CD4– NKT) in each subject (Fig. 4G). This analysis has revealed that the dominance of CD4+ over

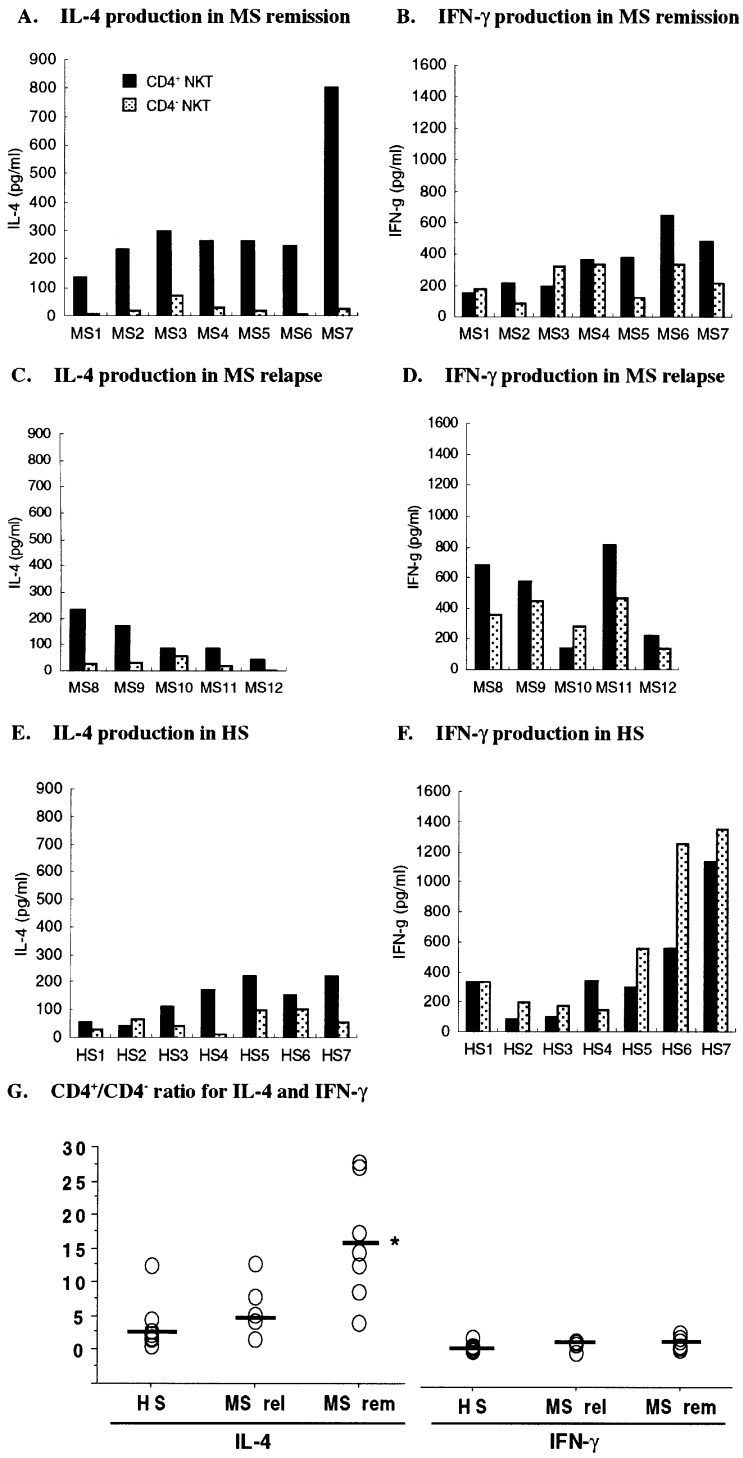

Fig. 4. Comparison of DN and CD4+ NKT line cells regarding the amounts of IL-4 and IFN-γ production. On days 20–30 after stimulation with α-GC, we sorted DN (CD4–) and CD4+ populations from the α-GC-stimulated line cells from seven MS-rem (MS1–MS7), five MS-rel (MS8–MS12) or seven HS (HS1–HS7). We stimulated the cells with microbeads coated with anti-CD3 mAb/anti-CD28 mAb *in vitro*. Two days later, we collected the supernatant, and measured the amount of IFN-γ and IL-4 by ELISA in duplicate or triplicate wells. (A) IL-4 detected in the supernatant of CD4+ or CD4– NKT line cells from MS-rem. (B) IFN-γ detected in the supernatant of CD4+ or CD4– NKT line cells from MS-rem. (C) IL-4 detected in the supernatant of CD4+ or CD4– NKT line cells from MS-rel. (D) IFN-γ detected in the supernatant of CD4+ or CD4– NKT line cells from MS-rel. (E) IL-4 detected in the supernatant of CD4+ or CD4– NKT line cells from HS. (F) IFN-γ detected in the supernatant of CD4+ or CD4– NKT line cells from HS. (G) CD4+/CD4– ratio for IL-4 and IFN-γ. In each subject (seven HS, seven MS-rem and five MS-rel), we divided the cytokine value for the CD4+ NKT line cells by that for CD4– NKT cells to evaluate the dominance of CD4+ over CD4– NKT cells. The CD4+/CD4– ratio for IL-4 was significantly elevated in MS-rem compared with HS or MS-rel (*$P < 0.01$), whereas the ratio for IFN-γ was not significantly different.

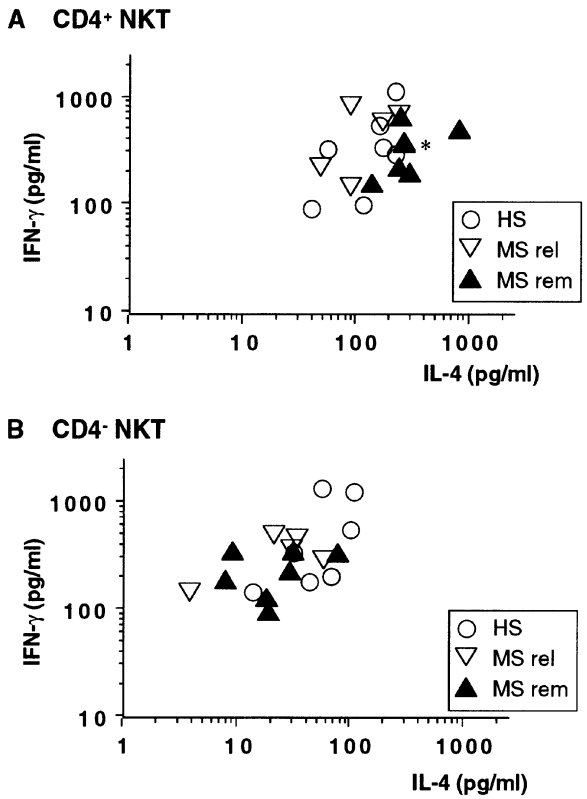

Fig. 5. Comparison of MS and HS in the cytokine profile of NKT cells. Here we plot the NKT line cell samples from each subject (seven HS, five MS-rel and seven MS-rem) according to the IL-4 and IFN-γ values detected in the culture supernatant. An asterisk represents the presence of two dots at virtually the same position.

CD4⁻ NKT line cells in IL-4 production is more remarkable in MS-rem, as compared to HS ($P < 0.01$). In contrast, there was no significant dominance for CD4+ line cells in IFN-γ production in either HS or MS-rem. We also examined the cytokine profile of the CD4+ or CD4⁻ NKT line cells expanded from five MS patients during relapse (MS-rel). The profile of the patients in relapse did not differ significantly from that of HS (Fig. 4C, D and G). This indicates that the CD4+ NKT cell dominance in IL-4 production is reverted during relapse of MS.

CD4+ NKT cells exhibit a T_h2 bias in MS in remission

We also evaluated the T_h1/T_h2 balance of the individual NKT line samples by plotting each one according to the levels of IL-4 and IFN-γ produced in the supernatant (Fig. 5). This analysis revealed that CD4⁻ and CD4+ NKT line cells from MS-rem are polarized in opposing directions with regard to IL-4 production. Namely, CD4+ NKT cells from MS-rem produced a larger amount of IL-4 than from HS, whereas CD4⁻ NKT cells from MS-rem produced a lower amount of IL-4 than from HS. To clarify if the opposing changes in IL-4 production are associated with an altered T_h1/T_h2 balance, we also calculated the IL-4/IFN-γ ratio for each sample (Fig. 6). We found that the IL-4/IFN-γ ratio for CD4+ NKT line cells is significantly increased in MS-rem, compared with HS ($P < 0.05$), confirming that CD4+ NKT line cells are T_h2-biased in MS-rem. In contrast to our

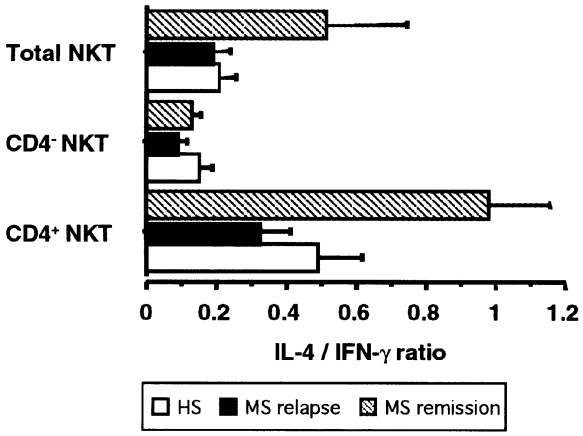

Fig. 6. Comparison of the IL-4/IFN-γ ratios in total, CD4⁻ and CD4+ NKT cells. We determined the IL-4/IFN-γ ratio for each NKT line cell sample to evaluate the possible occurrence of functional bias. The data represent mean ± SEM of seven samples from HS and MS-rem, and of five samples from MS-rel.

expectations, there was no T_h1 or T_h2 bias in CD4⁻ NKT line cells from MS-rem. This appeared to reflect that the CD4⁻ NKT cells from MS-rem were defective not only in IL-4, but also in IFN-γ production. Reflecting the T_h2 bias of CD4+ NKT line cells and a non-bias of CD4⁻ NKT line cells, the total NKT line cells showed a tendency for a T_h2 bias (Fig. 6). The CD4+ NKT samples derived from MS-rel tended to overlap with HS in the IL-4/IFN-γ plot, whereas CD4⁻ NKT samples from MS-rel overlapped with MS-rem (Fig. 5). Hence, the NKT samples from MS-rel were not biased for T_h1 or T_h2 (Fig. 6).

Comparison of CD1d tetramer analysis with anti-$V_\alpha24$/anti-$V_\beta11$ staining reveals a good correlation in two measurements

Although we have used anti-$V_\alpha24$/anti-$V_\beta11$ staining to evaluate the CD1d-restricted NKT cells in the present study, we were curious to know how reliable $V_\alpha24/V_\beta11$ co-expression is as a marker for the NKT cells. We additionally examined PBMC from seven HS and seven MS-rem, using a combination of α-GC-loaded CD1d tetramer (α-GC-tetramer) (31) and anti-$V_\beta11$ mAb. As shown in Fig. 7, the α-GC-tetramer was able to identify the small-sized population thought to be NKT cells. Moreover, the frequency of α-GC-tetramer+$V_\beta11^+$ cells was found to correlate well with that of $V_\alpha24^+V_\beta11^+$ cells, with a correlation coefficient of 0.990 for HS ($P < 0.0001$) and 0.998 for MS-rem ($P < 0.0001$). These data support the specificity of anti-$V_\alpha24$/anti-$V_\beta11$ staining for NKT cells.

Discussion

Although a reduction of CD1d-restricted NKT cells has been demonstrated in a number of disease conditions, including systemic sclerosis and MS (23,26–30), very little was known about the functional status of the NKT cells in autoimmune diseases. The functional analysis has been hampered not only because of technical problems handling such a small population as NKT cells, but also because one might have postulated that a functional change of NKT cells in autoimmune diseases may be, if any, trivial, due to the reduction in

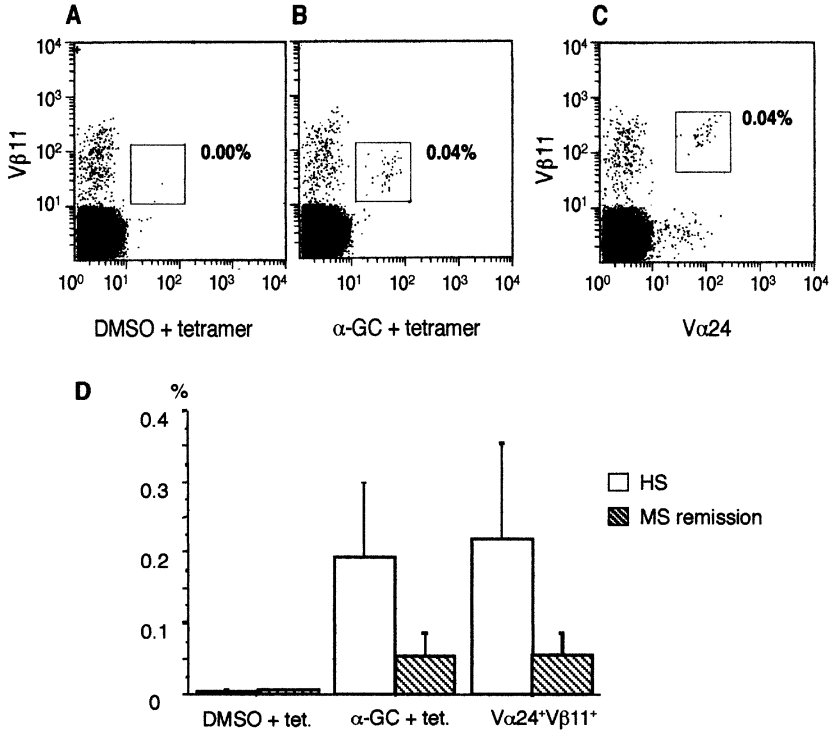

Fig. 7. Identification of NKT cells by CD1d tetramer staining. Here we stained the PBMC samples from seven HS and seven MS-rem with the combination of DMSO-treated CD1d tetramer and anti-V_β11 mAb (A), α-GC-loaded CD1d tetramer and anti-V_β11 mAb (B) or anti-V_α24 mAb and anti-V_β11 mAb (C). (D) Frequency of the NKT cells determined by tetramer/anti-V_β11 and by anti-V_α24/ anti-V_β11. The data represent mean ± SEM.

number. Moreover, despite the presence of CD4+ NKT cells, previous studies have mainly focused on DN (CD4–) NKT cells due to the relative abundance of the latter subset in number. However, CD1d tetramer-assisted studies of fresh NKT cells have recently revealed that CD4+ and CD4– NKT cells may represent distinct functional lineages (31,32); whereas CD4– NKT cells selectively produce T_h1 cytokines IFN-γ and tumor necrosis factor-α, CD4+ cells could potently produce both T_h1 and T_h2 cytokines. Moreover, the CD4– cells resemble NK cells in the ability to become primed for cytotoxic function by exposure to IL-2 and IL-12 (31), although CD4+ NKT cells do not. Here we examined the CD4– and CD4+ subsets from MS patients and HS in both the frequency and cytokine profile. Analysis of fresh PBMC and α-GC-stimulated line cells has indicated that CD4– and CD4+ NKT cells may be differentially altered in MS-rem in both of the parameters, and that remission and relapse of MS could be distinguished by the status of NKT cells. A key finding was that CD4+ NKT line cells derived from MS-rem would produce a larger amount of IL-4 compared with those from HS or from MS-rel. By evaluating the balance between IL-4 and IFN-γ, the CD4+ NKT line cells from MS-rem were found to be significantly biased for T_h2. We also showed that the T_h2 polarization of NKT cells would accompany a T_h2 bias of conventional T cells, indicating that NKT cells in MS patients have potentials to modulate T_h1-mediated autoimmune response, despite their reduction in number.

Although these results do not directly imply occurrence of the T_h2 bias of the corresponding CD4+ NKT subset *in vivo*, it indicates that the dysfunction of the regulatory network in MS could involve CD4+ NKT cells. Prior to our studies, analysis of α-GC-stimulated line cells (37,38) as well as freshly isolated NKT cells (31,32) has showed that CD4+ NKT cells are superior to CD4– NKT cells in the ability to produce IL-4 in healthy human subjects. Using RT-PCR, we also found that CD4+ (but not CD4–) NKT cells are a major source of IL-4 in healthy subjects, whereas both CD4+ and CD4– subsets produced IFN-γ (unpublished observation). Taking these results into consideration, we would conceive that our cytokine assay using NKT line cells reflect the *in vivo* cytokine profile of NKT cells in healthy subjects reasonably well, as it also showed the CD4+ cell predominance for IL-4 production in healthy subjects. Although we cannot exclude a possibility that the same assay might yield highly distorted results when applied for MS patients, we think it likely that CD4+ NKT cells may actually be T_h2-biased or ready to be polarized toward T_h2 *in vivo* in the remission phase of MS, but not during remission.

Of interest, we have recently observed that NK cells freshly isolated from MS-rem are significantly biased toward secreting IL-5 and would inhibit T_h1 cell induction *in vitro* (7). However, the ability of NK cells to produce the type 2 cytokine and to inhibit T_h1 induction was not appreciable during relapses, allowing us to speculate that IL-5-producing NK

cells may play a role in maintaining the remission of MS. It is possible that both NK cells and $CD4^+$ NKT cells may become polarized into the T_h2 direction with a common mechanism underlying the remission of MS. We could speculate that T_h1-promoting cytokines such as IL-12 may play a role in reverting the T_h2 bias in relapse, given that IL-12 is involved in the functional bias of NKT cells towards T_h1 (39,40).

With regard to the frequency of NKT cells, we showed the reduction of total NKT cells in the peripheral blood of MS with anti-$V_\alpha24$/anti-$V_\beta11$ staining and with α-GC-loaded CD1d tetramer staining. In support for our previous study (23), the numerical reduction was more remarkable in remission than relapse of MS. We also showed that it is largely restricted to the $CD4^-$ population and the $CD4^+$ NKT cells relatively spared. The relative persistence of $CD4^+$ NKT cells would contradict an argument that the T_h2 bias of $CD4^+$ NKT cells may be trivial if the cells were greatly reduced in autoimmune conditions.

In summary, this study showed that $CD4^+$ NKT line cells derived from autoimmune disease patients (MS-rem) could exhibit a T_h2 bias as compared to those from healthy individuals. In contrast, $CD4^-$ NKT line cells derived from the same patients were not functionally biased toward either T_h1 or T_h2. The present data allow us to speculate that $CD4^+$ NKT cells are uniquely biased, in order to play a role expected for this population, i.e. regulation of T_h1 autoimmunity via production of IL-4. Our results may have significant implications for the study of NKT cell biology and of immune-mediated diseases that are under control of NKT cells.

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Abbreviations

α-GC	α-galactosylceramide
APC	antigen-presenting cell
CNS	central nervous system
DN	double negative
EAE	experimental autoimmune encephalomyelitis
HS	healthy subjects
MS	multiple sclerosis
MS-rel	MS in relapse
MS-rem	MS in remission
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin

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