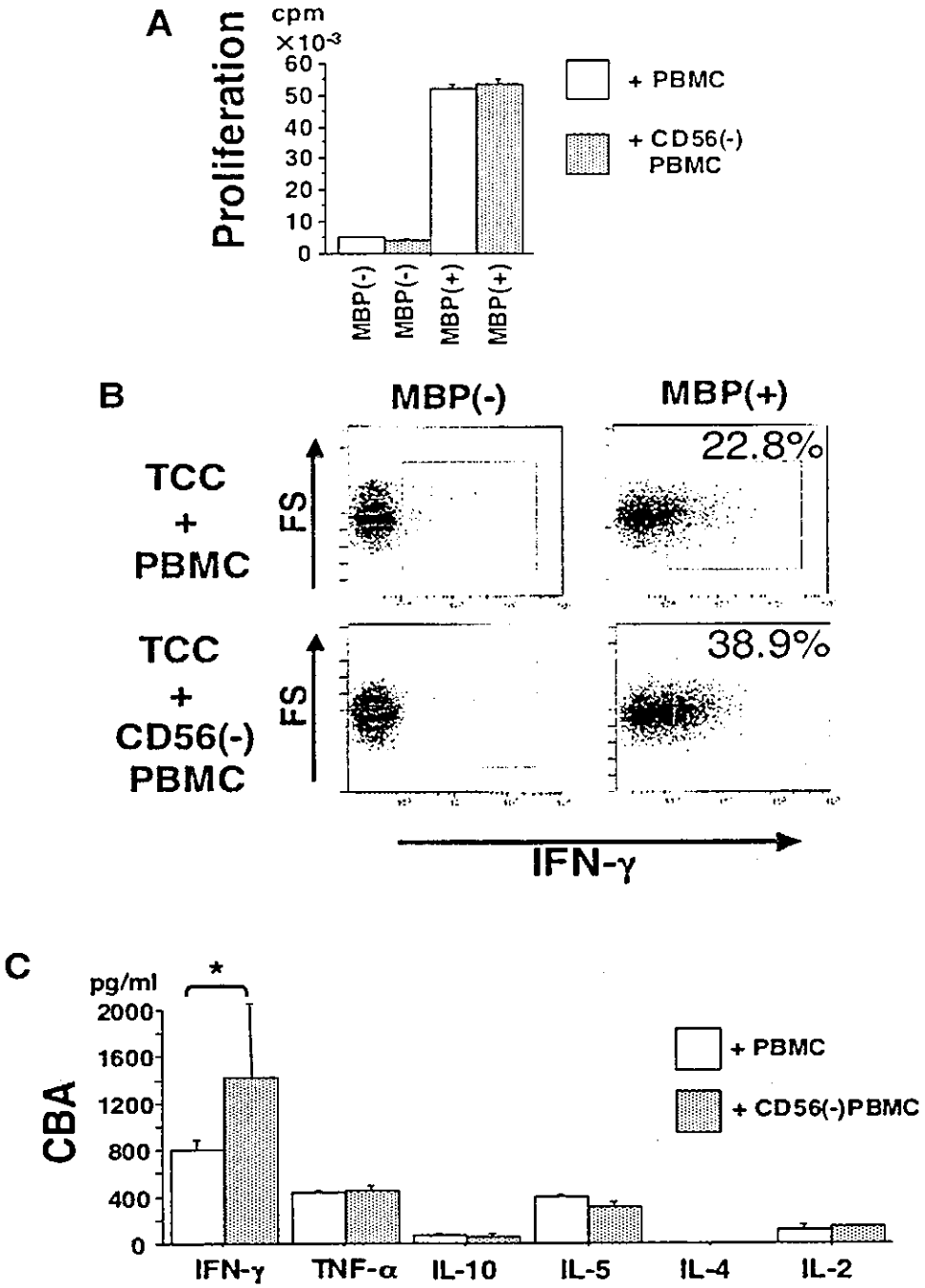


**Fig. 4** Depletion of NK cells augments the antigen-presenting potential of PBMCs from 'CD95$^+$ NK-high' multiple sclerosis. (**A**) Effect of NK-cell deletion on the proliferation of MBP-specific TCC. We established three MBP-specific TCC from a 'CD95$^+$ NK-high' patient, and evaluated the proliferative response of the clone cells to MBP (10 μg/ml) in the presence of fresh autologous PBMCs [+ PBMC] or NK-deleted PBMCs [+ CD56 (−) PBMC]. This is a representative result of three TCC, which yielded essentially the same results. Data represent mean ±SD of quadruplicate cultures. (**B**) Effect of NK cell deletion on IFN-γ secretion by the MBP-specific TCC. MBP-specific TCC were cultured with or without MBP for 8 h in the presence of autologous PBMCs (upper panels) or of the autologous PBMCs depleted for CD56$^+$ NK cells (lower panels). We then conducted the cytokine secretion assay to detect IFN-γ-positive cells. Red dots indicate IFN-γ-secreting cells among CD4$^+$CD3$^+$PI$^-$ cells; blue dots represent IFN-γ-negative CD4$^+$CD3$^+$PI$^-$ T cells. The values (%) represent the frequency of IFN-γ-secreting cells among CD4$^+$CD3$^+$PI$^-$ cells. We conducted the assay with three TCC, which yielded essentially the same results. FS = forward scatter. (**C**) Effect of NK cell depletion on cytokine release by TCC into culture medium. The TCC were stimulated with MBP for 48 h in the presence of autologous PBMCs or NK-depleted PBMCs. Then we measured the concentrations of IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2 in the supernatants, using ELISA and CBA. Both assays yielded essentially the same results, and here we show the result of a CBA assay. Data represent mean ± SD. The Mann–Whitney *U*-test was used for statistical analysis. $*P < 0.05$. We conducted the assay with three TCC, which yielded essentially the same results.

The present results show that multiple sclerosis patients in remission can be divided at least into two subgroups, 'CD95$^+$ NK-high' and 'CD95$^+$ NK-low', based on the frequency of CD95$^+$ cells among NK cells. Furthermore, our functional analysis combining NK cell deletion and stimulation with MBP has indicated that the two subgroups differ significantly with regard to the responsiveness of the MBP-specific memory T cells to MBP in the absence of NK cells. Namely, after deleting CD56$^+$ NK cells, we saw a rapid induction of IFN-γ-secreting, anti-MBP T cells in 'CD95$^+$ NK-high' multiple sclerosis, whereas such a rapid response to MBP was not seen in 'CD95$^+$ NK-low' multiple sclerosis or healthy subjects. This result is in harmony with the previous results that clonally expanded MBP-specific T cells can be detected in a majority of multiple sclerosis patients (Zhang *et al.*, 1994; Smeltz *et al.*, 1999), and indicates that patients with an increased number of the autoimmune T cells may have the 'CD95$^+$ NK-high' phenotype during remission. Thus, the frequency of CD95$^+$ NK cells correlates with the frequency of MBP-reactive memory T cells and may serve as a useful marker to evaluate the immunological status of multiple sclerosis during remission.

The role of NK cells in the regulation of MBP-specific T cells was further strengthened by the demonstration that deletion of CD16$^+$ cells also enabled detection of memory MBP-specific T cells. Because we confirmed that depletion of the CD16$^+$ cells would greatly reduce the number of NK cells but did not significantly reduce CD56$^+$CD3$^+$ NK T cells, the role of the NK T cells in the regulation was excluded.

We have previously described that the 'CD95$^+$ NK-low' phenotype could also be seen in multiple sclerosis patients during relapse. However, the 'CD95$^+$ NK-low' phenotype in MS-rel was not persistent, but the 'CD95$^+$ NK-high' phenotype could be regained in a month or so along with clinical recovery. This fact raised the possibility that 'CD95$^+$ NK-low' MS-rem may represent an active state of multiple sclerosis, contrary to our speculation. To evaluate this possibility, we examined three patients with MS-rem for the 'CD95$^+$ NK-high/low' phenotype every 4–6 weeks, and found that they maintained the 'CD95$^+$ NK-low' phenotype for longer than several months (data not shown). This is in a striking contrast to the transient appearance of the 'CD95$^+$ NK-low' phenotype during relapse. Together with the clinical observations that these patients were in a very stable condition with minimal neurological disability, we estimate the disease condition in 'CD95$^+$ NK-low' MS-rem to be truly inactive and distinct from MS-rel.

It is of note that IFN-γ-secreting T cells could be identified as early as 8 h after stimulation with MBP in the absence of NK cells. This result implies that the NK cells should interact with the autoimmune T cells shortly after antigen stimulation to regulate very early T cell response. To account for such a rapid regulation by NK cells, we speculate that the regulatory NK cells may detect the subtle change of the autoimmune T cells during the early stage of activation. At present, very little is known about the molecular basis of T cell–NK cell interaction. However, it is obvious that NK cells must interact with T cells in an antigen-non-specific fashion, as they do not express highly variable receptors like T cell antigen receptors. Our results indicate that attempts to identify the ligand and receptors involved in T cell–NK interactions are very rewarding.

It is currently speculated that activation of autoimmune T cells could occur in response to microbial proteins whose sequence has a significant homology to the self-peptide (Steinman, 2001). We predict that the increased MBP-reactive Th1 cells in the 'CD95$^+$ NK-high' patients will most likely respond to microbial peptides mimicking MBP from time to time. However, counter-regulatory NK cells would maintain the clinical silence by actively suppressing activation of the autoimmune T cells that might lead to destructive CNS inflammation (Fig. 5). We then imagine that the clinical silence in the 'CD95$^+$ NK-high' patients could readily be disrupted when NK cells are numerically or functionally altered by exogenous or endogenous factors independent of multiple sclerosis (Wu *et al.*, 2000). In contrast, the clinical remission in 'CD95$^+$ NK-low' multiple sclerosis appears to be stable, as they are expected to possess much lower numbers of MBP-specific memory T cells, which does not necessitate the active

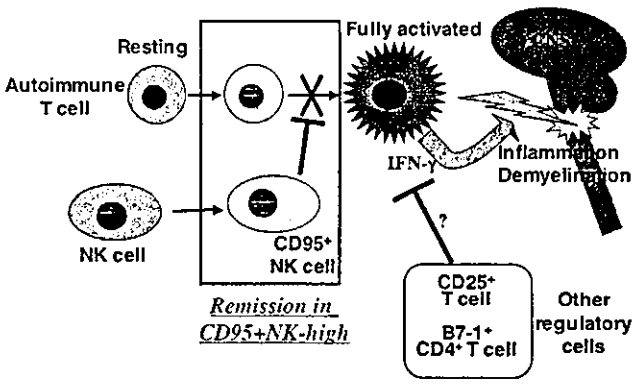


Fig. 5 The role of NK cells in 'CD95$^+$ NK-high' multiple sclerosis. As described in the text, the 'CD95$^+$ NK-high' patients are characterized by a concurrent increase of memory autoimmune T cells and CD95$^+$ NK cells. In the sense that memory autoimmune T cells cannot be detected in other patients in remission ('CD95$^+$ NK-low') even after NK cell depletion, we describe the immunological status of the 'CD95$^+$ NK-high' as a 'smouldering' state rather than 'remission'. Given that T cell recognition is much more promiscuous than previously anticipated, we imagine that autoimmune T cells in the 'CD95$^+$ NK-high' patients would respond to exogenous self-mimicking peptides from time to time. However, our results indicate that the CD95$^+$ NK cells could detect the early sign of T cell activation and then interact with autoimmune T cells to prohibit their full activation. Once this delicate control by NK cells is disrupted, the autoimmune T cells could be fully activated in response to the self-mimicking peptides. The fully activated T cells may be controlled by other regulatory cells such as CD4$^+$CD25$^+$ T cells (Sakaguchi *et al.*, 2001) or B7-1$^+$CD4$^+$ T cells (Kipp *et al.*, 2000). However, it is difficult to predict how efficiently the regulatory T cells may control the activated autoimmune T cells in individual cases.

engagement of regulatory NK cells. If these premises hold true, we may consider that the 'CD95$^+$ NK-high' patients are at a greater risk than 'CD95$^+$ NK-low' of developing relapses when exposed to potentially dangerous microbes that have cross-reactive epitopes. To describe the immunological status in 'CD95$^+$ NK-high', which seems to be more active than the 'CD95$^+$ NK-low', it might be appropriate to use the term 'smouldering' state rather than 'remission'.

After determining the presence of the 'CD95$^+$ NK-high' and 'CD95$^+$ NK-low' phenotypes in the patients with MS-rem, an important question might be whether the 'CD95$^+$ NK-high/-low' phenotype correlates with some clinical parameters or disease course. We speculated that 'CD95$^+$ NK-low' might be clinically less active than 'CD95$^+$ NK-high', when evaluated retrospectively. However, it might take time and would require a large number of patients to verify this postulate, taking the heterogeneity and chronic nature of the illness into consideration. Furthermore, it is of note that the 'CD95$^+$ NK-high' or '-low' phenotype appears to be interchangeable. For example, two of the patients who were examined for the memory T cell frequency showed the 'CD95$^+$ NK-low' phenotype in the first examination, but were found to have the 'CD95$^+$ NK-high' phenotype when examined 1 year later (Table 1). The phenotype switch in these patients was associated with an increase in the frequency of MBP-reactive memory T cells. We speculate that activity of multiple sclerosis may have been increased in these patients during the 1-year interval, although it is too early to draw any conclusions from the analysis of two patients.

Conversely, we have recently seen an opposing phenotype switch (from the 'CD95$^+$ NK-high' to 'CD95$^+$ NK-low') in two other patients. The frequency of CD95$^+$ cells among NK cells was >46.0% in both cases in the initial examinations, but the latest test showed normal values (27.4% and 10.0%). Although the patients appeared to be in the state of remission at the last examination, they developed serious signs of acute exacerbation 2 days later. As stated above, a transient switch from 'CD95$^+$ NK-high' to 'CD95$^+$ NK-low' could occur during relapse. Therefore, we speculate that the phenotype switch from 'high' to 'low' may be triggered by the very early events leading to clinical relapse. However, it is also possible that the reduction of the CD95$^+$ NK cells might have been triggered by multiple sclerosis-independent factors, such as infection or stress, and that this led to the occurrence of the relapse in these patients. This speculation is supported by the fact that a number of physiological conditions can alter NK cell number and/or function, and that CD95$^+$ NK cells tend do die more rapidly in culture than CD95$^-$ NK cells (our unpublished data). In future, it will be worthwhile to examine more systematically whether the phenotype switch may be the earliest marker to detect occurrence of relapse.

As Japanese neurologists have traditionally stressed that multiple sclerosis in Japan might be quite unique in immunopathology, it is theoretically possible that the regulatory function of CD95$^+$ NK cells reflects the uniqueness of Japanese multiple sclerosis and that the T cell–NK cell interaction is not operative in Caucasian multiple sclerosis. However, recent studies suggest that the frequency of pure optic-spinal form of multiple sclerosis linked with Japanese patients (Misu *et al.*, 2002) is drastically declining, possibly due to change in life-style or environmental factors in Japan (Yamamura, 2002; Houzen *et al.*, 2003). Reflecting this fact, the patients randomly recruited in this study did not have optic-spinal multiple sclerosis, and all had brain lesions similar to those found in Western multiple sclerosis. We therefore speculate that our experimental results will be reproduced in Caucasian patients in the future.

In summary, we have revealed that multiple sclerosis patients in remission have either 'CD95$^+$ NK-high' or 'CD95$^+$ NK-low' phenotype, and that 'CD95$^+$ NK-high' patients have a higher frequency of memory autoimmune T cells and have more active multiple sclerosis than 'CD95$^+$ NK-low' patients. Our *ex vivo* assay has demonstrated that 'CD95$^+$ NK-high' patients possess NK cells that actively inhibit activation of memory autoimmune T cells. In the sense that clinical silence depends on the functional regulatory NK cells, the condition of 'CD95$^+$ NK-high' is thought to be so unstable, as could be expressed by the term 'smouldering'. As such, evaluation of the NK cell functions and phenotypes in multiple sclerosis gives us a new insight into the autoimmune pathogenesis of multiple sclerosis, encouraging further efforts to clarify the NK cell–T cell interactions.

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

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*Keywords*: α-galactosylceramide, CD4+ NKT cell, immunoregulation, multiple sclerosis, $T_h2$ bias

## 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 $V_\beta$ 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 $2 \times 10^6$/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 ($1 \times 10^6$) 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.

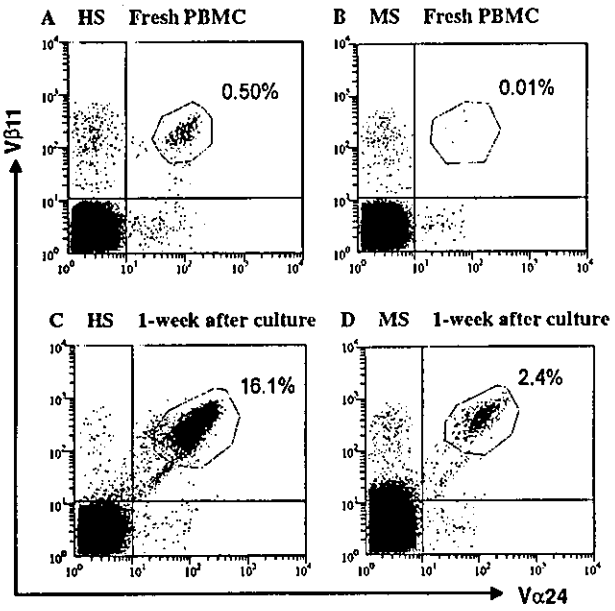


**Fig. 1.** Identification of $V_\alpha24^+V_\beta11^+$ NKT cells in PBMC and α-GC-stimulated culture. Representative flow fluorometric data for $V_\alpha24^+V_\beta11^+$ NKT cells are shown here. Freshly isolated PBMC (A and B) or the α-GC-stimulated cultures on day 7 (C and D) were stained and analyzed 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.

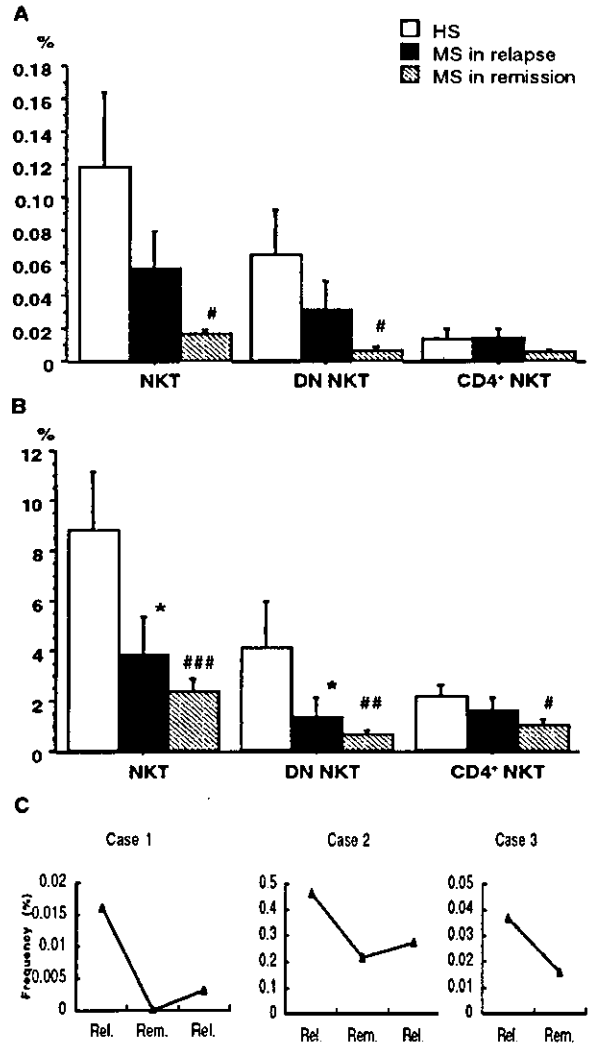


**Fig. 2.** The frequency of the total, DN and CD4+ NKT cells in MS and HS. (A) Freshly isolated PBMC. We obtained the PBMC samples from 15 HS, 12 MS-rel and 27 MS-rem. They were stained with the combination of anti-CD4–PE–Texas Red, anti-CD8–PE–cyanin 5.1, anti-$V_\alpha24$–FITC and anti-$V_\beta11$–PE 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.

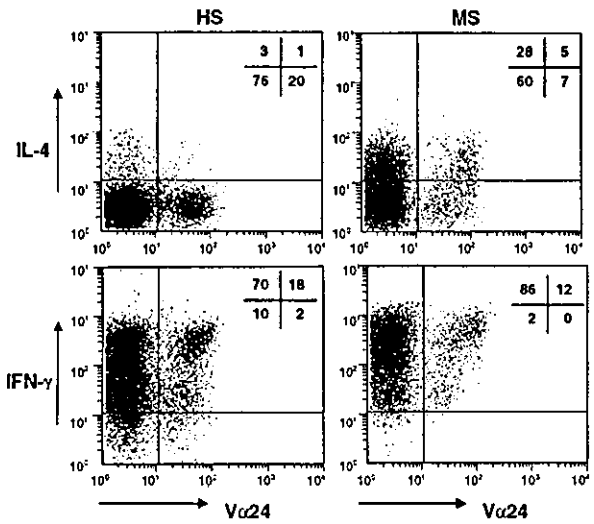


**Fig. 3.** Cytokine-secreting cells in α-GC-stimulated line cells on day 20. We used α-GC-stimulated line cells on day 20 for cytokine secretion assay with the cell-surface affinity matrix technique. One million of the line cells from HS or MS-rem were stimulated with phorbol myristate acetate (25 ng/ml) 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 × $10^4$ 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

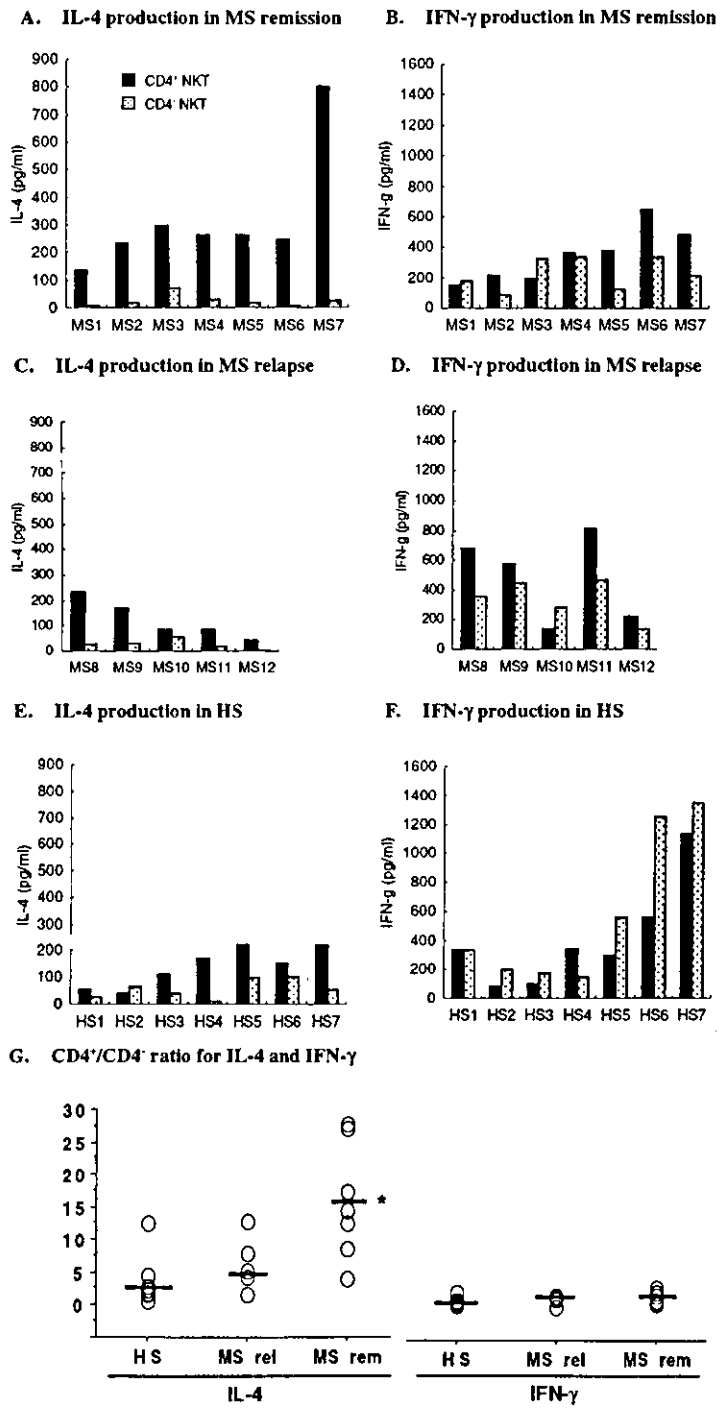


**Fig. 4.** Comparison of DN and CD4+ NKT line cells regarding the amounts of IL-4 and IFN-γ production. On days 20–30 after stimulation with α-GC, we sorted DN (CD4-) and CD4+ populations from the α-GC-stimulated line cells from seven MS-rem (MS1–MS7), five MS-rel (MS8–MS12) or seven HS (HS1–HS7). We 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.

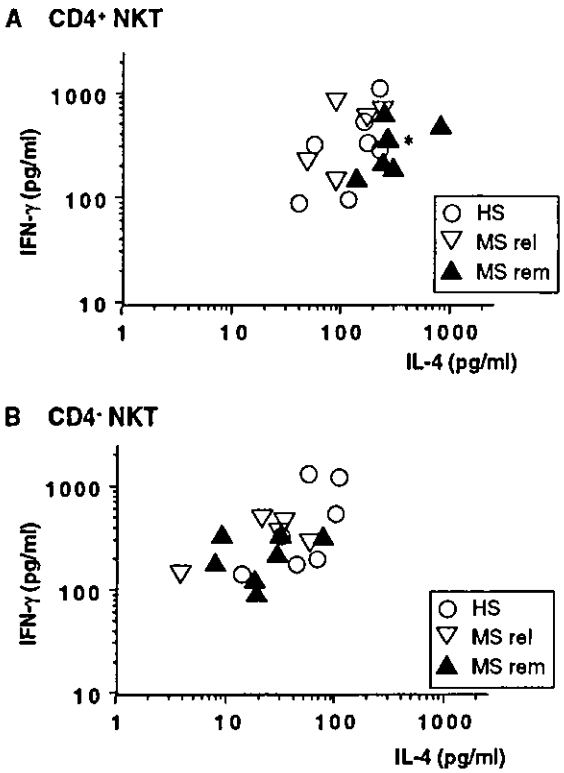


**Fig. 5.** Comparison of MS and HS in the cytokine profile of NKT cells. Here we plot the NKT line cell samples from each subject (seven HS, five MS-rel and seven MS-rem) according to the IL-4 and IFN-γ values detected in the culture supernatant. An asterisk represents the presence of two dots at virtually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

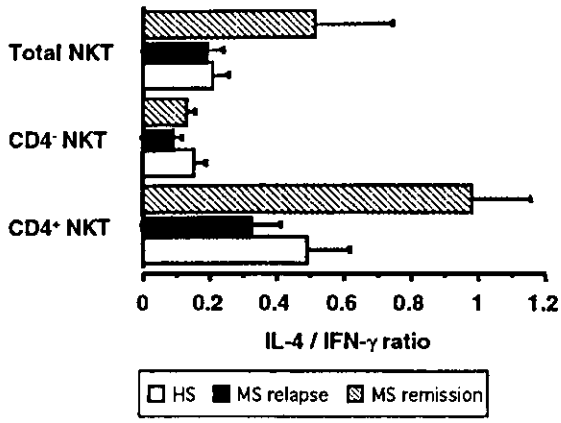


**Fig. 6.** Comparison of the IL-4/IFN-γ ratios in total, CD4⁻ and CD4⁺ NKT cells. We determined the IL-4/IFN-γ ratio for each NKT line cell sample to evaluate the possible occurrence of functional bias. The data represent mean ± SEM of seven samples from HS and MS-rem, and of five samples from MS-rel.

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

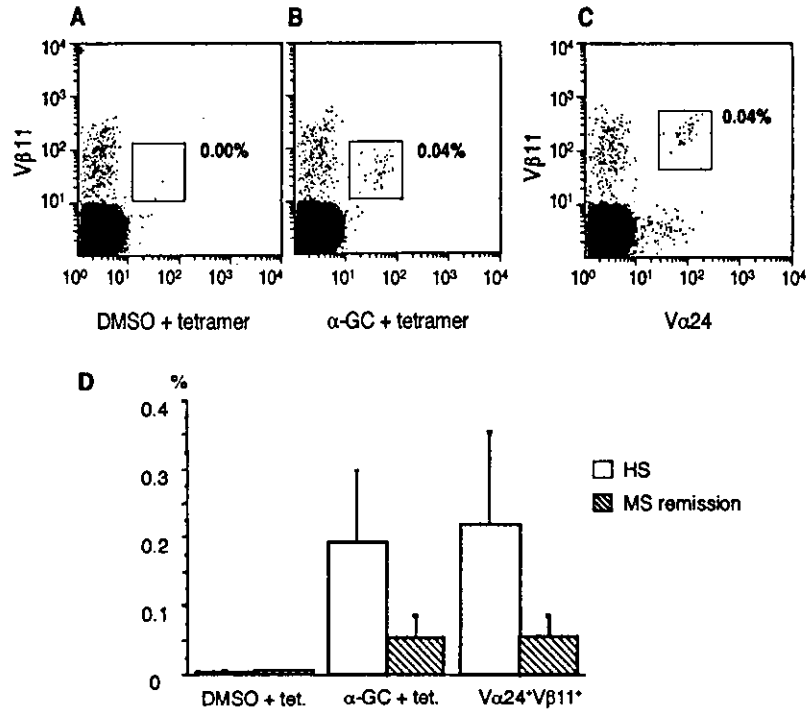


**Fig. 7.** Identification of NKT cells by CD1d tetramer staining. Here we stained the PBMC samples from seven HS and seven MS-rem with the combination of DMSO-treated CD1d tetramer and anti-$V_\beta$11 mAb (A), α-GC-loaded CD1d tetramer and anti-$V_\beta$11 mAb (B) or anti-$V_\alpha$24 mAb and anti-$V_\beta$11 mAb (C). (D) Frequency of the NKT cells determined by tetramer/anti-$V_\beta$11 and by anti-$V_\alpha$24/ anti-$V_\beta$11. 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.

## Acknowledgements

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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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# Neuropeptide Y (NPY) Suppresses Experimental Autoimmune Encephalomyelitis: $NPY_1$ Receptor-Specific Inhibition of Autoreactive Th1 Responses In Vivo[1]

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**Prior studies have revealed that the sympathetic nervous system regulates the clinical and pathological manifestations of experimental autoimmune encephalomyelitis (EAE), an autoimmune disease model mediated by Th1 T cells. Although the regulatory role of catecholamines has been indicated in the previous works, it remained possible that other sympathetic neurotransmitters like neuropeptide Y (NPY) may also be involved in the regulation of EAE. Here we examined the effect of NPY and NPY receptor subtype-specific compounds on EAE, actively induced with myelin oligodendrocyte glycoprotein 35–55 in C57BL/6 mice. Our results revealed that exogenous NPY as well as NPY $Y_1$ receptor agonists significantly inhibited the induction of EAE, whereas a $Y_5$ receptor agonist or a combined treatment of NPY with a $Y_1$ receptor antagonist did not inhibit signs of EAE. These results indicate that the suppression of EAE by NPY is mediated via $Y_1$ receptors. Furthermore, treatment with the $Y_1$ receptor antagonist induced a significantly earlier onset of EAE, indicating a protective role of endogenous NPY in the induction phase of EAE. We also revealed a significant inhibition of myelin oligodendrocyte glycoprotein 35–55-specific Th1 response as well as a Th2 bias of the autoimmune T cells in mice treated with the $Y_1$ receptor agonist. Ex vivo analysis further demonstrated that autoimmune T cells are directly affected by NPY via $Y_1$ receptors. Taken together, we conclude that NPY is a potent immunomodulator involved in the regulation of the Th1-mediated autoimmune disease EAE.** *The Journal of Immunology,* **2003, 171: 3451–3458.**

Experimental autoimmune encephalomyelitis (EAE)[3] is an animal autoimmune disease that can be induced with sensitization against CNS components such as myelin oligodendrocyte glycoprotein (MOG) (1, 2). Because the neurological signs of paralysis can be monitored continuously and because the pathological findings characterized by focal mononuclear cell infiltrates and demyelinating lesions resemble those found in multiple sclerosis (MS), this representative autoimmune disease model is widely used. It is established that EAE is mediated by $CD4^+$ Th1 T cells producing IFN-$\gamma$ and TNF-$\alpha$ in response to the peptide of the CNS components. In support of this consensus, a number of studies have proven that polarizing autoimmune Th1 cells toward Th2 directions (3–7) leads to suppression of the clinical and pathological manifestations of EAE. These findings indicate that human Th1-mediated diseases such as MS could also be treated or prevented with the Th2-inducing protocols effective in suppression of EAE. Thus, molecular mechanisms controlling the Th1/Th2 balance needs to be further elucidated in terms of the regulation of autoimmunity.

It is now well established that the immune system and the nervous system are connected bidirectionally (8–10). Although much remains to be investigated, several lines of evidence suggest that the sympathetic nervous system (SNS) provides a major pathway for neuroimmune interactions. Indeed, a role for catecholamines such as norepinephrine and epinephrine in SNS-mediated immunoregulation has been implicated in various conditions (11–15). Regarding the modulation of autoimmunity, it was previously demonstrated that depletion of SNS transmitters by chemical sympathectomy enhances the severity of EAE (11, 12). Because $\beta$-adrenoceptor agonists protect against EAE (13) and catecholamines modulate several immunological functions critical to the pathogenesis of EAE (14), the enhancement of EAE by chemical sympathectomy has largely been attributed to the depletion of catecholamines. However, although neuropeptide Y (NPY) is also released from SNS terminals innervating lymphatic tissues (16, 17), no previous studies have explored the possibility that depletion of other SNS transmitters such as NPY may contribute to these findings.

NPY is a 36-aa peptide. This amidated peptide is abundant in neurons and can be detected in all parts of the body. NPY regulates a variety of physiological activities, including energy balance and feeding, anxiety, neuroendocrine secretion, neuronal excitability, and vasoconstriction (18, 19). NPY exerts its pleiotropic functions through the activation of several G-protein coupled NPY receptor subtypes (18). Accumulating evidence indicates that NPY receptor subtypes mediate the differential actions of NPY (18) and that they are differentially expressed in the mammalian tissues. Whereas expression of $Y_2$ and $Y_5$ receptor is highly restricted to the CNS,

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[3] Abbreviations used in this paper: EAE: experimental autoimmune encephalomyelitis; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NPY, neuropeptide Y; PLP, proteolipid protein; SNS, sympathetic nervous system.



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$Y_4$ receptors are selectively expressed in the periphery. In contrast, $Y_1$ receptors are rather ubiquitously expressed; their presence has been reported in brain, heart, kidney, gastrointestinal tract, endothelial cells, and leukocytes (18, 19).

Of note, NPY can be found in the storage vesicles of the sympathetic nerve terminals innervating lymph nodes, spleens, and the bone marrow of various species (19). Furthermore, $Y_1$ receptors were demonstrated on rat PBMC (20, 21). These results suggested a role for NPY in neuroimmune interactions. In support of this hypothesis, two independent studies previously showed that NPY significantly modifies the cytokine profile of T helper clone cells in vitro (22, 23). Namely, Levite (23) reported NPY converts the cytokine profile of Th1 clones to a Th0 type in vitro, whereas Kawamura et al. (22) showed that NPY inhibits the IFN-γ production by Th1 clones as well as that of freshly isolated spleen T cells. However, despite the potential of NPY to induce a Th2 shift in vitro, it remains unclear whether NPY may alter the cytokine profile of Th1 cells in vivo. This prompted us to investigate a possible role of NPY in the regulation of EAE mediated by Th1 cells.

To explore the role of NPY in vivo, we immunized female C57BL/6 (B6) mice with $MOG_{35-55}$ and treated them with NPY and/or NPY receptor subtype-selective compounds every other day. Here we report that exogenous NPY significantly suppresses the clinical course of EAE and that this effect is mediated through the activation of $Y_1$ receptors expressed by T cells. Our experiments have revealed that suppression of IFN-γ production by $MOG_{35-55}$-specific Th1 cells and the concomitant Th2 bias account for the suppression of EAE.

## Materials and Methods

### *Mice and reagents*

Female B6 mice were purchased from CLEA Laboratory Animals (Tokyo, Japan), and female SJL/J mice were purchased from Charles River Japan (Tokyo, Japan). The animals were kept under specific pathogen-free conditions and were subjected to experiments at 6–10 wk of age. Rat $MOG_{35-55}$ (amino acid sequence, MEVGWYRSPFSRVVHLYRNGK) was synthesized at Chiron Technologies (Clayton, Victoria, Australia), and proteolipid protein (PLP) 139–151 (amino acid sequence, HCLGKWL GHPDKF) at Toray Research Center (Tokyo, Japan). IFA and heat-killed *Mycobacterium tuberculosis* H37Ra were obtained from Difco (Detroit, MI), and pertussis toxin was obtained from List Biological Laboratories (Campbell, CA). NPY was purchased from Sigma-Aldrich (St. Louis, MO). A $Y_1$ receptor agonist, [$F^7$,$P^{34}$]NPY, and a $Y_5$ receptor agonist, [$Ala^{31}$,$Aib^{32}$]NPY, were generated as previously described (24, 25). Another $Y_1$ receptor agonist, [D-$His^{26}$]NPY (26), was a gift from Schering (Kenilworth, NJ). Receptor specificity of these compounds was achieved by replacing certain amino acids at specific positions that are critical for the structural interaction of native NPY with different NPY receptor subtypes (for details see Table I). The $Y_1$ receptor antagonist BIBO3304, a small nonpeptide compound (27), was kindly provided by Boehringer Ingelheim (Biberach, Germany).

### *Immunization*

Active EAE was induced in B6 mice as described previously (6, 7). Briefly, the mice were challenged in the tail base with an emulsion containing 100 μg of $MOG_{35-55}$ and 500 μg of *M. tuberculosis* in IFA. Directly after the immunization and 48 h later, the mice were injected i.p. with 500 ng of pertussis toxin. SJL/J mice were immunized s.c. with an emulsion containing 100 μg of $PLP_{139-151}$ and 1000 μg of *M. tuberculosis* in IFA. They were injected with 200 ng of pertussis toxin shortly after immunization.

### *Clinical assessment*

Mice were observed daily for clinical signs of EAE. Disease severity was scored and evaluated as follows: 0 = normal; 1 = weakness of the tail and/or paralysis of the distal half of the tail; 2 = loss of tail tonicity; 3 = partial hind limb paralysis; 4 = complete hind limb paralysis; 5 = forelimb paralysis or moribund; 6 = death. Cumulative scores were calculated for an individual mouse by summing up the daily scores.

### *Application of NPY and NPY receptor subtype-specific compounds*

NPY and the receptor subtype-specific compounds were diluted in PBS. The animals were injected every second day with NPY and/or these compounds throughout the experiment, unless otherwise stated. Control mice were injected with 200 μl of PBS on alternate days. To treat mice with a combination of NPY and the $Y_1$ receptor antagonist BIBO3304 on alternate days, we injected NPY and the antagonist on the same day (NPY injection followed by BIBO3304) and gave two injections of PBS to control mice.

### *Measurement of $MOG_{35-55}$-specific IgG1 and IgG2a titers*

ELISA plates were coated with 10 μg/ml $MOG_{35-55}$ in PBS overnight at 4°C. After blocking with 3% BSA in PBS, serial dilutions of the serum from animals at day 40 after immunization, or normal mice or PBS were added to the plates. $MOG_{35-55}$-specific Abs were detected, using biotin-labeled anti-IgG1 and anti-IgG2a Abs. After adding streptavidin-peroxidase and a substrate, Ab concentrations were estimated on the basis of dilutions/OD curves.

### *$MOG_{35-55}$-specific T cell proliferation assay*

After immunization with $MOG_{35-55}$, the animals were treated every second day with the indicated compounds from day 0 to day 10 after immunization. The mice were sacrificed at day 10 and inguinal and popliteal lymph nodes (LN) were removed. Total LN cells were suspended in RPMI 1640 supplemented with $5 \times 10^{-5}$ M 2-ME, 2 mM L-glutamine, 100 U/100 mg/ml penicillin/streptomycin, and 1% syngeneic mouse serum (standard medium). We incubated the cells in 96-well round-bottom plates at $1 \times 10^6$/well for 72 h (37°C, 5% $CO_2$ atmosphere) in the presence of $MOG_{35-55}$ (1, 10, or 100 μg/ml). Incorporation of [$^3$H]thymidine (1 μCi/well) for the final 16 h of the culture was determined with a β-1205 counter (Pharmacia, Uppsala, Sweden).

To determine whether the suppressive effects of a $Y_1$ receptor agonist, [D-$His^{26}$]NPY, are due to its interaction with T cells or with APC, T cells were isolated from the LN using a standard nylon wool column procedure. The LN cells were obtained from $MOG_{35-55}$-primed mice treated with [D-$His^{26}$]NPY or PBS. They were applied to the nylon wool column and incubated for 1 h at 37°C (5% $CO_2$ atmosphere), and the T cells were harvested from the column by gently rinsing with RPMI 1640 containing 5% FCS. The LN cells that had been x-irradiated with 4000 rad were used

Table I. *Amino acid sequence of NPY and receptor subtype-specific NPY analogs*

| Peptide | Preference | Amino Acid Sequence | Alteration | Reference |
|---|---|---|---|---|
| NPY | $Y_{1-6}$ receptor | YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY | | |
| [$F^7$,$P^{34}$]NPY | $Y_1$ receptor | YPSKPD**F**PGEDAPAEDLARYYSALRHYINLITR**P**RY[a] | Amino acids in positions 7 and 34 are replaced | 24 |
| [D-$His^{26}$]NPY | $Y_1$ receptor | YPSKPDNPGEDAPAEDLARYYSALR**H**YINLITRQRY | L-His in position 26 is replaced by D-His | 26 |
| [$Ala^{31}$,$Aib^{32}$]NPY | $Y_5$ receptor | YPSKPDNPGEDAPAEDLARYYSALRHYINL**AB**RQRY | Amino acids in positions 31 and 34 are replaced by a synthetic dipeptide | 25 |

[a] Replaced amino acid residues are bold and underlined as shown.

as APC. T cells ($5 \times 10^5$/well) and APC ($5 \times 10^5$/well) were than cocultured in 96-well round-bottom plates in the presence or absence of $MOG_{35-55}$. Cytokine assay was conducted as described below for the supernatants harvested at 48 h. Cell proliferation was determined by measuring the incorporation of [$^3$H]thymidine (1 $\mu$Ci/well) in the final 16 h of 72-h cultures.

*Cytokine assay*

To evaluate the effect of $Y_1$ receptor stimulation on the cytokine secretion, LN cells from the $MOG_{35-55}$-immunized, NPY-treated mice were suspended in the standard medium and cultured in 96-well round-bottom plates at $1 \times 10^6$/well for 48 h in the presence of $MOG_{35-55}$. The concentrations of IFN-$\gamma$ and IL-4 in the supernatants were determined by using a sandwich ELISA. The assays were performed according to the protocol provided by BD PharMingen (San Diego, CA). All the reagents, including recombinant mouse cytokines and Abs, were purchased from BD PharMingen.

*Anti-CD3 stimulation of splenocytes derived from naive mice*

For the stimulation of the Ag receptor complex of T cells, 96-well round-bottom plates were coated with 1 $\mu$g/ml anti-CD3 mAb (clone 2C11) (BD PharMingen) overnight. After three washings with PBS, splenocytes ($1 \times 10^6$/well) from untreated, naive animals were added and incubated in the standard medium for 48 h in the presence of various concentrations of [D-His$^{26}$]NPY. IFN-$\gamma$ levels in the supernatants were detected with the sandwich ELISA.

*Induction of passive EAE in SJL/J mice*

At day 10 after immunization with $PLP_{139-151}$, the total spleen and draining LN cells were prepared from the mice and stimulated with the PLP peptide (30 $\mu$g/ml) in the standard medium. The cells were harvested 96 h after culture, and $1.6 \times 10^7$ of the cells were injected i.p. into each recipient that had been x-irradiated (300 rad) shortly before cell transfer. The recipient mice were further treated with pertussis toxin on the day of cell transfer and 2 days later (200 ng for each i.p. injection).

*In vitro T helper cell differentiation*

Spleen T helper cells were polarized for either Th1 or Th2 direction according to the protocol described by others (28). In brief, $CD4^+CD44^{low}$ naive T cells were purified from the spleen of young B6 mice by using the magnetic beads (Dynal, Oslo, Norway), and the cells were stimulated with anti-CD3 (2 $\mu$g/ml) and anti-CD28 (1 $\mu$g/ml) under Th1- or Th2-inducing conditions. Namely, Th1 cells were induced in the presence of mouse IL-12 (5 ng/ml) and anti-IL-4 mAb (HB188; 10 $\mu$g/ml), whereas Th2 cells were induced in the presence of mouse IL-4 (1000 U/ml), anti-IFN-$\gamma$ (HB170; 5 $\mu$g/ml), and anti-IL-12 (3 $\mu$g/ml). Three days later, the cells were fed with the fresh medium supplemented with 100 U/ml IL-2 in addition to the cytokines and Abs used in the primary stimulation. Eight days later, the cells were harvested and subjected to RNA preparation.

*RT-PCR and real time PCR*

RT-PCR was used to determine the transcription level of NPY $Y_1$ receptor in the LN cells from $MOG_{35-55}$-sensitized animals or in the nylon wool-purified spleen T cells from naive mice. Homogenized brain tissues from naive mice served as controls. Total RNA was extracted from these samples using RNABee (Tel-Test, Friendswood, TX). RNA (5 $\mu$g) was subjected to reverse transcription with the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA), and 35 cycles of PCR were conducted using *Taq*DNA polymerase and GeneAmp PCR system 9700 (Perkin-Elmer, Applied Biosystems, MA). Each cycle of PCR amplification comprised denaturation (95°C for 5 min), annealing (54°C for 30 s), and amplification (72°C for 60 s). The products of these reactions were analyzed by 2% gel electrophoresis. Primers used were as follows: $Y_1$ receptor sense, CTTCGGGGAGACCATGTGCAAACTGAATC; $Y_1$ receptor antisense, AGGAGAGTCGTGTAAGACAG; GAPDH sense, AACGACC CCTTCATTGAC; GAPDH antisense, TTCACGACATACTCAGCAC. Real time PCR was conducted by using the Light Cycler quantitative PCR system (Roche Molecular Biochemicals, Mannheim, Germany). We used a commercial kit (Light Cycler-FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals) according to the manufacturer's instructions.

*Statistical analysis*

We used the Mann-Whitney test to analyze the differences in the clinical score of treatment vs control group. Data for cytokines and proliferative responses were subjected to overall two-way ANOVA. When there was a significant difference, a Fisher post hoc test was implemented. The statistical analysis was performed using SPSS for Windows.

## Results

*NPY inhibits actively induced EAE in a dose-dependent manner*

To investigate a possible effect of NPY on actively induced EAE, we immunized female B6 mice with $MOG_{35-55}$ to actively induce EAE. The mice were injected with 0.01–50 $\mu$g/kg NPY i.p. on alternate days from the day of immunization (day 0) until the termination of the experiments. The selection of NPY dosages is based on previous studies (29). We found that the continuous, alternate day treatment with NPY inhibits the clinical severity of EAE in a dose-dependent manner (Fig. 1). The maximum disease score was significantly inhibited when the mice were treated with 50 $\mu$g/kg (but not 0.01 or 1.0 $\mu$g/kg) of NPY (Fig. 1*A* and Table II). However, the cumulative disease score was effectively suppressed at both 1 and 50 $\mu$g/kg (Fig. 1*B*).

*The inhibitory action of NPY is due to $Y_1$ receptor activation in vivo*

Given that differential actions of NPY are mediated through distinct receptor subtypes (18, 19), we sought to elucidate which NPY receptor subtypes are involved in the EAE-inhibitory action of NPY. To this aim, we used various NPY receptor subtype-specific compounds. Lymphoid cell expression of $Y_1$ receptor has been

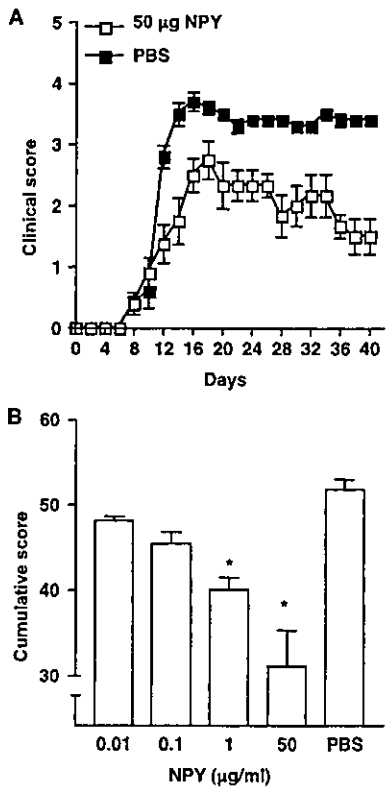


**FIGURE 1.** Effect of NPY on actively induced EAE. EAE was induced in female B6 mice by an immunization with $MOG_{35-55}$ in CFA as described in *Materials and Methods*. *A*, Repetitive treatment with NPY (50 $\mu$g/kg) suppresses the clinical course of EAE as compared with sham-treated animals (PBS). *B*, Treatment with various NPY dosages induced a dose-dependent inhibition of EAE as assessed by cumulative disease scores. Statistical analysis reveals a significant inhibition of the cumulative disease score at 1 $\mu$g/kg ($p = 0.0196$) and 50 $\mu$g/kg ($p = 0.0176$) vs control mice injected with PBS. One representative experiment is shown ($n = 5$ for each group) and data are expressed as mean ± SEM. *, Significant differences between NPY and controls (PBS). Further statistical analysis is shown in Table II.

Table II. *Effect of NPY/receptor-specific analogs on EAE actively induced in wild-type B6*[a]

| Treatment | Day of Onset | Maximum Score | Cumulative Score |
|---|---|---|---|
| NPY, 50 μg/kg | 10.7 ± 2.67 | 2.67 ± 0.34* (0.001)[b] | 31.2 ± 7.23* (0.006) |
| [D-His[26]]NPY ($Y_1$R agonist), 0.01 μg/kg | 13.0 ± 0.58 | 2.87 ± 1.03 | 30.8 ± 7.71* (0.032) |
| [F[7],P[34]]NPY ($Y_1$R agonist), 1.0 μg/kg | 13.2 ± 1.00 | 3.00 ± 0.50 (0.032) | 35.3 ± 7.28* (0.032) |
| BIBO 3304 ($Y_1$R antagonist), 100 μg/kg | 8.5 ± 0.50* (0.032) | 3.88 ± 0.32 | 52.5 ± 7.51 |
| [Ala[31],Aib[32]]NPY ($Y_5$R agonist), 50 μg/kg | 10.8 ± 0.49 | 3.90 ± 0.10 | 52.2 ± 1.82 |
| Control (PBS) | 11.2 ± 0.80 | 4.20 ± 0.20 | 54.5 ± 1.24 |

[a] Representative experiments in wild-type B6 mice treated with NPY and various receptor-specific analogs. Experimental procedures are described in *Materials and Methods*. Data represent mean ± SEM at day 40 of the total mice in each group ($n$ = 5). Significant differences between NPY/analog and controls (PBS) are indicated by asterisks (Mann-Whitney test).
[b] Numbers in parentheses are $p$ values.

recently reported (20, 21). In a first step, we treated $MOG_{35-55}$-immunized mice with a combination of the amount of NPY found to consistently inhibit EAE (50 μg/kg) and 100 μg/kg of the $Y_1$ receptor antagonist BIBO3304. Interestingly, blocking $Y_1$ receptors with BIBO3304 abrogated the suppressive effect of NPY on EAE (Fig. 2*A*). This indicates that NPY probably inhibits clinical signs of EAE via $Y_1$ receptors. To further clarify this point, we treated the mice with a novel $Y_1$ receptor agonist, [D-His[26]]NPY (Table I). Preliminary experiments showed that this compound is very potent and that a smaller dose (0.1 μg/kg to 0.01 μg/kg) than that for NPY effectively suppresses EAE. Due to a limited amount of the compound available, we treated the mice with 0.01 μg/kg of [D-His[26]]NPY on alternate days until the end of the experiment. As shown in Fig. 2*B* and Table II, this $Y_1$ receptor agonist significantly down-regulated the clinical course of EAE, further supporting the role of $Y_1$ receptor in the NPY-mediated suppression of EAE. We also examined the effect of another $Y_1$ receptor agonist, [F[7],P[34]]NPY, on EAE at 0.01, 0.1, and 1 μg/kg. Unlike [D-His[26]]NPY, [F[7], P[34]]NPY was not effective at 0.01 or 0.1 μg/kg. However, treatment with 1 μg/kg [F[7],P[34]]NPY every other day significantly ameliorated clinical signs of EAE (Table II). In contrast, treatment with a selective $Y_5$ receptor agonist, [Ala[31],Aib[32]]NPY, did not show any effect on the clinical course of EAE (Table II). Taken together, these experiments strongly indicate that exogenous NPY suppresses the clinical signs of EAE through the activation of $Y_1$ receptors.

Next we asked whether endogenous NPY plays a role in the natural course of EAE. To answer this question, we evaluated the clinical course of EAE in mice treated with the $Y_1$ receptor antagonist BIBO3304. Blocking $Y_1$ receptors with BIBO3304 led to a significantly earlier onset of disease (Table II), although severity of EAE after onset was not significantly altered. This indicates that endogenous NPY prevents premature development of EAE by interfering with the induction of $MOG_{35-55}$-specific autoimmune T cells, but it is inefficient to modulate the effector phase of EAE.

### *$Y_1$ receptor agonist inhibits induction phase of EAE*

We attempted to treat the mice with the $Y_1$ receptor agonist [D-His[26]]NPY after appearance of the first clinical signs of EAE. However, the treatment protocols starting after onset of clinical manifestations did not significantly alter the clinical course of EAE (data not shown), indicating that $Y_1$ receptor stimulation could not modify the effector phase of EAE. In contrast, alternate day administration of the D-His[26] compound during the induction phase of EAE (from day 0 to 10) after sensitization significantly inhibited the development of EAE (Fig. 3). In fact, the induction phase treatment (days 0–10) was as efficient as the long term treatment

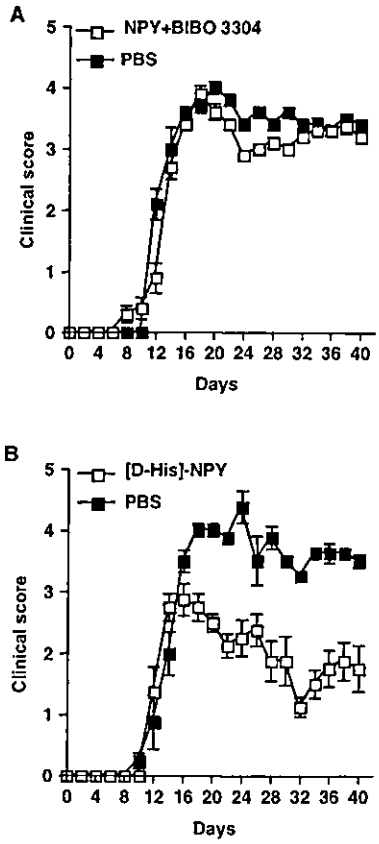


**FIGURE 2.** Receptor specificity of the suppressive effect of NPY. *A*, The suppressive effect of NPY is abrogated when NPY is administered in combination with the $Y_1$ receptor antagonist BIBO3304 (100 μg/kg). *B*, The novel and highly selective $Y_1$ receptor agonist [D-His[26]]NPY (0.01 μg/kg) induced a similar suppression of EAE as seen with NPY (Fig. 1*A*). Data represent mean ± SEM. Additional disease parameters are analyzed in Table I.

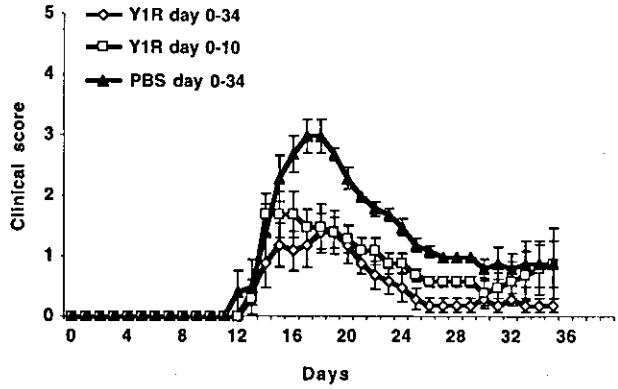


**FIGURE 3.** [D-His$^{26}$]NPY treatment from day 0 to day 10 significantly suppresses development of EAE. The B6 mice sensitized with $MOG_{35-55}$ were treated i.p. with [D-His$^{26}$]NPY (0.01 $\mu$g/kg) every other day either throughout the experiment (Y1R day 0–34) or during the induction phase (Y1R day 0–10). Compared with control mice that were given PBS on alternate days, the mice treated with [D-His$^{26}$]NPY showed milder clinical course. The effect of the treatment from day 0 to day 10 is comparable with the continuous treatment from day 0 to 34. Data represent mean ± SEM. Each treatment group consists of five mice.

(days 0–34) covering both induction and effector phases. This result implies that $Y_1$ receptor stimulation leads to the inhibition of induction, but not effector phase of EAE.

*EAE suppression is associated with an inhibition of $MOG_{35-55}$-specific Th1 response*

NPY has been demonstrated to alter the cytokine profile of in vitro established Th1 clones toward Th2 directions (22, 23). We therefore speculated that the inhibitory action of NPY on EAE might be due to a modulation of the Th1/Th2 balance resulting from a Th2 bias of $MOG_{35-55}$-reactive T cells. To explore this possibility, we first measured serum levels of IgG1 and IgG2a isotypes of anti-$MOG_{35-55}$ Abs at day 40 after immunization. It is generally accepted that elevation of Ag-specific IgG2a Ab results from the augmentation of a Th1 immune response to the Ag, whereas a higher level of IgG1 Ab reflects a stronger Th2 response to the Ag. Fig. 4*A* demonstrates that the treatment with NPY and the $Y_1$ receptor agonists remarkably inhibits anti-$MOG_{35-55}$ IgG2a titers, but they do not significantly alter IgG1 titers. Consequently, the IgG1-IgG2a ratio was significantly elevated in mice treated with either NPY or the $Y_1$ receptor agonists, indicating that the suppression of EAE after NPY treatment is associated with a Th2 bias of $MOG_{35-55}$-reactive autoimmune T cells (Fig. 4*B*).

*Treatment with the $Y_1$ receptor agonist inhibits the ex vivo production of IFN-$\gamma$ by $MOG_{35-55}$-specific T cells*

To further characterize the immunomodulatory properties of NPY in vivo, we isolated the draining LN cells at day 10 from mice treated with [D-His$^{26}$]NPY and from control mice treated with PBS and stimulated the lymphoid cells with $MOG_{35-55}$ in vitro. We compared these two groups with respect to the levels of IFN-$\gamma$ and IL-4 in the culture supernatant and cell-proliferative responses. We found that in vivo treatment with the $Y_1$ receptor agonist significantly inhibited the production of IFN-$\gamma$ on in vitro stimulation with $MOG_{35-55}$ (Fig. 5*A*). [D-His$^{26}$]NPY seemed to slightly inhibit the proliferation of $MOG_{35-55}$-specific T cells as well (Fig. 5*B*), but it was not statistically significant. IL-4 concentrations were below the detection level. These results indicate that the inhibition of IFN-$\gamma$ production by $MOG_{35-55}$-specific T cells may underlie

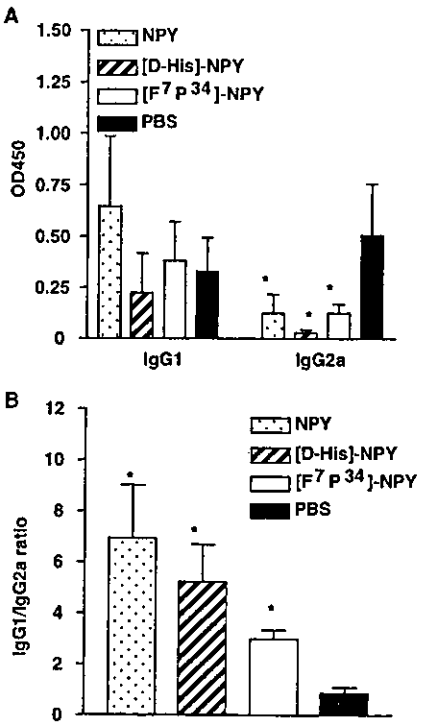


**FIGURE 4.** Analysis of anti-$MOG_{35-55}$ Abs of IgG1 and IgG2a isotype after treatment with NPY and $Y_1$ receptor agonists. *A*, NPY ($p$ = 0.0430) and the $Y_1$ receptor agonists [F$^7$,P$^{34}$]NPY ($p$ = 0.0091) and [D-His$^{26}$]NPY ($p$ = 0.0147) induced a significant inhibition of IgG2a. *B*, Assessment of the IgG1-IgG2a ratio revealed that NPY ($p$ = 0.0309) and the $Y_1$ receptor agonists [F$^7$,P$^{34}$]NPY ($p$ = 0.0054) and [D-His$^{26}$]NPY ($p$ = 0.0417) would induce a Th2 bias of T cell response to $MOG_{35-55}$. Serum samples ($n$ = 5 per condition) were obtained at day 40 after immunization and were analyzed as indicated in *Material and Methods*. Data represent mean ± SEM; *, significant differences between NPY/analogs and controls (PBS).

the Th2 deviation (a higher IgG1-IgG2a ratio) provoked by the $Y_1$ receptor agonist.

*NPY treatment alters autoimmune T cells but not APC*

To obtain insights into the altered Th1/Th2 balance through the activation of $Y_1$ receptors, we explored whether T cells or APCs are the major target of NPY. To address this question, we separated T cells from animals treated in vivo with either [D-His$^{26}$]NPY (treated) or PBS (untreated). The T cells were mixed with irradiated LN cells from treated or untreated mice, serving as APC, and then stimulated with 100 $\mu$g/ml $MOG_{35-55}$ in vitro. Despite whether T cells from untreated mice (untreated T cells) were reconstituted with treated or untreated APC, they responded equally well to $MOG_{35-55}$ with regard to the production of IFN-$\gamma$ (Fig. 6*A*, Columns 3 and 4). However, when T cells from treated mice (treated T cells) were used for reconstitution (Fig. 6*A*, Columns 1 and 2), IFN-$\gamma$ production was remarkably reduced regardless of the source of the APC (two ANOVA, $p$ = 0.001). However, cell proliferation responses were not significantly different among the reconstituted populations (Fig. 6*B*). These results demonstrate that the in vivo effect of [D-His$^{26}$]NPY is mediated by the selective alteration of the T cell function to secrete IFN-$\gamma$ but not of APC.

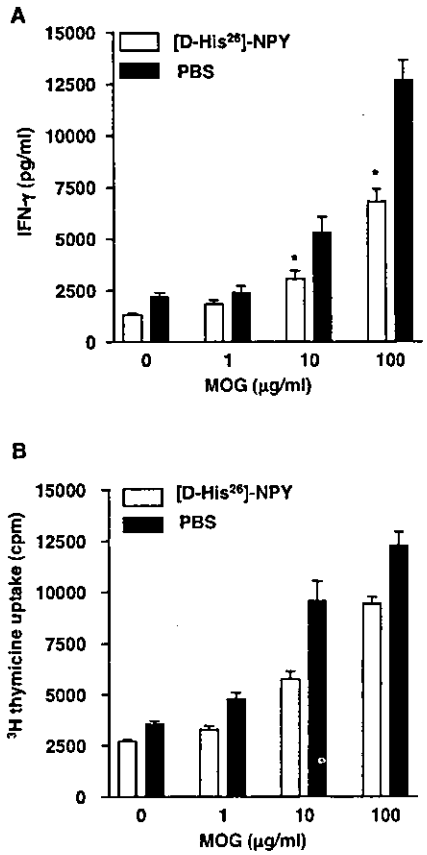


FIGURE 5. Comparison of $MOG_{35-55}$-specific T cell responses after in vivo treatment with [D-$His^{26}$]NPY and PBS. *A*, In vivo treatment with the $Y_1$ receptor agonist [D-$His^{26}$]NPY significantly inhibits the ability of $MOG_{35-55}$-specific T cells to secrete IFN-γ on Ag challenge (treatment vs control; 100 μg $MOG_{35-55}$, $p < 0.0001$). *B*, Tendency toward inhibition of the proliferative response of $MOG_{35-55}$-specific T cells. Statistical analysis revealed no significant differences (ANOVA). Popliteal and inguinal LN cells from treated and control animals were incubated in the presence of $MOG_{35-55}$ for 48 h. IFN-γ was detected by ELISA, and proliferation was determined by estimating the uptake of [$^3$H]thymidine. Pooled data from three independent experiments is shown ($n = 9$). Error bars, SEM; *, significant differences.

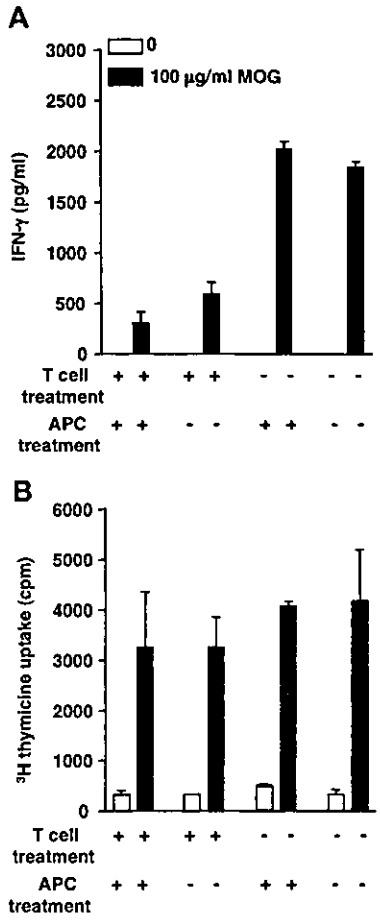


FIGURE 6. Effect of [D-$His^{26}$]NPY on primed T cells and APC. T cells and APC were isolated from animals treated with either [D-$His^{26}$]NPY in vivo (treated) or PBS (untreated) and then coincubated in the presence of $MOG_{35-55}$ in vitro. *A*, Significant inhibition of the IFN-γ secretion is observed only in T cells from treated animals (two-way ANOVA: treated T cells vs untreated T cells, $p = 0.001$). Coincubation with either treated or untreated APC revealed no statistically significant differences. *B*. The proliferative response is not significantly different, although there is a tendency toward a decreased proliferation on treatment. Pooled data from two independent experiments is shown ($n = 6$). Error bars represent SEM.

*Mouse T cells express $Y_1$ receptor mRNA and respond to the $Y_1$ receptor agonist in vitro*

The in vivo results presented above strongly suggest that NPY acts on EAE via direct activation of $Y_1$ receptors expressed on the $MOG_{35-55}$-specific autoimmune T cells. To verify this further, we examined whether mouse T cells express the $Y_1$ receptor. As shown in Fig. 7, RT-PCR enabled us to detect the expression of $Y_1$ receptor mRNA in $MOG_{35-55}$-sensitized LN cells (Fig. 7*A*) and in spleen T cells isolated from naive mice (Fig. 7*B*). We also examined expression levels of the $Y_1$ receptor in T cells polarized in vitro toward Th1 or Th2, according to the described method (28). We saw no significant difference between Th1 and Th2 cells regarding the $Y_1$ receptor expression.

To further determine the functional significance of these findings, we stimulated spleen T cells with plate-bound anti-CD3 mAb in the presence of different concentrations of [D-$His^{26}$]NPY ($10^{-12}$–$10^{-8}$ M), and measured IFN-γ in the supernatant. The results indicate that the $Y_1$ receptor agonist significantly inhibits the secretion of IFN-γ on stimulation with anti-CD3 Ab in a dose-dependent manner (Fig. 8). These data further support that the Th2 bias found in vivo is mediated through the activation of functional $Y_1$ receptors expressed on autoimmune T cells.

*Effect of the $Y_1$ receptor agonist on EAE induced in SJL/J mice*

Furthermore, we asked whether the Y1 receptor agonist might also modulate acute EAE actively induced with $PLP_{139-151}$ in SJL/J mice. We found that the continuous treatment from day 0 to day 30 significantly suppressed clinical EAE, regarding the maximum clinical score: D-$His^{26}$-treated mice, 1.8 ± 0.52 vs PBS-treated mice, 3.2 ± 0.37 ($p < 0.02$). Administration of D-$His^{26}$ during the induction phase (from day 0 to day 10) also reduced the clinical severity of EAE as compared with treatment with PBS. It was interesting to know whether the treatment during the induction phase may inhibit the generation of encephalitogenic T cells reactive to $PLP_{139-151}$. To answer this question, we isolated $PLP_{139-151}$-sensitized lymphoid cells from D-$His^{26}$- or PBS-treated mice at day 10 after immunization and stimulated the cells in vitro with $PLP_{139-151}$. The activated T cells were adoptively transferred to naive SJL/J mice to induce passive EAE as described in *Materials and Methods*. Our protocol induced very serious EAE in the recipients ($n = 5$ for each