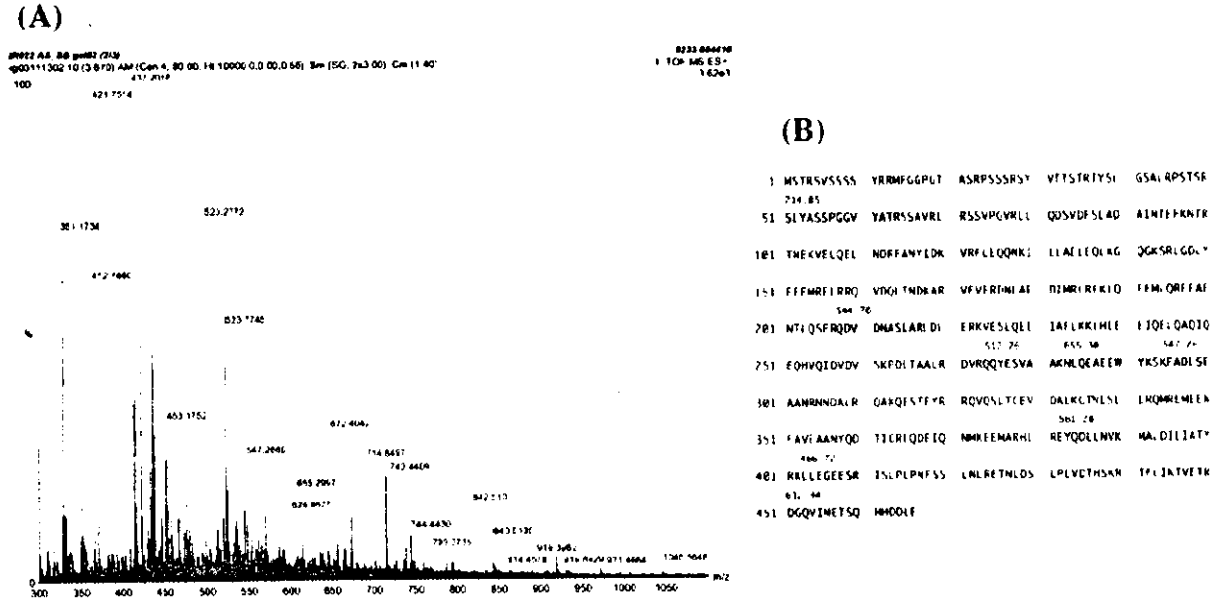

Figure 9. Mass spectrometry analysis of the 14-3-3ε isoform binding proteins in cultured human astrocytes. Spots no. 1 and no. 2 labeled with the rbt-14-3-3ε probe (Figure 8A, a and b) were excised from the gel, trypsanized, and processed for nanoESI MS/MS analysis. **A:** The spectra of nanoESI MS/MS analysis of spot no. 1. Each peak indicates individual peptide fragments. The position of several peaks was automatically numbered on the spectra. Peptides derived from the autolytic fragments of trypsin (eg, 412, 421, and 523) were omitted to be processed for further analysis. The peptide fragments were selected for MS analysis in order of their signal intensity. **B:** Amino acid sequence of human vimentin. Eight peptide fragments of spot no. 1 identified by nanoESI MS/MS analysis (**shadowed**) showed a perfect match with the amino acid sequence encompassing residues 51 to 466 of vimentin. The number indicated on each fragment represents the position in the horizontal axis of the spectra (**A**).

forms except for σ, whose levels were elevated in a cell growth-dependent manner.

Differential Expression of 14-3-3 Isoforms in Reactive Astrocytes in Demyelinating Lesions of MS

To investigate the differential expression of seven 14-3-3 isoforms in MS lesions, the brain, spinal cord, and optic nerve of four progressive MS patients (no. 791, no. 744, no. 609, and no. 544) and 12 non-MS control cases were processed for immunohistochemistry using a panel of isoform-specific antibodies. In chronic active and inactive demyelinating lesions of MS, the majority of GFAP$^+$ hypertrophic astrocytes intensely expressed β, ϵ, ζ, and η isoforms, whereas a small population of reactive astrocytes displayed immunoreactivities against γ, θ, and σ isoforms (Table 2; Figure 3, a to e; Figure 4 a and b). Reactive astrocytes immunoreactive against the 14-3-3 protein exhibited the most dense accumulation at the lesion edge, although they were widely distributed in demyelinating lesions and in the normal appearing white matter. A glial scar was also intensely labeled with the antibodies against β, ϵ, ζ, and η isoforms (ϵ shown in Figure 3e and the others not shown). In MS and non-MS brains, a major population of cerebral cortical neurons constitutively expressed high levels of β, γ, ζ, and η isoforms, and to a lessor degree, θ isoform, whereas they hardly showed immunoreactivity for the σ isoform, and a small population of cerebral cortical neurons in MS and non-MS brains occasionally expressed weak immunoreactivity for the ϵ isoform, although these findings varied among brains for different cases (Table 2; Figure 4, c to f; and Figure 6). Disrupted, distorted, and swollen axons found in the active demyelinating lesions of MS exhibited strong immunoreactivity against γ and ζ isoforms (γ shown in Figure 5a and the other not shown).

A very small population of reactive astrocytes in demyelinating lesions of MS, which occasionally showed a binucleated morphology, intensely expressed the σ isoform, whose expression was not detected in cultured human astrocytes (Figure 5c). A number of reactive astrocytes that appeared in the ischemic lesions of cerebral infarction expressed strong immunoreactivity against ϵ, ζ, and η isoforms (Table 2; Figure 5b), and the σ isoform was again strongly expressed in a very small number of reactive astrocytes (Figure 5d). The immunoreactivity against the η isoform was often concentrated in the nuclear region of reactive astrocytes in MS lesions (not shown) and the ischemic lesions (Figure 6; a to f). Furthermore, some GFAP$^+$ astrocytes occasionally identified in the brains of schizophrenia and neurologically normal patients expressed ϵ and σ isoforms at variable levels (Table 2; Figure 6, b and e). CD68$^+$ macrophages and microglia, with the greatest accumulation identified in the center and edge of active demyelinating lesions of MS and necrotic lesions of cerebral infarction, expressed β, ζ, and η isoforms, whereas they did not show substantial immunoreactivity against ϵ, θ, or σ isoforms (Table 2; Figure 6c). CD3$^+$ lymphocytes found in the perivascular cuffs of active MS lesions expressed variable immunoreactivities for β and ζ isoforms (not shown). A substantial

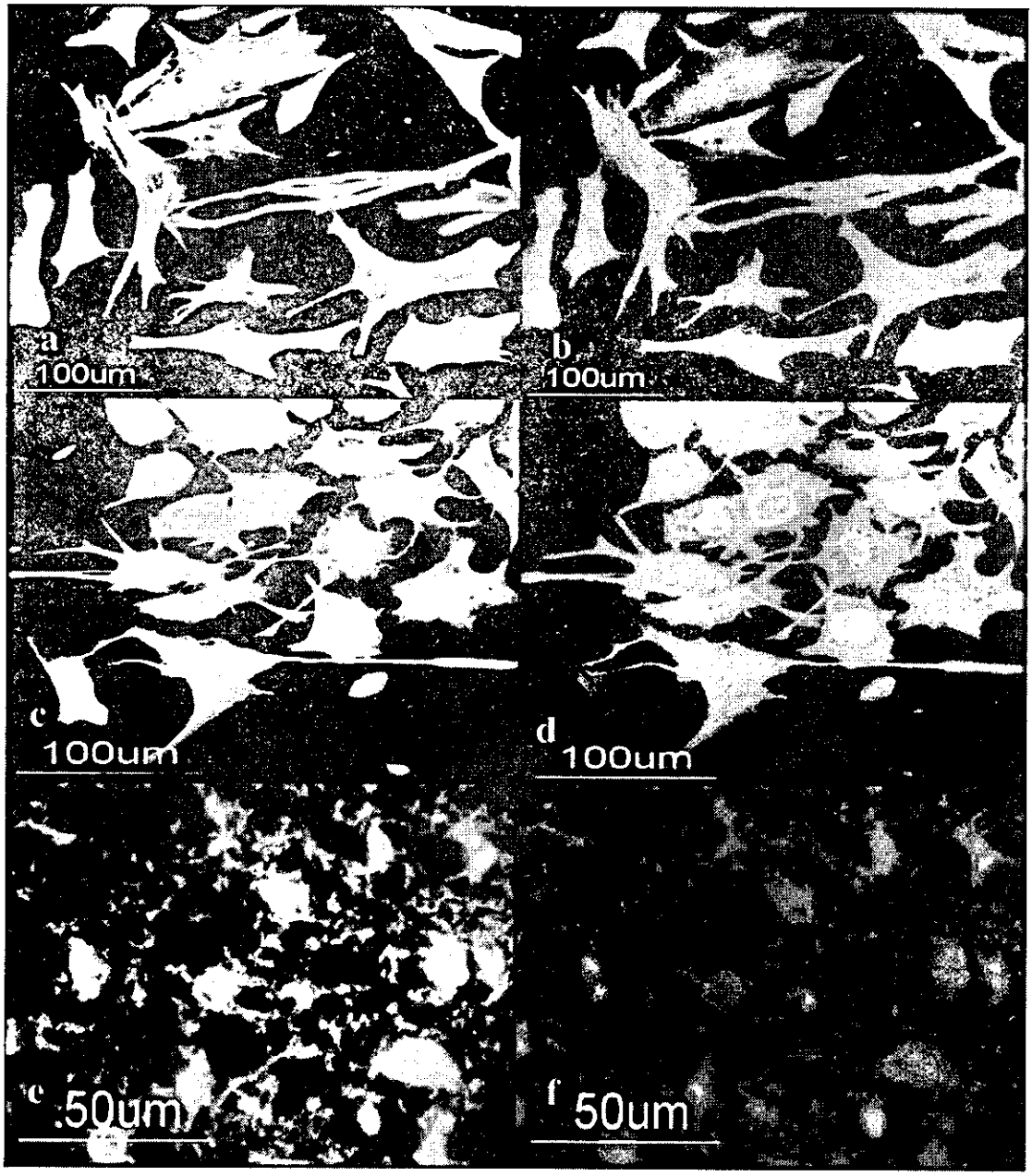

Figure 10. Co-expression of the 14-3-3 ε isoform and vimentin in cultured human astrocytes and reactive astrocytes in chronic demyelinating lesions of MS. Cultured human astrocytes and MS brain tissues were processed for double immunolabeling with anti-vimentin antibody and ε isoform-specific antibody or anti-GFAP antibody followed by labeling with fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies. **a** to **f** represent cultured human astrocytes (AS BW) (**a–d**); no. 7-11, chronic active demyelinating lesions in the subcortical white matter of the frontal lobe (**e**, **f**); vimentin (**a**, **c**, **e**); ε (**b**, **f**); and GFAP (**d**).

population of oligodendrocytes, which survived in chronic active demyelinating lesions of MS and ischemic lesions of cerebral infarction, expressed intense immunoreactivity against θ isoform (Table 2; Figure 5, e and f; and Figure 6d). These results suggest that markedly up-regulated expression of the ε isoform is the most reliable marker for identifying reactive astrocytes in MS and non-MS brains. Co-expression of the ε isoform and GFAP was verified in reactive astrocytes in MS lesions (Figure 7; a to c) and cultured human astrocytes (Figure 7; d to f) by double immunolabeling.

Binding of the 14-3-3ε Isoform to Vimentin and GFAP in Cultured Human Astrocytes

To identify the binding partner of the 14-3-3 protein in human astrocytes, we performed a protein overlay anal-

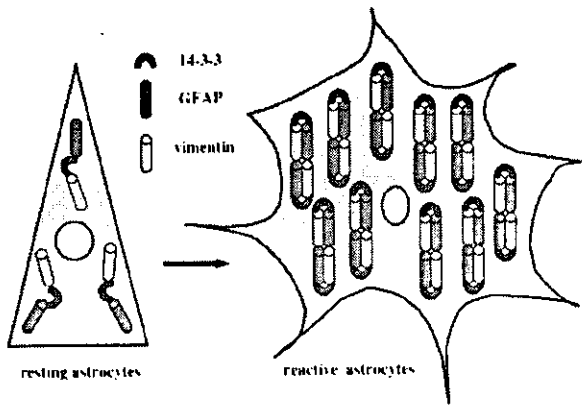

Figure 11. Putative role of the 14-3-3 protein in reactive gliosis in MS. Reactive gliosis is characterized by hypertrophy and proliferation of astrocytes associated with enhanced expression of GFAP (green) and vimentin (orange), which are co-polymerized in assembled filaments. Cultured human astrocytes expressed β, γ, ε, ζ, η, and θ isoforms, whose levels were markedly up-regulated under the growth-promoting culture condition, in which the 14-3-3 protein (red) interacted with vimentin (orange) and GFAP (green). These observations suggest that the 14-3-3 protein (red) might act as an adaptor that connects vimentin (orange) and GFAP (green) in reactive astrocytes at the site of demyelinating lesions in MS.

ysis using recombinant human 14-3-3ε protein with the Xpress tag (rh14-3-3ε) as a probe. Human astrocytes were incubated in 10% FBS-containing culture medium. Total protein extract was separated on a two-dimensional PAGE gel (Figure 8A, a) and transferred onto a polyvinylidene difluoride membrane (Figure 8A, b to h). The rh14-3-3ε probe strongly reacted with several spots on the blot, among which two major 54-kd spots were designated spots no. 1 and no. 2 (Figure 8A, b). In contrast, the rhISG15 probe did not react with these spots, excluding nonspecific binding of rh14-3-3ε via the Xpress tag (Figure 8A, h). Spots no. 1 and no. 2 were excised from the original gels, trypsinized, and processed for nanoESI-MS/MS analysis (Figure 9A). Among the peaks detected, eight peptide fragments derived from spot no. 1 and six from spot no. 2 showed a perfect match with the amino acid sequence covering residues 51 to 466 of human vimentin (Figure 9B), suggesting that these spots correspond to nearly full-length vimentin. Intense vimentin immunoreactivity was also identified in reactive astrocytes in demyelinating lesions of MS (Figure 3f). Furthermore, anti-vimentin monoclonal antibody reacted with spots no. 1 and no. 2, although this antibody labeled three additional, more acidic spots having smaller molecular weights (Figure 8A, c). The latter might represent post-translationally modified isoforms or degradation products of vimentin. Because vimentin is heavily phosphorylated at multiple serine residues in various mesenchymal cells, the phosphorylation state was characterized by repeated relabeling of the blot with three different antibodies specific for phosphorylated serine epitopes of vimentin. Phosphorylated Ser-39-, Ser-72-, and Ser-83-specific antibodies strongly reacted with spots no. 1 and no. 2, along with three additional spots unlabeled with rh14-3-3ε, suggesting that these serine residues are not involved in the interaction of the ε isoform with vimentin (Figure 8A, d to f). Protein overlay analysis using the rh14-3-3ε probe identified a distinct spot, designated spot no. 3 (Figure 8A, b). This spot was labeled with anti-GFAP antibody, indicating that GFAP is another binding partner of the 14-3-3 protein (Figure 8A, g). A more acidic spot having a smaller molecular weight immunoreactive for GFAP and weakly labeled with rh14-3-3ε might represent a post-translationally modified isoform or a degradation product of GFAP (Figure 8A, b and g). Vimentin and GFAP were detected in the immunoprecipitates of cultured human astrocyte protein extract, when the lysate was incubated with the ε, β, or ζ isoform-specific antibody (Figure 8B, top and bottom panels, lanes 1 to 3, 6 to 8). In contrast, only marginal bands were found in those with normal rabbit IgG (Figure 8B, top and bottom panels, lanes 4 and 9). Co-expression of the ε isoform with vimentin and GFAP was verified in cultured human astrocytes (Figure 10, a to d) and in reactive astrocytes in demyelinating lesions of MS (Figure 10, e and f) by double immunolabeling.

Discussion

The present study showed that seven 14-3-3 isoforms are differentially expressed in reactive astrocytes in demyelinating lesions of MS. Human astrocytes in culture also expressed β, γ, ε, ζ, η, and θ isoforms whose levels were markedly elevated under the growth-promoting culture condition. In demyelinating lesions of MS, the majority of GFAP$^+$ hypertrophic astrocytes intensely expressed β, ε, ζ, and η isoforms, although the expression of these isoforms was found in reactive astrocytes appearing in non-MS brains. Previous studies showed that the σ isoform expression is confined to differentiated squamous epithelial cells.[19,38] However, we found that some reactive astrocytes in MS and non-MS brains intensely expressed this isoform. Neurons constitutively expressed β, γ, ζ, and η isoforms but they did not constantly express ε or σ isoforms. Macrophages and microglia in MS and non-MS lesions intensely expressed β, ζ, and η isoforms, but they did not express ε, θ, or σ isoforms. A substantial population of oligodendrocytes, surviving in active demyelinating lesions of MS and ischemic lesions of cerebral infarction, intensely expressed the θ isoform, consistent with the expression of this isoform in the white matter of the developing rat CNS.[7] These observations are in agreement with our previous findings that the 14-3-3 protein is expressed not only in neurons but also in astrocytes, microglia, and oligodendrocytes in mouse brain cell cultures.[26] The present observations suggest that up-regulated expression of the ε isoform could be used as an immunohistochemical marker to identify reactive astrocytes at least in demyelinating lesions of MS and ischemic lesions of cerebral infarction. However, Lewy bodies in the Parkinson's disease brain[30] and a minor population of neurons in MS and non-MS brains express the ε isoform, indicating that this isoform is not astrocyte-specific.

The biological role of ε and σ isoforms in human astrocyte function remains unknown. Increasing evidence indicates that isoform-specific function regulates the devel-

opment and differentiation of neural and nonneural cells. Particularly, the ε isoform plays a role in the regulation of various cellular signaling events. The 14-3-3ε gene is deleted in the patients with Miller-Dieker syndrome, a human neuronal migration disorder presenting with the most severe form of lissencephaly (LIS) associated with facial abnormalities.[39] ε Isoform-deficient mice are defective in neuronal migration during brain development.[40] The multimolecular complex composed of the ε isoform, LIS1 and nudE nuclear distribution gene E homolog-like 1 (NUDEL) regulates the activity of dynein, a cytoplasmic motor protein, suggesting a role of ε in neuronal migration.[40] Somatic homozygous deletion of the 14-3-3ε gene is frequently found in small cell lung cancers, supporting the idea that the ε isoform serves as a tumor suppressor gene.[41] The 14-3-3ε isoform, by binding to the intracellular domain of the p75 neurotrophin receptor (NTR) in a NGF-dependent manner, promotes p75NTR-associated cell death executor (NADE)-mediated apoptosis.[42] During apoptosis, the ε protein is cleaved by caspase-3 at a cleavage site located in the C-terminal hydrophobic tail, where the amino acid sequence is highly variable among different 14-3-3 isoforms.[43] The ε isoform interacts with cdc25A and cdc25B phosphatases, key enzymes required for cell-cycle progression by activating cyclin-dependent kinases.[44] Phosphorylation-dependent interaction of the ε isoform with heat shock transcription factor HSF1 restricts the location of HSF1 in the cytoplasm by keeping it in an inactive form.[45] The ε isoform catalyzes the depolymerization and unfolding of mitochondrial precursor proteins in an ATP-dependent manner.[46] Based on these observations, we propose that the ε isoform plays a regulatory role in proliferation, apoptosis, and stress responses in reactive astrocytes.

The σ isoform constitutes a component of the G_2/M cell-cycle checkpoint machinery.[47] Exposure of the cells to DNA-damaging agents results in p53-dependent induction of the σ isoform, which in turn arrests the cells in the G_2/M phase by sequestering the cdc2-cyclin B1 complex in the cytoplasm.[48] Therefore, σ isoform-deficient cells are unable to maintain cell-cycle arrest.[47] Selective down-regulation of the σ isoform because of the hypermethylation of CpG islands in its promoter region is responsible for the malignant transformation of breast cancer cells,[49] whereas reduced expression of the σ isoform allows human epidermal keratinocytes to escape replicative senescence.[50] These observations raise the possibility that a population of reactive astrocytes with strong immunoreactivity against the σ isoform might represent the cells responding to DNA damage at the site of demyelinating lesions in MS and ischemic lesions of cerebral infarction.

Reactive gliosis is characterized by hypertrophy and proliferation of astrocytes associated with enhanced expression of GFAP and vimentin, accompanied by increased production of growth factors, cytokines, neuropeptides, and extracellular matrix molecules.[51,52] Astrocytes play a role in the repair of the blood-brain barrier, protection of neurons from glutamate excitotoxicity, and enhancement of neuronal survival by supplying neurotrophic factors.[53] On the other hand, reactive astrocytes strongly inhibit neurite outgrowth by forming glial scars after CNS injury and inflammation.[53,54] Through protein overlay and nanoESI-MS/MS analysis, we showed that vimentin is the major 14-3-3 protein-interacting protein expressed in cultured human astrocytes. Consistent with previous observations,[55,56] we identified vimentin expression in reactive astrocytes in demyelinating lesions of MS. Astrocytes isolated from vimentin-deficient mice possess an abnormal filamentous network of GFAP.[57,58] Furthermore, mice lacking vimentin and GFAP do not form proper glial scars after CNS injury, indicating that the type III IF family proteins play a pivotal role in cytoskeletal organization in astrocytes.[59]

In our study, the rh14-3-3ε probe strongly reacted with two distinct spots named no. 1 and no. 2 among five phosphovimentin-immunoreactive spots on the blot. Vimentin was immunoprecipitated with the ζ and β isoforms along with ε. These observations suggest that the interaction between vimentin and the 14-3-3 protein is not isoform-specific, and that the 14-3-3 protein-binding domain in vimentin might not include phosphorylated Ser-39, Ser-72, and Ser-83 epitopes. Protein overlay analysis identified GFAP as another binding partner of the 14-3-3ε isoform. Immunoprecipitation experiments verified the interaction between GFAP and the ε, ζ, or β isoform. However, a different spot strongly immunoreactive against GFAP but much weakly labeled with rh14-3-3ε was identified on the two-dimensional gel blot. This suggests that a substantial pool of cytoplasmic vimentin and GFAP proteins steadily interact with the 14-3-3 protein in human astrocytes.

Our observations raise the possibility that the 14-3-3 protein acts as an adaptor that connects vimentin and GFAP in cultured human astrocytes (Figure 11). Previous studies showed that vimentin and GFAP are co-expressed and co-polymerized in assembled filaments in astrocytes,[58,60] supporting the view that the 14-3-3 protein not only bridges vimentin and GFAP one by one, but also bundles both of them in the same assembled filaments. All these proteins are expressed at much higher levels in reactive astrocytes, which require more efforts to coordinate the IF network compared with resting astrocytes (Figure 11). Several other binding partner candidates for vimentin in astrocytes include αB-crystallin, which inhibits the *in vitro* assembly of GFAP,[61] and the multiple endocrine neoplasia type 1 (MEN1) gene product named menin, which binds to vimentin and GFAP in glioma cells.[62] The 14-3-3γ isoform interacts with F-actin and Raf kinase in cultured mouse astrocytes, indicating its role in cytoskeletal rearrangement during cell growth and division.[63,64] Importantly, a recent study using COS-7 cells overexpressing the 14-3-3 protein showed that phosphorylated vimentin binds to the 14-3-3 protein and limits the interaction of 14-3-3 with other 14-3-3-binding partners, thereby modulating Raf-dependent intracellular signaling.[65] This study also found that vimentin does not have typical consensus 14-3-3-binding motifs.[65] However, a close interaction of the 14-3-3 protein with phosphorylated vimentin affects the phosphorylation and dephosphorylation state of vimentin.[65] Site-specific phosphorylation of vimentin and GFAP is mediated by

a range of protein kinases, including Rho kinase, cdc2 kinase, Ca^{2+} calmodulin-dependent kinase II, protein kinases A and C, and Aurora-B kinase.[60,66–69] They coordinately regulate dynamic equilibrium between the assembly and disassembly of IF proteins during mitosis.[60,66–69] Furthermore, these kinases are identified as binding partners for the 14-3-3 protein.[1–3] Therefore, our observations suggest that the 14-3-3 protein plays a role in the organization of IF proteins and IF-related kinases during conversion from resting astrocytes to reactive astrocytes. A role for 14-3-3 protein in IF dynamics is supported by our preliminary observations that suggest the effects of difopein,[70] a specific inhibitor of 14-3-3 protein/ligand interaction, on the morphological characteristics of cultured human astrocytes.

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The regulatory role of natural killer cells in multiple sclerosis

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Summary

Multiple sclerosis is a chronic demyelinating disease of presumed autoimmune pathogenesis. The patients with multiple sclerosis typically shows alternating relapse and remission in the early stage of illness. We previously found that in the majority of multiple sclerosis patients in a state of remission, natural killer (NK) cells contain unusually high frequencies of the cells expressing CD95 (Fas) on their surface (>36.0%). Here we report that in such 'CD95$^+$ NK-high' patients, NK cells may actively suppress potentially pathogenic autoimmune T cells that can mediate the inflammatory responses in the CNS. Using peripheral blood mononuclear cells (PBMCs) derived from 'CD95$^+$ NK-high' or 'CD95$^+$ NK-low' multiple sclerosis in a state of remission, we studied the effect of NK cell depletion on the memory T cell response to myelin basic protein (MBP), a major target antigen of multiple sclerosis. When we stimulated PBMCs of the 'CD95$^+$ NK-high' multiple sclerosis after depleting CD56$^+$ NK cells, a significant proportion of CD4$^+$ T cells (1/2000 to 1/200) responded rapidly to MBP by secreting interferon (IFN)-γ, whereas such a rapid T cell response to MBP could not be detected in the presence of NK cells. Nor did we detect the memory response to MBP in the 'CD95$^+$ NK-low' multiple sclerosis patients in remission or healthy subjects, regardless of whether NK cells were depleted or not. Depletion of cells expressing CD16, another NK cell marker, also caused IFN-γ secretion from MBP-reactive CD4$^+$ T cells in the PBMCs from 'CD95$^+$ NK-high' multiple sclerosis. Moreover, we showed that NK cells from 'CD95$^+$ NK-high' multiple sclerosis could inhibit the antigen-driven secretion of IFN-γ by autologous MBP-specific T cell clones *in vitro*. These results indicate that NK cells may regulate activation of autoimmune memory T cells in an antigen non-specific fashion to maintain the clinical remission in 'CD95$^+$ NK-high' multiple sclerosis patients.

Keywords: multiple sclerosis; myelin basic protein; NK cell; NK2; T cell–NK cell interaction

Abbreviations: CBA = cytokine bead array; HLA = human leukocyte antigen; IFN = interferon; IL = interleukin; MBP = myelin basic protein; MS-rel = multiple sclerosis in relapse; MS-rem = multiple sclerosis in remission; NK = natural killer; NK2 = NK type 2; OVA = ovalbumin; PBMCs = peripheral blood mononuclear cells; PI = propidium iodide; PLP = proteolipid protein; TCC = T-cell clone; TNF = tumour necrosis factor

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Introduction

Multiple sclerosis is a chronic neurological disease the pathology of which is characterized by multiple foci of inflammatory demyelinating lesions accompanying a variable degree of axonal changes (Bjartmar and Trapp, 2001). Regarding the pathogenesis of multiple sclerosis, studies have indicated that autoimmune T cells targeting myelin components play a crucial role in mediating the inflammatory process, particularly in the early stages of relapsing–remitting multiple sclerosis (Steinman, 2001). A number of laboratories have studied the properties of potentially pathogenic autoimmune T cell clones (TCC) reactive to myelin antigens such as myelin basic protein (MBP) and proteolipid protein (PLP), which have been derived from the peripheral blood of multiple sclerosis (Ota *et al.*, 1990; Pette *et al.*, 1990; Martin *et al.*, 1991; Ohashi *et al.*, 1995). The large majority of the TCC are CD4$^+$ and produce T helper type 1 (Th1) cytokines

such as interferon (IFN)-γ after recognizing the myelin peptide bound to human leukocyte antigen (HLA)-DR molecules. These results are consistent with the idea that the inflammatory process of multiple sclerosis is triggered by invasion of autoimmune Th1 cells into the CNS, and that exogenous or endogenous factors altering the Th1/Th2 balance may influence the disease activity. The relevance of this postulate is actually supported by clinical observations that Th2-inducing medications, such as copolymer-1, are beneficial for multiple sclerosis (Duda *et al.*, 2000; Neuhaus *et al.*, 2000), and that administration of IFN-γ showed deleterious effects on multiple sclerosis in previous clinical trials (Panitch *et al.*, 1987).

Although there are a number of candidate target antigens for multiple sclerosis, MBP is thought to be a primary target for autoimmune T cells, at least in some patients (Bielekova *et al.*, 2000). It is of note that MBP- or PLP-specific TCC can be established not only from multiple sclerosis, but also from peripheral blood of healthy subjects, which raised the intriguing issue as to how healthy subjects are protected from self-attack by the potentially pathogenic autoimmune Th1 cells. Although much remains to be clarified, studies in the last decade have showed that regulatory cells are involved in prevention of or recovery from autoimmune diseases in rodent (Das *et al.*, 1997; Zhang *et al.*, 1997; Olivares-Villagomez *et al.*, 1998; Sakaguchi *et al.*, 2001). This allows us to speculate that regulatory cells may contribute to protecting healthy subjects from developing autoimmune diseases such as multiple sclerosis, or to prohibiting acute attacks or enhancing the recovery from clinical exacerbations in patients with relapsing–remitting multiple sclerosis.

Whereas regulatory cells constitute various lymphoid populations, substantial evidence supports that natural killer (NK) cells play significant roles in protecting against autoimmune diseases (Zhang *et al.*, 1997; Matsumoto *et al.*, 1998; Smeltz *et al.*, 1999). In fact, it has previously been demonstrated that NK cell depletion augments the severity of a model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (Zhang *et al.*, 1997; Matsumoto *et al.*, 1998), which can be induced by sensitization against CNS myelin component. Given that autoimmune Th1 cells would mediate the pathology of EAE, we propose a possible involvement of NK cells in suppressing autoimmune Th1 cells in multiple sclerosis.

With the hypothesis that NK cells may contribute to maintaining the remission in relapsing–remitting multiple sclerosis, we have previously examined the cytokine production and surface phenotype of NK cells freshly isolated from the peripheral blood mononuclear cells (PBMCs) of multiple sclerosis in remission (MS-rem) or relapse (MS-rel) (Takahashi *et al.*, 2001). The results demonstrate that NK cells in MS-rem (but not MS-rel) are characterized by a remarkable elevation of interleukin (IL)-5 mRNA and a decreased expression of IL-12Rβ2 mRNA, as well as a higher percentage of $CD95^+$ cells among the $CD56^+$ NK cells. These features of the cells are reminiscent of NK type 2 (NK2) cells, which can be induced *in vitro* in the presence of IL-4 and of anti-IL-12 antibodies (Peritt *et al.*, 1998). The NK2 cells induced from PBMCs of healthy subjects inhibit the generation of IFN-γ-secreting Th1 cells from the PBMCs of the same subjects (Takahashi *et al.*, 2001), leading us to postulate that NK2-like cells detected in MS-rem may play a regulatory role. While the NK2-like features were found to be lost in patients at acute relapsing state, they tended to be restored along with clinical recovery. Obviously, these results do not imply that clinically diagnosed MS-rem represents a homogeneous condition. In fact, the parameters characteristic for NK2-like cells (i.e. up-regulation of IL-5 mRNA and an increased frequency of $CD95^+$ cells) showed a substantial variance in MS-rem, indicating their heterogeneity.

More recently, we have noticed that MS-rem can be divided into two subgroups, '$CD95^+$ NK-high' and '$CD95^+$ NK-low', according to the frequency of $CD95^+$ cells among NK cells. Here, we demonstrate that these two groups significantly differ in the responsiveness to MBP *ex vivo* in an NK-cell-depleted condition. Namely, NK-deleted PBMCs from '$CD95^+$ NK-high' multiple sclerosis responded rapidly to MBP, as assessed by the frequency of IFN-γ-secreting $CD4^+$ T cells at 8 h after stimulation with MBP, whereas those from the '$CD95^+$ NK-low' or from healthy subjects responded only marginally. Moreover, we showed that NK cells from a '$CD95^+$ NK-high' multiple sclerosis could inhibit the antigen-driven secretion of IFN-γ by MBP-specific TCC established from the same patient. These results demonstrate, for the first time to our knowledge, that NK cell depletion leads to augmentation of memory T cell response to an autoantigen in human, and that an elaborate interplay between NK cells and MBP-specific memory T cells may be involved in the regulation of multiple sclerosis in '$CD95^+$ NK-high' patients.

Material and methods

Subjects

To clarify the heterogeneity among patients with MS-rem regarding NK cell phenotype, we first examined 30 patients with MS-rem (male/female = 11/19; aged 37.7 ± 11.1 years) for the lymphoid cell expression of CD95. As a control for multiple sclerosis, we examined 26 healthy sex- and age-matched subjects (male/female = 11/15; aged 39.9 ± 12.2 years). Furthermore, for a new cohort of 14 patients with MS-rem (male/female = four/10; aged 39.2 ± 10.7 years) (Table 1) and 14 healthy subjects (male/female = five/nine; aged 35.3 ± 8.0 years), we conducted the cytokine secretion assay as well as flow cytometer analysis for the frequency of $CD95^+$ NK cells. Two of the patients were examined again after a 1-year interval.

Written informed consent was obtained from all patients and healthy volunteers and the study was approved by the Ethics Committee of the National Center of Neuroscience (NCNP). All patients fulfilled standard criteria for the diagnosis of relapsing–remitting multiple sclerosis (Poser *et al.*, 1983; McDonald *et al.*, 2001). The clinical status of multiple sclerosis (MS-rem or MS-rel) was operationally determined as described previously (Takahashi *et al.*, 2001). In brief, we selected MS-rem patients for study who had been clinically stable without any immunosuppressive medications for >3 months, and had shown no sign of new lesions as assessed by a recent MRI scan with gadolinium enhancement. None of our patients represented the pure optic-spinal form of multiple sclerosis (Misu *et al.*, 2002), which may be rather unique to Japanese populations.

Table 1 *List of the PBMC samples examined for the frequency of memory Th1 cells*

Information on patients			
PBMC code	Age (years)/sex	$CD95^+$ NK frequency	EDSS#
#1	43/M	High	2.5
#2	30/F	High	2.5
#3	53/M	High	1.0
#4	39/F	High	3.5
#5	28/F	High	1.0
#6*	35/M	Low	2.0
#7**	57/F	Low	3.0
#8	31/M	Low	1.0
#9	29/F	Low	3.0
#10	38/F	Low	2.0
#11	59/F	High	3.5
#12*	36/M	High	2.0
#13**	58/F	High	3.0
#14	33/F	High	6.5
#15	29/F	Low	1.0
#16	45/F	Low	4.0

The samples marked with * or ** are derived from the same patients, with an interval of 1 year between samples. The phenotype of both of these patients changed from '$CD95^+$ NK-low' to '$CD95^+$ NK-high'. M = male; F = female; EDSS = Expanded Disability Status Scale.

Reagents

Anti-CD3-FITC or -ECD, anti-CD4-PC5, anti-CD8-FITC, anti-CD16-Phytoerythrin, and anti-CD56-PC5 or -PE mAbs were purchased from IMMUNOTECH (Marseille, France). Anti-CD57-FITC, anti-CD69-PE, anti-CD94-FITC, anti-CD95-FITC, -Cych or -PE, anti-CD158a-FITC, anti-NKB1-FITC, and anti-HLA-DR-FITC mAbs were purchased from BD PharMingen (San Jose, CA, USA). Human MBP was purified with a modification of previously described methods (Deibler *et al.*, 1972, 1995).

Cell preparation and NK cell deletion

Shortly after drawing peripheral blood, PBMCs were separated by density gradient centrifugation with Ficoll-Hypaque™ PLUS (Amersham Biosciences, Uppsala, Sweden). They were washed three times in phosphate-buffered saline (PBS), and resuspended at 1×10^6 cells/ml in AIM-V culture medium (Invitrogen Corp., Carlsbad, CA, USA) containing 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Rockville, MD, USA). NK cells were depleted from the PBMCs with either CD56- or CD16-MicroBeads (Miltenyi Biotech, Gradbach, Germany), following the protocol provided by the manufacturer.

T cell clones

$CD4^+$ TCC were generated from a '$CD95^+$ NK-high' multiple sclerosis patient (HLA-DRB1*1502) by repeated selection against human whole MBP with modification of a previously described method (Pette *et al.*, 1990). The TCC proliferated and secreted Th1 cytokines specifically in response to MBP, and the proliferative response and cytokine production was greatly reduced in the presence of antibodies against HLA-DR. The DR-restricted clone cells were grown in AIM-V medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

T-cell stimulation with MBP

To assess the presence of memory MBP-reactive T cells in the peripheral blood, fresh PBMCs or NK-deleted PBMCs were stimulated for 8 h with 10 µg/ml MBP in 96-well round-bottomed plates at 2×10^5 cells/well, and then analysed for the presence of IFN-γ-secreting cells using the cytokine secretion assay. To evaluate the regulatory function of NK cells from '$CD95^+$ NK-high' multiple sclerosis, resting cells of MBP-specific TCC (2×10^4 cells/well) were stimulated with 10 µg/ml MBP in the presence of X-irradiated (5000 rad) autologous total PBMCs or $CD56^+$ NK-deleted PBMCs (1×10^5 cells/well) for 8 h prior to the cytokine secretion assay, and for 60 h to determine the proliferation of the TCC. To assess cell proliferation, we counted incorporation of [^{3}H]thymidine (1 µCi/well) during the final 12 h with a beta-1205 counter (Pharmacia, Uppsala, Sweden).

Cytokine secretion assay

We used a commercial kit from Miltenyi Biotech to identify T cells secreting IFN-γ. The principle of this assay has been described previously (Manz *et al.*, 1995). Briefly, cells were stained with IFN-γ capture antibody 8 h after stimulation with MBP or ovalbumin (OVA), then washed and cultured again for 45 min. They were stained with PE-conjugated IFN-γ detection antibody, together with anti-human CD3-FITC and -CD4-PC5, then washed and resuspended in PBS containing propidium iodide (PI) (BD PharMingen). Samples were analysed using flow cytometry.

Cytokine bead array

The levels of IL-2, -4, -5, -10, tumour necrosis factor (TNF)-α and IFN-γ in the culture supernatants were measured by cytokine bead array (CBA) (BD PharMingen), in which six bead populations with distinct fluorescence intensities are coated with capture antibodies specific for each cytokine (Cook *et al.*, 2001). The cytokine capture beads were mixed with the PE-conjugated detection antibodies and then incubated with recombinant standards or supernatant samples to form sandwich complexes. After washing the beads, sample data were acquired using the flow cytometer and were analysed with the BD CBA Analysis Software® (BD PharMingen).

Results

An increased frequency of $CD95^+$ NK cells distinguishes a subgroup of multiple sclerosis

As we have reported previously (Takahashi *et al.*, 2001), whereas proportions of $CD3^-$ $CD56^+$ NK cells in fresh PBMCs weakly express CD95 on their surface, the frequency of $CD95^+$ NK cells is significantly elevated in MS-rem as compared with healthy subjects or MS-rel. We have further noticed that MS-rem can be divided into two subgroups according to the frequency (%) of $CD95^+$ cells among NK cells (Fig. 1A; see also the left panels in Figs 1B and 2A, showing the distinction between $CD95^+$ and $CD95^-$ cells). When we determined the mean + 2 SD value for healthy subjects (35.86%) as an upper boundary for healthy subjects,

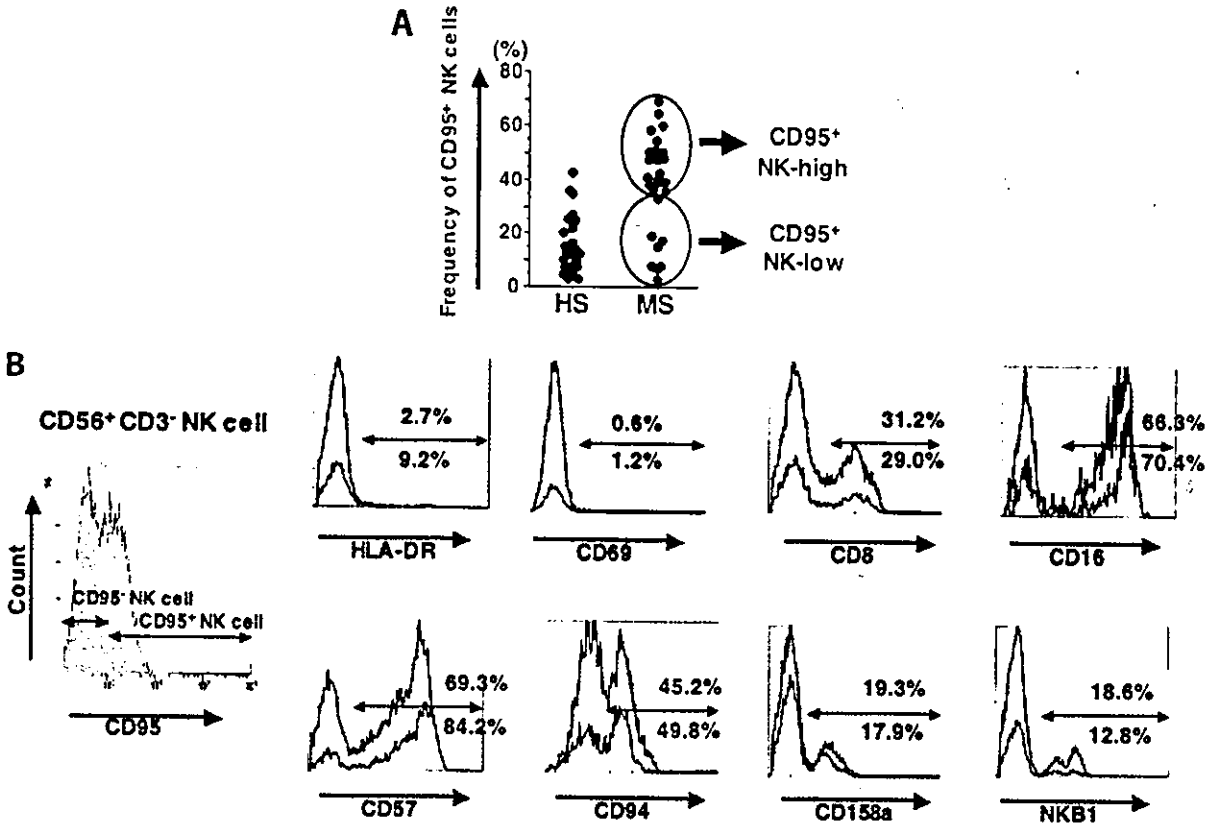

Fig. 1 Characterization of CD95$^+$ NK cells from 'CD95$^+$ NK-high' multiple sclerosis. (A) Multiple sclerosis patients in remission (MS-rem) can be subgrouped into 'CD95$^+$ NK-high' and 'CD95$^+$ NK-low'. Freshly isolated PBMCs from 26 healthy subjects or 30 MS-rem were stained with the combination of anti-CD3-FITC, -CD56-PC5 and -CD95-PE, and evaluated for the frequency of CD95$^+$ cells in the CD56$^+$CD3$^-$ NK cell population (the fluorescence intensity for CD95 expression is shown in the histograms in B and Fig. 2A). Note that flow fluorocytometric analysis was completed within 2 h after drawing blood in order to avoid spontaneous death of CD95$^+$ cells. (B) Comparison of CD95$^+$ versus CD95$^-$ NK cells in the expression of various surface molecules. We stained the PBMCs from 'CD95$^+$ NK-high' patients with the panel of antibodies for surface molecules expressed by NK cells. Red lines represent the histogram gated for CD95$^-$ NK cells and blue lines for CD95$^+$ NK cells. Values in red and in blue represent the positive percentage in CD95$^-$ and CD95$^+$ cells, respectively. As indicated, CD95$^+$ NK cells did not differ significantly from CD95$^-$ NK cells in the staining pattern for each antibody regarding the proportion of positive cells as well as the mean fluorescence intensity. Shown are the results of a representative case.

three-quarters of MS-rem had a percentage value higher than this boundary. We defined these patients in remission with a higher frequency of CD95$^+$ cells in NK cells as 'CD95$^+$ NK-high' multiple sclerosis, and the rest as 'CD95$^+$ NK-low' (Fig. 1A). In contrast to CD56$^+$ NK cells, CD3$^+$CD56$^-$ T cells and CD3$^+$CD56$^+$ NK T cells were not different between healthy subjects and multiple sclerosis patients as regards the frequency of CD95$^+$ cells (data not shown), which directed our attention to the analysis of CD56$^+$ NK cells.

Because NK cells from MS-rem were found to express a larger amount IL-5 mRNA, and since they were neither defective in cytolytic function nor reduced in number (Takahashi *et al.*, 2001), we hypothesized that the CD95 expression may reflect an activation state of the NK cells. To test this hypothesis, we compared the CD95$^+$ and CD95$^-$ NK populations derived from 'CD95$^+$ NK-high' patients by flow cytometry. Histogram plot analysis for the proportion of positive cells and for mean fluorescence intensity showed that the two populations are analogous in the expression of HLA-DR, CD69, CD8, CD16, CD57, CD94, CD158a and NKB1 (Fig. 1B). Whereas HLA-DR and CD69 molecules are regarded as cell activation markers, few populations of CD95$^+$ NK cells from multiple sclerosis or healthy subjects expressed these molecules. These results do not support the idea that the CD95$^+$ NK cells are in a state of activation, nor do they indicate that the CD95$^+$ cells represent a unique subset of monoclonal or oligoclonal origin. It has recently been suggested that CD56^{bright} NK cells may represent a distinct subset (Jacobs *et al.*, 2001). However, we saw no difference in the proportion of CD56^{bright} cells between CD95$^+$ and CD95$^-$ NK cells (data not shown).

CD56$^+$ NK cell depletion induces the rapid activation of MBP-reactive memory T cells in PBMCs from 'CD95$^+$ NK-high' multiple sclerosis

We have previously shown that the CD95$^+$ NK cells found in multiple sclerosis patients resemble the NK cells that can be induced in culture in the presence of IL-4 and anti-IL-12

mAb [referred to as 'NK2-like cells' according to the definition by Peritt *et al.* (1998)]. We also found that Peritt's NK2 cells induced *in vitro* inhibited the induction of IFN-γ-secreting T cells from peripheral T cells after stimulation with phorbol myristate acetate and ionomycin (Takahashi *et al.*, 2001). Based on these observations, we speculated that NK cells might prohibit Th1 cell activation in the remission of multiple sclerosis in an antigen-non-specific manner, and contribute to maintaining the remission. However, it remained an open question as to whether the NK2-like cells found in MS-rem would indeed regulate pathogenic autoimmune T cells *in vivo*. To investigate functions of NK cells in MS-rem, we evaluated the effect of NK cell depletion on the peripheral T cell response to MBP, a major target antigen of multiple sclerosis (Bielekova *et al.*, 2000). In brief, we depleted $CD56^+$ cells from the PBMCs with a magnetic sorter, and then stimulated the NK-deleted populations as well as whole PBMCs with MBP *in vitro* for 8–24 h. Subsequently, we detected the antigen-responsive T cells based on the secretion of IFN-γ (Manz *et al.*, 1995). The preparatory experiments revealed that 8 h of stimulation provides an optimal condition yielding a low background (0–0.03%). This novel assay enables us to selectively detect memory-type Th1 cells that can respond rapidly to antigen, whereas previous assays that depend on long-term cultures (Pette *et al.*, 1990; Martin *et al.*, 1992) evaluate not only memory but also naive T cells. Of note, there is a general consensus that peripheral blood of multiple sclerosis patients contains MBP-reactive T cells that are activated and/or differentiated into memory T cells (Allegretta *et al.*, 1990; Martin *et al.*, 1992; Zhang *et al.*, 1994; Lovett-Racke *et al.*, 1998; Scholz *et al.*, 1998).

We examined 16 PBMC samples from 14 MS-rem patients (nine samples from '$CD95^+$ NK-high', and seven from '$CD95^+$ NK-low') and 14 healthy subjects (see Table 1). When freshly isolated PBMCs were stimulated with MBP before NK cell depletion, four MS-rem and five healthy subjects samples showed a marginal response to MBP (0.01–0.03% increase of IFN-γ-positive cells among $CD4^+$ T cells). We did not find any significant response to MBP with the other PBMC samples. In contrast, when cells were stimulated with MBP after deleting $CD56^+$ NK cells, a significant response with a stimulatory index >3 was detected in seven of the nine '$CD95^+$ NK-high' samples, and a marginal response was detected in two (Fig. 2A and B). Of note, none of the NK-deleted samples from the '$CD95^+$ NK-low' patients and healthy subjects showed a definitive response to MBP. The difference for the '$CD95^+$ NK-high' versus the '$CD95^+$ NK-low' or healthy subjects was statistically significant (Fig. 2B). These *ex vivo* experiments have revealed that the '$CD95^+$ NK-high' patients may possess a higher number of T cells that can rapidly respond to MBP (MBP-specific memory T cells), compared with '$CD95^+$ NK-low' MS-rem or healthy subjects. In other words, they provide strong evidence for clonal expansion of memory autopathogenic T cells in the '$CD95^+$ NK-high' patients. However, as we could demonstrate an increase of the memory autoimmune T cells only after depleting NK cells, we interpreted that the potentially hazardous autoimmune T cells are being controlled by counter-regulatory NK cells in the '$CD95^+$ NK-high' patients. Of note, previous studies relying on alternative assays have revealed the presence of MBP-reactive T cells with activated and/or memory phenotypes at similar high frequencies in not all, but a major portion, of multiple sclerosis patients (Allegretta *et al.*, 1990; Zhang *et al.*, 1994; Bieganowska *et al.*, 1997; Lovett-Racke *et al.*, 1998; Scholz *et al.*, 1998; Illés *et al.*, 1999).

We conducted the same assay with a foreign antigen OVA in three of the '$CD95^+$ NK-high' (PBMC codes #3, #4 and #5 in Table 1) and one of the '$CD95^+$ NK-low' samples (#6). However, OVA-reactive T cells could not be detected in any sample of the fresh or NK-deleted PBMCs (data not shown). Because NK cells cannot discriminate T cells with different antigen specificities, the negative response to OVA in the four multiple sclerosis patients was interpreted to mean that they do not possess clonally expanded memory T cells reactive to OVA.

Depletion of $CD16^+$ NK cells also allows detection of MBP-reactive memory T cells in PBMCs from '$CD95^+$ NK-high' multiple sclerosis

Although we used anti-CD56 magnetic beads to deplete NK cells in the above experiments, the method would also deplete $CD3^+CD56^+$ NK T cells that may possibly play a role in the regulation of autoimmunity. To evaluate the possible contribution of $CD3^+CD56^+$ NK T cells, we next depleted NK cells from PBMCs from two '$CD95^+$ NK-high' patients on the basis of their expression of CD16. We found that after treatment with CD16-MicroBeads, almost all of $CD56^+$ NK cells are deleted, but $CD56^+CD3^+$ NKT cells remain largely untouched (Fig. 3A). However, like $CD56^+$-cell-deleted PBMCs, the $CD16^+$-cell-deleted PBMCs responded to MBP, as assessed by the induction of IFN-γ-secreting $CD4^+$ T cells (Fig. 3B). The responses found in the two patients were considered significant with regard to both percentage increase of IFN-γ-secreting cells (0.08% and 0.04%) and the stimulatory index (9.0 and 5.0) obtained after MBP stimulation. This result indicates that responsible cells to regulate autoimmune T cells in '$CD95^+$ NK-high' multiple sclerosis are not $CD56^+CD3^+$ NK T cells but NK cells.

Unfortunately, it remains unclear whether only $CD95^+$ NK cells play a regulatory role in '$CD95^+$ NK-high' multiple sclerosis or whether $CD95^-$ cells could also exhibit regulatory functions in the patients. We attempted to compare directly the function of $CD95^+$ and $CD95^-$ populations. However, isolation of $CD95^+$ NK cells with a cell sorter invariably induced cell activation as revealed by the expression of various activation markers. Furthermore, the isolated cells tended to die rapidly, probably due to CD95 ligation by the antibody (data not shown).

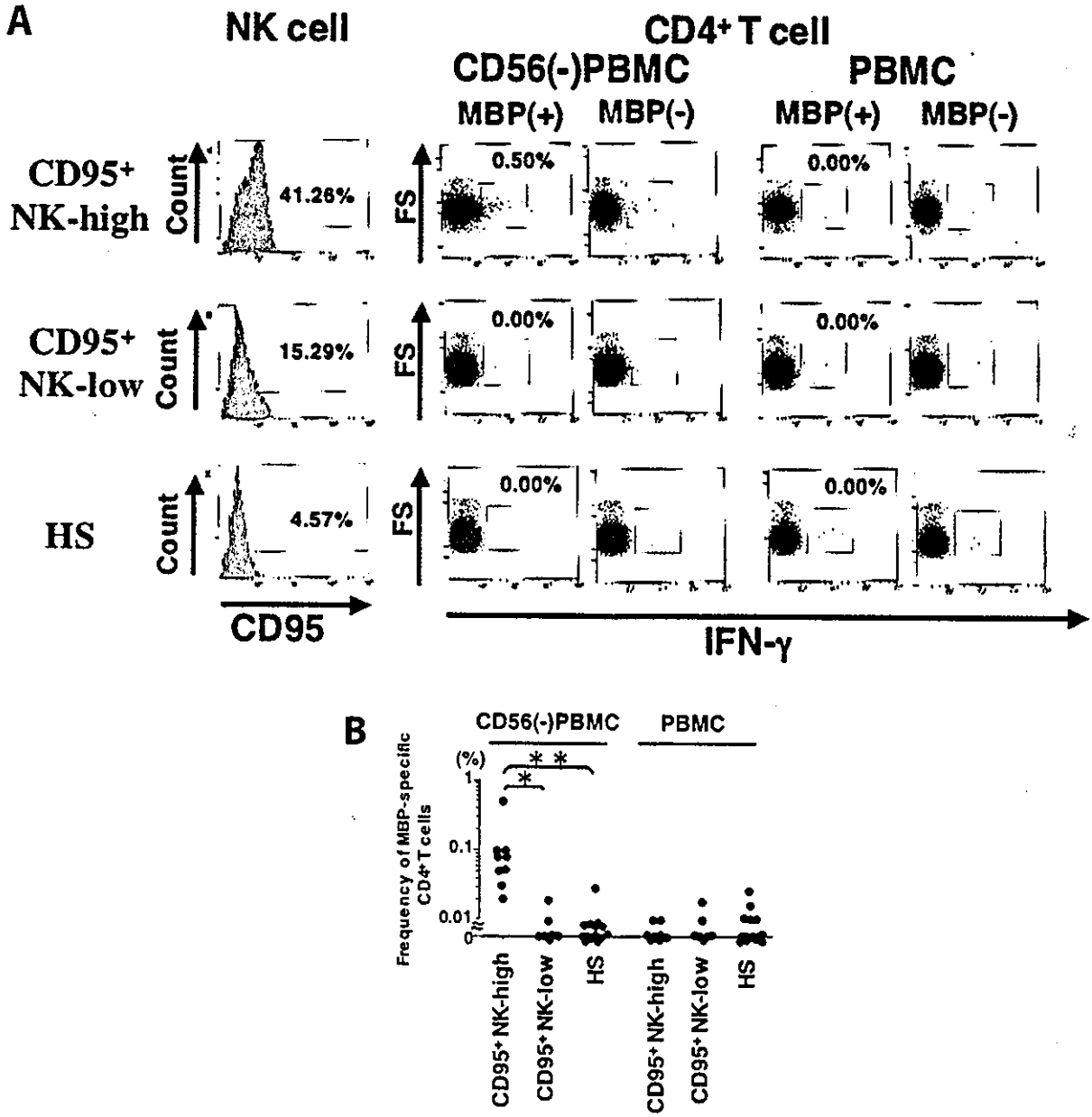

Fig. 2 Evidence for the role of NK cells in the regulation of MBP-reactive memory T cells in 'CD95$^+$ NK-high' multiple sclerosis. (A) IFN-γ secretion assay for NK-cell-deleted PBMCs and freshly isolated PBMCs. Whole PBMCs or PBMCs depleted for CD56$^+$ NK cells [CD56(−) PBMC] from the 'CD95$^+$ NK-high' multiple sclerosis (n = 9), 'CD95$^+$ NK-low' multiple sclerosis (n = 7) or healthy subjects (n = 14) were stimulated with 10 μg/ml of human MBP for 8 h for the IFN-γ secretion assay. The cells were also stained with anti-CD4-PC5 and -CD3-FITC, and the CD4$^+$CD3$^+$ and PI$^-$ cells were gated for analysis. Here we show representative results from 'CD95$^+$ NK-high' (*top*), 'CD95$^+$ NK-low' (*middle*) and healthy subjects (*bottom*). The IFN-γ-secreting CD4$^+$ T cells are shown as red dots; blue dots represent IFN-γ-négative cells. The histograms demonstrate the level of CD95 expression on the fresh CD56$^+$ NK cells from each individual, and the attached values show the frequency of CD95$^+$ cells. (B) Frequency (%) of MBP-reactive memory T cells among CD4$^+$ T cells. By using the cytokine secretion assay, we determined the frequency of IFN-γ-positive cells among CD4$^+$ T cells in each individual after culture with or without MBP. Here we plot the Δ% values [(%) with MBP − (%) without MBP], which represent the frequency of MBP-reactive CD4$^+$ T cells in each subject. Kruskal–Wallis test with Scheffe's F *post hoc* test was used for statistical analysis. $^*P < 0.05$; $^{**}P < 0.02$.

NK cells from 'CD95$^+$ NK-high' multiple sclerosis inhibit IFN-γ production by MBP-reactive T cell clones

To analyse how the NK cells from 'CD95$^+$ NK-high' multiple sclerosis efficiently control autoimmune T cell responses, we established three MBP-specific TCC from a 'CD95$^+$ NK-high' patient. These TCC proliferated and secreted IFN-γ, TNF-α, IL-2 and IL-5 in response to MBP presented by irradiated, fresh autologous PBMCs. Using the proliferation response and cytokine secretion by

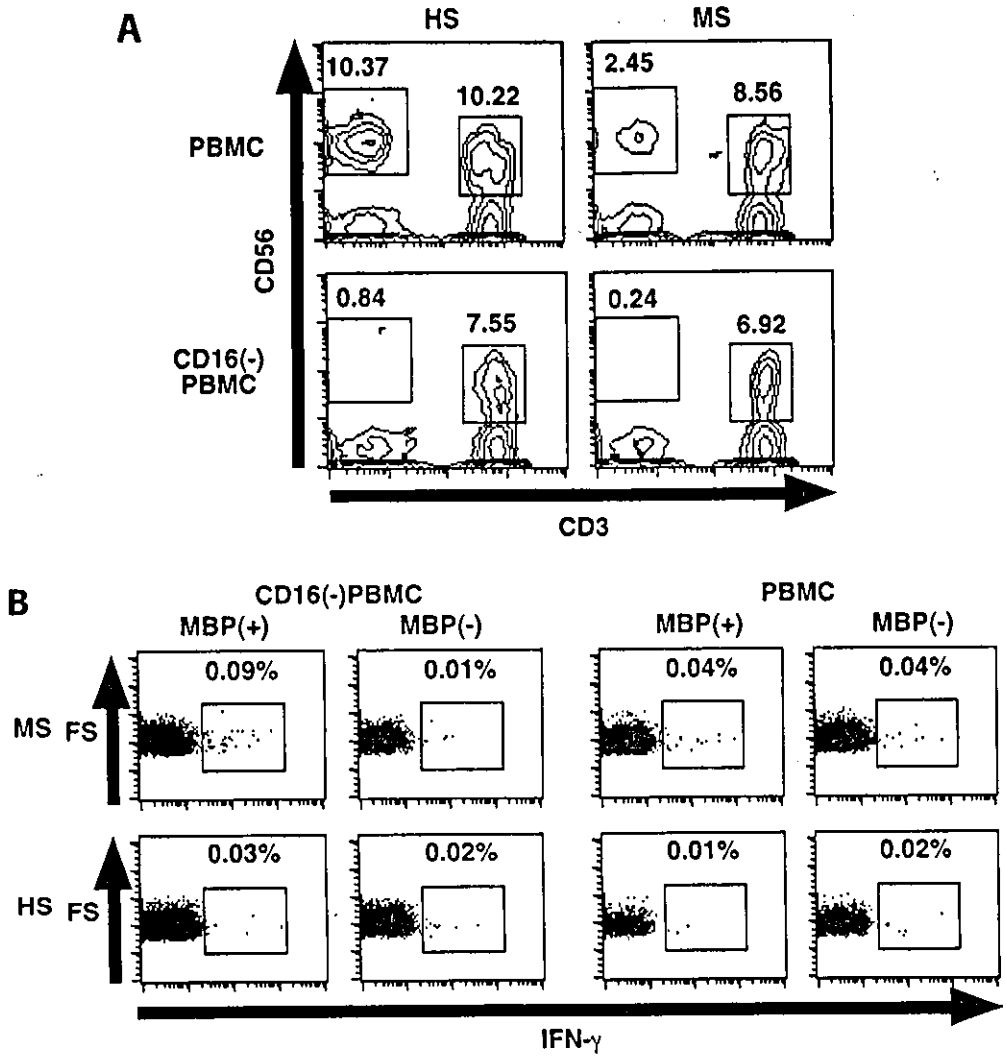

Fig. 3 Depletion of CD16$^+$ cells also allows detection of MBP-specific memory T cells in 'CD95$^+$ NK-high' multiple sclerosis. (**A**) Changes in the frequency of CD56$^+$ NK cells and CD56$^+$ NKT cells after deleting CD16$^+$ cells. Using CD16 microbeads, we deleted CD16$^+$ cells from PBMCs from two 'CD95$^+$ NK-high' patients and from two healthy subjects. The cells were stained with anti-human CD3-FITC and anti-CD56-PC5 to check the proportion of CD56$^+$ NK cells and CD56$^+$ T cells before and after CD16$^+$ cell depletion (upper versus lower panels). Shown are the results of a representative pair of multiple sclerosis and healthy subjects. (**B**) CD16$^+$-cell-depleted PBMCs from 'CD95$^+$ NK-high' multiple sclerosis responded rapidly to MBP. Using the same PBMC samples (CD16$^+$ or CD16$^-$), we conducted the IFN-γ secretion assay as described in Fig. 2A. This figure shows the result of the representative pair of multiple sclerosis patients and healthy subjects.

the TCC as read-out, we compared the whole PBMCs and the NK cell-deleted PBMCs for the ability to present whole MBP to the autologous TCC. We found that the whole PBMCs did not differ from the NK-deleted PBMCs in the ability to induce MBP-driven proliferation of TCC (Fig. 4A). However, the proportion of IFN-γ-secreting T cells among the TCC increased significantly when the NK cell-depleted PBMCs were used as antigen presenting cells (APC) (Fig. 4B). We also noticed a significant elevation of IFN-γ in the culture supernatant along with the increase of IFN-γ-secreting T cells (Fig. 4C). However, neither TNF-α nor IL-2 production was enhanced by NK cell depletion. These results support the view that NK cells from 'CD95$^+$ NK-high' multiple sclerosis regulate autoimmune T cells by inhibiting the T cell production of IFN-γ.

Discussion

It is generally held that relapse of multiple sclerosis represents the destructive CNS inflammation triggered by recently activated autoimmune T cells. In other words, pathogenic autoimmunity is apparently active during clinical relapse, which can be objectively defined by clinical status as well as MRI findings. In contrast, remission of multiple sclerosis, which is chiefly determined by exclusion of active inflammation in the CNS, may probably cover a wider range of disease states.

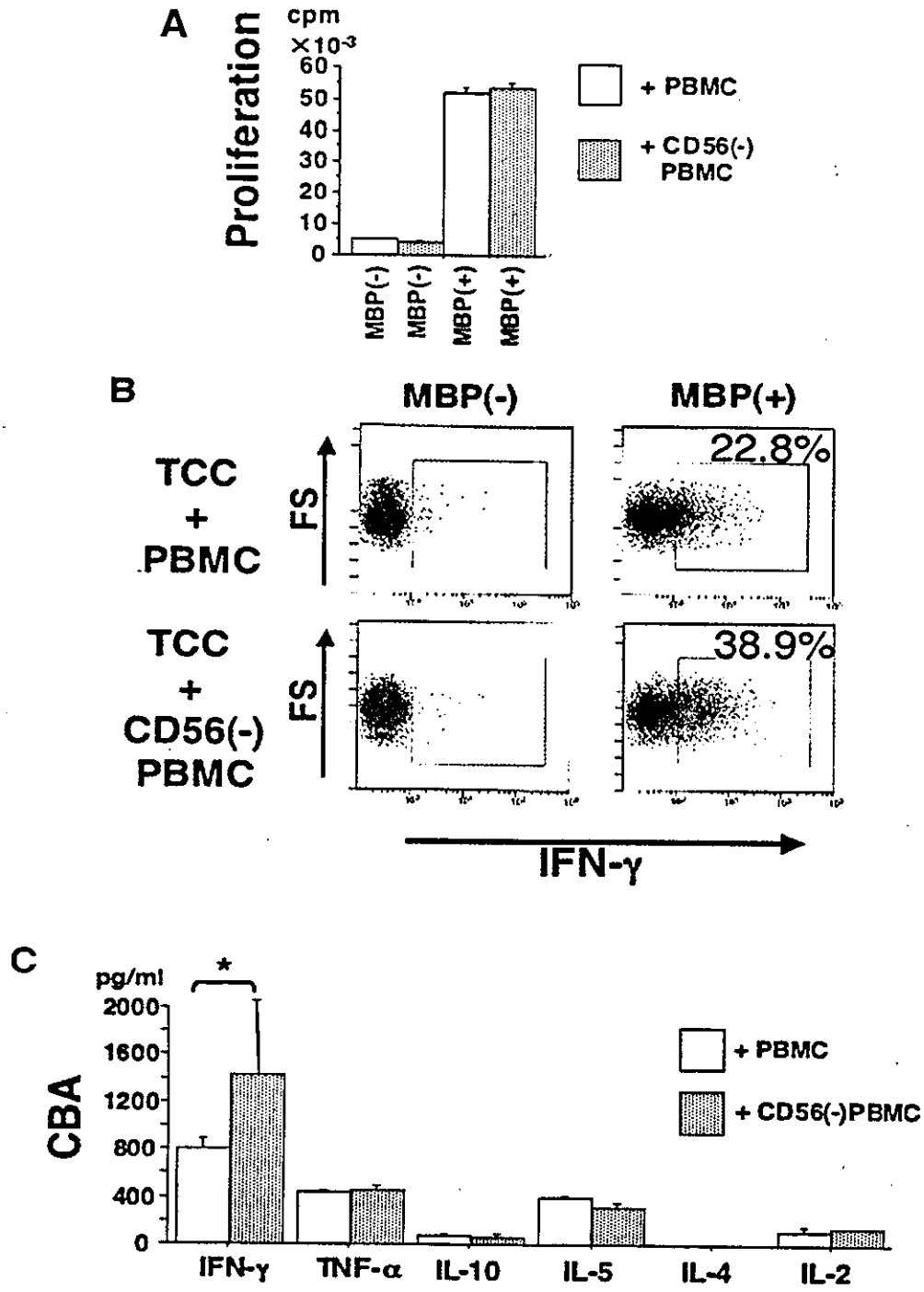

Fig. 4 Depletion of NK cells augments the antigen-presenting potential of PBMCs from 'CD95$^+$ NK-high' multiple sclerosis. (A) Effect of NK-cell deletion on the proliferation of MBP-specific TCC. We established three MBP-specific TCC from a 'CD95$^+$ NK-high' patient, and evaluated the proliferative 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

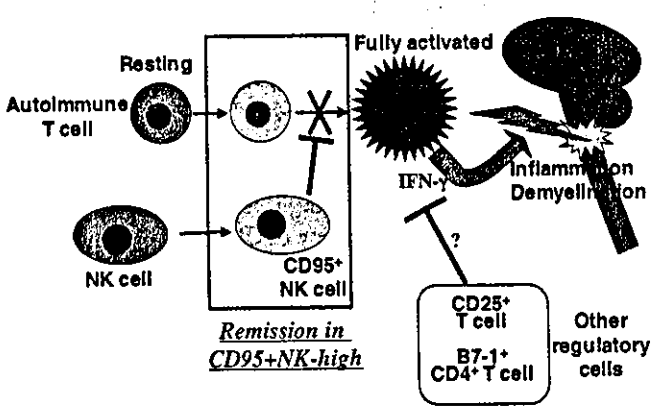

Fig. 5 The role of NK cells in 'CD95$^+$ NK-high' multiple sclerosis. As described in the text, the 'CD95$^+$ NK-high' patients are characterized by a concurrent increase of memory autoimmune T cells and CD95$^+$ NK cells. In the sense that memory autoimmune T cells cannot be detected in other patients 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.

Acknowledgements

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Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice

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Abstract

Non-obese diabetic (NOD) mice develop diabetes mediated by pathogenic T-helper type 1 (Th1) cells. Vα14 Natural killer (NKT) cells are a unique lymphocyte subtype implicated in the regulation of autoimmunity and a good source of protective Th2 cytokines. We recently developed a Th2-skewing NKT cell ligand, OCH. OCH, a sphingosine truncated derivative of α-galactosylceramide (α-GC), stimulates NKT cells to selectively produce Th2 cytokines. Here we show that OCH prevented the development of diabetes and insulitis in NOD mice. The suppression of insulitis by OCH was more profound compared to α-GC. Infiltration of T cells, B cells and macrophages into islets is inhibited in OCH-treated NOD mice. OCH-mediated suppression of diabetes is associated with Th2 bias of anti-islet antigen response and increased IL-10 producing cells among islet-infiltrating leukocytes. Considering the non-polymorphic and well conserved features of the CD1d molecule in mice and humans, these findings not only support the proposed role of NKT cells in the regulation of self-tolerance but also highlight the potential use of OCH for therapeutic intervention in type I diabetes.

Keywords: NKT cells; Autoimmune disease; Type I diabetes; NOD mice; Glycolipid

1. Introduction

Non-obese diabetic (NOD) mice develop spontaneous autoimmune (type I) diabetes (T1D) very similar to the human disease. In female mice, insulitis usually begins at 3 to 5 weeks of age, eventually leading to β-cell destruction and overt diabetes by 4 to 6 months of age. Autoimmune destruction of β-cells is preceded by infiltration of pancreatic islets by macrophages, B cells and T cells. The capacity to transfer disease by islet specific T cells purified from diabetic NOD mice or T cell clones demonstrates the key role of T cells in the pathogenesis of diabetes. Th1 type CD4 cells, which preferentially secrete IFN-γ and TNF-α and CD8 T cells, have been implicated in the development of diabetes in NOD mice [1,2]. In parallel with these effector T cells, regulatory cells including $CD4^+CD25^+$ T cells have been suggested to inhibit the development of diabetes. Although the mechanisms of suppressive effect of these regulatory T cells are not fully understood, it is believed that an imbalance between autoreactive effector T cells and regulatory T cells may trigger the development of destructive insulitis and T1D [3]. Previous studies indicate that the β-cell-destructive immune response in NOD mice is biased toward Th1 and treatment with Th2 cytokines such as IL-4 or IL-10 have been shown to prevent the onset of spontaneous diabetes [4–8].

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Natural killer T (NKT) cells are a unique subset of T cells that coexpress receptors of the NK lineage and α/β T cell receptor (TCR) [9–11]. NKT cells express an invariant TCRα chain (encoded by a Vα14-Jα281 rearrangement in mice and a homologous Vα24-Jα15 rearrangement in humans). Unlike conventional T cells that recognize peptides in association with MHC, NKT cells recognize glycolipid antigens such as α-galactosylceramide (α-GC) bound by the non-polymorphic MHC class I-like protein CD1d [12]. One striking feature of NKT cells is their capacity to secrete large amounts of cytokines including IL-4 and IFN-γ in response to TCR ligation. Although the precise function of NKT cells remains to be elucidated, evidence indicates that NKT cells play critical roles in the regulation of autoimmune responses [13–15]. Abnormalities in the number and function of NKT cells have been observed in patients with autoimmune diseases as well as in a variety of mouse strains that are genetically predisposed for development of autoimmune diseases. The putative involvement of NKT cells in the control of islet β-cell reactive T cells in NOD mice was suggested by prevention of diabetes following infusion of NKT cell-enriched thymocyte preparations [16] and by over-expression of NKT cells in Vα14-Jα281 transgenic NOD mice [17]. Moreover, several recent studies have investigated the effect of treating NOD mice with α-GC [18–21]. When started at around 3 or 4 weeks of age, repeated injections at least once a week delayed the onset and reduced the incidence of diabetes by inducing Th2 bias of autoreactive T cells. We have recently developed a synthetic glycolipid ligand, OCH, which stimulates NKT cells to selectively produce IL-4. OCH is a synthetic glycolipid, sphingosine truncation analogue of α-GC [22]. Administration of OCH inhibited experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) by inducing Th2 bias of autoreactive T cells [22,23]. These findings led us to examine the effect of OCH on the development of diabetes in NOD mice.

In the present study, we show that OCH can inhibit the development of insulitis and diabetes in NOD mice by inducing a Th2 bias of autoreactive T cells. These results imply that targeting NKT cells with OCH could be an attractive means for intervention in T1D.

2. Materials and methods

2.1. Mice

C57BL/6(B6) mice were purchased from CLEA Laboratory Animal Corp. (Tokyo, Japan). NOD/Shi mice were obtained from CLEA Japan (Tokyo, Japan). The animals were kept under specific pathogen-free conditions. We followed the guidelines for the use and care of laboratory animals of National Institute of Neuroscience, NCNP.

2.2. In vitro responses of NKT cells to α-GC or OCH

Splenocytes of naïve B6 mice were cultured with α-GC or OCH in RPM I1640 medium supplemented with 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/mg/ml penicillin/streptomycin, and 1% syngenic mouse serum for 72 h. 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). The levels of IL-4, IL-10 and IFN-γ in culture supernatant were measured by a standard sandwich ELISA, using purified and biotinylated antibody pairs and standards from BD PharMingen (San Jose, CA).

2.3. In vivo responses of NKT cells to α-GC or OCH

NOD mice were injected with 100 μg/kg of OCH or α-GC intraperitoneally and serum were collected after 2 h, 6 h and 24 h after injection. Serum levels of IL-4, IL-10 and IFN-γ were measured by ELISA.

2.4. Assessment of diabetes and evaluation of insulitis

Diabetes was assessed by monitoring glucose levels every week in the blood using GLU-W (Fujifilm, Kanagawa, Japan). Mice with two consecutive positive blood glucose measurements greater than 250 mg/dl were considered diabetic. For histological evaluation of insulitis, mice were killed and pancreata were removed and fixed with 4% paraformaldehyde. Sections were stained with hematoxylin and eosin (HE). Multiple HE stained pancreatic sections were scored. Insulitis was graded as follows: grade 0, no inflammation; grade 1, peri-insulitis but no intra-insulitis; grade 2, mild intra-insulitis (cell infiltration in the area less than 25% of an islet); grade 3, moderate intra-insulitis (cell infiltration in the area more than 25% but less than 50% of an islet); and grade 4, severe intra-insulitis (cell infiltration in the area more than 50% of an islet).

2.5. Immunohistochemistry

Immunohistochemistry was performed on 15-mm-thick adjacent serial sections according to the avidin–biotin–peroxidase complex (ABC) method using ABC Elite (Vector) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen. Primary antibodies were used as follows; mouse CD4 (RM4-5, BD PharMingen), mouse CD8a (53-6.7, BD PharMingen), mouse B220 (RA3-6B2, BD PharMingen), mouse F4/80 antigen (A3-1, Serotec), mouse CD45 (sc-1121, Santa Cruz

Biotechnology Inc.), mouse interleukin-4 (11B11, ATCC), mouse interleukin-10 (JES-2A5), and mouse interferon-γ (XMG1.2, BD PharMingen). The biotinylated goat anti-mouse antibody or the biotinylated rabbit anti-goat antibody (Vector, Burlingame, CA, USA) was used as a secondary antibody. Cell infiltration was graded as follows: no infiltration, peri-infiltration, cell infiltration in peri-islet area but no intra-islet; mild infiltration, cell infiltration in less than 50% of the area of an islet; severe infiltration, cell infiltration in more than 50% of the area of an islet. For the quantification of cytokine staining, more than 100 CD45 positive cells per mice (five animals in each group) were evaluated for the IL-4 or IL-10 staining.

2.6. In vivo glycolipids treatment

α-GC and OCH were synthesized as described previously [24]. Synthetic glycolipids were used to treat NOD mice. Starting from 5 weeks of age, mice were injected intraperitoneally twice per week with either OCH or α-GC at a dose of 100 μg/kg. The control mice were injected with vehicle alone (10% DMSO in PBS).

2.7. Measurement of autoantigen specific IgG1 and IgG2a

Anti-glutamic acid decarboxylase (GAD) was measured by ELISA as described previously [19,25]. GAD (Sigma) (1 mg/ml) was coated onto ELISA plates (Sumitomo Bakelite, Co., Ltd, Tokyo, Japan) at 4 °C overnight. After blocking with 1% bovine serum albumin in PBS, serially diluted serum samples were added onto GAD-coated wells. For detection of anti-GAD antibodies, the plates were incubated with biotin-labeled anti-IgG1 and anti-IgG2a (Southern Biotechnology Associates, Inc., Brimingham, AL) or anti-IgG antibody (CN/Cappel, Aurora, OH) for 1 h and then incubated with streptavidin-peroxidase. After adding a substrate, the reaction was evaluated and antibody titers were calculated on the basis of dilution/absorbance curves. To control the experiments, dilutions of anti-GAD-positive serum from diabetic NOD mice were used as the control samples. Based on the standard values of the control samples, the relative value for each test sample was displayed.

3. Results

3.1. OCH induces selective production of Th2 cytokines in NOD mice

NOD mice have been reported to exhibit the defect in the number and the function of NKT cells [26]. We first examined whether OCH stimulates proliferation and selective IL-4 production in NOD mice. Spleen cells from NOD mice proliferated in response to in vitro stimulation with OCH and produced significant amounts of IL-4 and IL-10, although OCH was less active in inducing cell proliferation and the cytokine production compared with α-GC (Fig. 1A). In contrast, IFN-γ was barely detectable in response to OCH stimulation, whereas α-GC induces massive IFN-γ production (Fig. 1A). We next examined whether OCH treatment in vivo also induces the selective Th2 cytokines. We injected OCH or α-GC intraperitoneally in NOD mice and measured the serum level of IL-4, IL-10 and IFN-γ by ELISA. Consistent with in vitro data, OCH injection induced a rise in IL-4 and IL-10 (Fig. 1B, left and right panels) along with much less increase in the levels of IFN-γ (Fig. 1B, middle panel). In contrast, injection of α-GC induced the production of IL-4, IL-10 and IFN-γ. These results suggest that OCH preferentially induces Th2 cytokines in NOD mice similar to B6 mice.

3.2. OCH treatment prevents diabetes and insulitis in NOD mice

IL-4 and IL-10 have been reported as important protective cytokines for diabetes in NOD mice [4–8,16]. Thus, we tested whether multiple injections of OCH to NOD mice can modulate the development of diabetes. As shown in Fig. 2A, treatment of OCH starting at 5 weeks of age significantly delayed the onset and reduced the incidence of diabetes from 75% to 27% in female NOD mice at 30 weeks of age. α-GC inhibited the development of diabetes as well as previously described [18–21].

Next we examined HE-stained sections from pancreata of mice treated with either glycolipid ligands or vehicle twice per week from the age of 5 weeks. As shown in Fig. 2B, significantly greater percentages of islets were free of insulitis from OCH-treated mice than those from the control mice. Fifty percent of islets were free of infiltrating cells in OCH-treated mice, at which time less than 20% of islets were from vehicle-treated mice. The percentage of islets affected by severe insulitis (grade 4) was significantly lower ($p = 0.023$, Dunnett's multiple comparison tests) and the frequency of intact islet (grade 0) was significantly higher ($p = 0.0038$, Dunnett's multiple comparison tests) in OCH-treated mice than in α-GC treated mice. The mean score of insulitis of α-GC- or OCH-treated mice was significantly lower than vehicle-treated mice. Typical histological appearance was shown in Fig. 2C. These results indicate that the inhibitory effect of insulitis by OCH is stronger than α-GC even though OCH was about less active in inducing NKT cell proliferation (Fig. 1A). We further examined whether a particular subset of cells are preferentially affected by OCH treatment at the age of