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Intrathecal activation of the IL-17/IL-8 axis in opticospinal multiple sclerosis

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Summary

There are two distinct subtypes of multiple sclerosis in Asians, opticospinal (OS-multiple sclerosis) and conventional (C-multiple sclerosis). In OS-multiple sclerosis, selective and severe involvement of the optic nerves and spinal cord is characteristic, though its mechanisms are unknown. The present study aimed to find out possible differences in the cytokine/chemokine profiles in CSF between OS-multiple sclerosis and C-multiple sclerosis and to delineate the relationships between these profiles and neuroimaging and pathological features. Sixteen cytokines/chemokines, namely interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1β (MIP-1β), were measured simultaneously in CSF supernatants from 40 patients with relapsing–remitting multiple sclerosis (20 OS-multiple sclerosis and 20 C-multiple sclerosis) at relapse and 19 control patients with spinocerebellar degeneration (SCD), together with intracellular production of IFN-γ and IL-4 in CSF $CD4^+$ T cells. In CSF supernatants relative to controls, IL-17, MIP-1β, IL-1β and IL-13 were only significantly increased in OS-multiple sclerosis patients, while TNF-α was only significantly increased in C-multiple sclerosis patients, using a cut-off level of 1 pg/ml. IL-8 was significantly elevated in both OS-multiple sclerosis and C-multiple sclerosis patients. MCP-1 was significantly decreased in both OS-multiple sclerosis and C-multiple sclerosis patients, while IL-7 was only significantly decreased in C-multiple sclerosis patients. IL-17, IL-8 and IL-5 were significantly higher in OS-multiple sclerosis patients than in C-multiple sclerosis patients. The increases in IL-17 and IL-8 in OS-multiple sclerosis were still significant even after exclusion of the patients undergoing various immunomodulatory therapies. Assays of intracellular cytokine production revealed that both the IFN-γ^+IL-4^- T-cell percentage and intracellular IFN-γ/IL-4 ratio in CSF cells were significantly greater in C-multiple sclerosis patients than in controls. Contrarily, OS-multiple sclerosis patients showed not only a significantly greater percentage of IFN-γ^+IL-4^- T cells than controls but also a significantly higher percentage of IFN-γ^-IL-4^+ T cells than C-multiple sclerosis patients. Among the cytokines elevated in multiple sclerosis, only IL-8 showed a significant positive correlation with the Expanded Disability Status Scale of Kurtzke score. Both the length of the spinal cord lesions on MRI and the CSF/serum albumin ratio had a significant positive correlation with IL-8 and IL-17 in multiple sclerosis, in which the spinal cord lesions were significantly longer in OS-multiple sclerosis than in C-multiple sclerosis. Three of six spinal cord specimens from autopsied OS-multiple sclerosis cases demonstrated numerous myeloperoxidase-positive neutrophils infiltrating necrotic lesions. These findings strongly suggest that in OS-multiple sclerosis, in addition to the Th1 cell upregulation seen in C-multiple sclerosis, intrathecal activation of the IL-17/IL-8 axis inducing heavy neutrophil infiltration contributes to extensive spinal cord lesion formation.

Keywords: multiple sclerosis; cytokine; chemokine; cerebrospinal fluid; neutrophil

Abbreviations: C-multiple sclerosis = conventional form of multiple sclerosis; EAE = experimental allergic encephalomyelitis; EDSS = Expanded Disability Status Scale of Kurtzke; G-CSF = granulocyte colony-stimulating factor; IFN = interferon; IL = interleukin; LP = lumbar puncture; MCP-1 = monocyte chemoattractant protein-1; MIP-1β = macrophage

inflammatory protein-1β; NMO = neuromyelitis optica; OS-multiple sclerosis = opticospinal form of multiple sclerosis; PBL = peripheral blood lymphocyte; SCD = spinocerebellar degeneration; TNF = tumour necrosis factor

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Introduction

Multiple sclerosis is a chronic inflammatory disease of the CNS characterized by macrophage and lymphocyte infiltration, demyelination, axonal injury and loss of neurological function. It has been hypothesized, but not yet proven, to be caused by an autoimmune mechanism targeting CNS myelin.

Cytokines are soluble proteins that mediate and regulate interactions between cells of the immune system, and are key mediators of autoimmune attack against CNS myelin (Huang *et al*., 1999). In multiple sclerosis, many prior studies have documented that proinflammatory (Th1 type) cytokines such as interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-2 and IL-12 (p40) are involved in the onset and perpetuation of the disease, while, in contrast, anti-inflammatory (Th2 type) cytokines such as IL-4, IL-10 and transforming growth factor-β are downregulated during phases of disease activity and upregulated in phases of disease remission (Panitch *et al*., 1987; Sharief and Hentges, 1991; Sharief and Thompson, 1993; Link *et al*., 1994; Rieckmann *et al*., 1994; Matusevicius *et al*., 1996; Navikas and Link, 1996; Navikas *et al*., 1996; Monteyne *et al*., 1997; Fassbender *et al*., 1998; Link, 1998; Huang *et al*., 1999; van Boxel-Dezaire *et al*., 1999).

It has been hypothesized that the progression of multiple sclerosis, and perhaps even its induction, may be causally related to dysregulation of the balance between Th1 and Th2 cytokines (Olsson, 1995). However, other reports have suggested that the Th1/Th2 cytokine paradigm for multiple sclerosis is an oversimplification, and that various other immune cells, including Th2, $CD8^+$ T and B cells, are involved in the complex and heterogeneous mechanisms in this condition (Laman *et al*., 1998; Hemmer *et al*., 2002; Lassmann and Ransohoff, 2004). Multiple cytokines often function as complexes, with the function of one inducing the function of another in a cascade effect. In this regard, multiplexed fluorescent bead-based immunoassays, which have a dynamic range of standard curves and require only small amounts of material for simultaneous measurements of numerous cytokines and chemokines, are more useful than enzyme-linked immunosorbent assay (ELISA) methods (Vignali, 2000; Kellar *et al*., 2001; de Jager *et al*., 2003).

Multiple sclerosis is rare in Asians but, when it does appear, the destruction of the optic nerves and spinal cord is striking (Kira, 2003). We previously reported the existence of two subtypes of multiple sclerosis in Japanese, the opticospinal form (OS-multiple sclerosis) that shows selective and severe involvement of the optic nerves and spinal cord, and the conventional form (C-multiple sclerosis) that shows disseminated lesions in the CNS including the cerebrum, cerebellum and brainstem (Kira *et al*., 1996). The two subtypes have different clinical and neuroimaging features, and immunogenetic backgrounds (Kira *et al*., 1996; Yamasaki *et al*., 1999; Kira, 2003). OS-multiple sclerosis has distinct features, such as a higher age at onset, marked female preponderance and a higher Kurtzke's Expanded Disability Status Scale (EDSS) score (Kurtzke, 1983), resulting from severe visual impairment and marked spinal cord dysfunction compared with C-multiple sclerosis. Severe inflammatory destruction has been suggested in OS-multiple sclerosis, because of the occasionally higher cell counts and protein amounts in the CSF, as well as long swollen lesions extending over several vertebral segments on spinal cord MRI (Kira, 2003). Furthermore, pathological studies have revealed not only demyelination, but also axonal loss, necrosis, cavity formation, thickened vessel wall and capillary proliferation in OS-multiple sclerosis lesions (Shiraki, 1965; Ikuta *et al*., 1982; Tabira and Tateishi, 1982). These clinico-pathological features of OS-multiple sclerosis in Asians are similar to those of a relapsing–remitting form of Devic's neuromyelitis optica (NMO) in a Western population (Wingerchuk *et al*., 1999; Cree *et al*., 2002; Lucchinetti *et al*., 2002). Considerable overlap and common mechanisms between the two conditions are supposed, as seen in the recent discovery of NMO-IgG, commonly found in both (Lennon *et al*., 2004); however, the immune mechanisms responsible for such distinct clinico-pathological features remain unknown.

Although many studies have been published on cytokine/chemokine alterations in multiple sclerosis, subtype-related alterations have been poorly characterized. We previously reported that OS-multiple sclerosis showed a significant Th1/Tc1 shift through relapse and remission phases in peripheral blood lymphocytes (PBLs), while C-multiple sclerosis only showed a significant Th1 shift during a relapse phase (Horiuchi *et al*., 2000; Wu *et al*., 2000; Ochi *et al*., 2001). In CSF, except for one report disclosing a greater increase in macrophage migration inhibitory factor (MIF) in OS-multiple sclerosis than in C-multiple sclerosis (Niino *et al*., 2000), OS-multiple sclerosis-related changes in CSF cytokine/chemokine profiles have not been investigated, probably because of the low concentrations of cytokines/chemokines and the fragility and limited numbers of CSF cells.

Therefore, in the present study, we attempted to uncover OS-multiple sclerosis-related cytokine/chemokine alterations in CSF that could explain the distinct neuroimaging and pathological features. First, we simultaneously measured 16 cytokines/chemokines, namely IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IFN-γ, TNF-α, granulocyte colony-stimulating factor (G-CSF),

Table 1 *Demographic features of OS-multiple sclerosis and C-multiple sclerosis patients*

	CSF supernatants assays		Intracellular cytokine production assays	
	OS-multiple sclerosis	C-multiple sclerosis	OS-multiple sclerosis	C-multiple sclerosis
No. of patients	20	20	11	13
Sex (F/M)	19/1	16/4	11/0	11/2
Age at onset (years)	44.2 ± 16.9*	25.8 ± 8.9	48.5 ± 21.8*	30.2 ± 15.2
Age at LP (years)	51.0 ± 15.1*	31.6 ± 9.6	54.6 ± 18.3*	37.2 ± 14.3
Duration of disease (years)	6.3 ± 5.4	6.1 ± 7.1	6.1 ± 5.0	7.2 ± 8.3
No. of relapses	7.3 ± 5.1	6.1 ± 5.7	5.7 ± 4.0	5.9 ± 6.0
EDSS	5.5 ± 2.3*	4.0 ± 1.8	5.0 ± 2.2	4.4 ± 1.8
CSF				
Cell count (/μl)	3.6 ± 8.0	4.2 ± 6.8	0.8 ± 1.2	1.8 ± 1.7
Total protein (mg/dl)	43.0 ± 22.5	37.7 ± 23.8	37.4 ± 15.2	39.6 ± 18.3
CSF/serum albumin ratio (10^{-4})	90.0 ± 44.0*	58.0 ± 41.2	91.5 ± 41.4*	52.0 ± 24.1
IgG index	0.674 ± 0.179	0.837 ± 0.414	0.843 ± 0.694	0.853 ± 0.306

Values are expressed as the mean ± SD. The CSF cell count and total protein amount in the control patients used for the CSF supernatant assays were 0.6 ± 0.6/μl and 28.6 ± 9.5 mg/dl, respectively, while those used for the intracellular cytokine production assays were 0.8 ± 1.1/μl and 33.5 ± 9.6 mg/dl, respectively. The numbers of patients whose CSF/serum albumin ratio and IgG index were measured were 14 in OS-multiple sclerosis and 16 in C-multiple sclerosis in the supernatants assays and eight in OS-multiple sclerosis and eight in C-multiple sclerosis in the intracellular cytokine production assays. OS-multiple sclerosis = opticospinal form of multiple sclerosis; C-multiple sclerosis = conventional form of multiple sclerosis; LP = lumbar puncture; EDSS = Expanded Disability Status Scale of Kurtzke. *$P < 0.05$.

monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1β (MIP-1β), in the CSF from OS-multiple sclerosis and C-multiple sclerosis patients at relapse using a multiplexed fluorescent bead-based immunoassay. Secondly, we examined the intracellular production of IFN-γ and IL-4 in CSF $CD4^+$ T cells from OS-multiple sclerosis and C-multiple sclerosis patients by flow cytometry. Thirdly, we analysed relationships among CSF cytokine/chemokine changes and clinical and spinal cord MRI findings, as well as neuropathological findings for autopsied spinal cord specimens from multiple sclerosis patients.

Materials and methods

Patients

Cytokine and chemokine assays of CSF supernatants were performed using CSF from multiple sclerosis patients exclusively at the time of clinical relapse (within 30 days of the onset of acute or subacute exacerbation). For these assays, 40 patients with relapsing–remitting multiple sclerosis [five males and 35 females; age at examination: 41.5 ± 15.9 years (mean ± SD), range: 18–89] who were diagnosed with multiple sclerosis based on McDonald's diagnostic criteria (McDonald *et al*., 2001) at the Department of Neurology, Kyushu University Hospital were enrolled in this study. In addition, 19 patients with spinocerebellar degeneration (SCD) (nine males and 10 females; age: 59.4 ± 10.9 years, range: 32–80) were used as controls. The multiple sclerosis patients were clinically classified into two subtypes: OS-multiple sclerosis and C-multiple sclerosis, as described previously (Kira *et al*., 1996). Briefly, patients who showed a relapsing–remitting course and had both optic nerve and spinal cord involvement without any clinical evidence of disease in either the cerebrum or cerebellum were considered to have OS-multiple sclerosis. Patients who showed minor brainstem signs, such as transient double vision and nystagmus, in addition to opticospinal involvement, were included in this subtype. All other patients who showed multiple involvement of the CNS, including the cerebrum and cerebellum, were considered to have C-multiple sclerosis. The disability status of the patients was scored by one of the authors (T.I.) throughout the study, according to the EDSS (Kurtzke, 1983). The mean times from symptom onset to lumbar puncture (LP) were 13.9 days (range: 1–30 days) for OS-multiple sclerosis and 12.8 days (range: 1–30 days) for C-multiple sclerosis; the two did not differ significantly. Of the 40 multiple sclerosis patients, five C-multiple sclerosis and 10 OS-multiple sclerosis patients had received IFN-β or high-dose corticosteroids at the time of LP. The demographic features of the patients are summarized in Table 1. The ages at onset and LP, the EDSS score and CSF/serum albumin ratio were significantly higher in OS-multiple sclerosis than in C-multiple sclerosis ($P = 0.0000064$, 0.0000049, 0.019 and 0.023, respectively), while the disease duration, number of relapses, CSF cell count, total protein amount and IgG index did not differ significantly between the two.

For analysis of intracellular IFN-γ and IL-4 in CSF cells, 24 patients with multiple sclerosis (two males and 22 females; age: 45.2 ± 18.2 years, range: 21–89) who fulfilled McDonald's criteria and 12 control patients with various other non-inflammatory neurological diseases (seven males and five females; age: 52.4 ± 17.2 years, range: 20–75) were enrolled. In the multiple sclerosis group, 11 had OS-multiple sclerosis and 13 had C-multiple sclerosis. Of these, 21 were relapsing–remitting multiple sclerosis (11 OS-multiple sclerosis and 10 C-multiple sclerosis) and three were secondary progressive multiple sclerosis (three C-multiple sclerosis). The control group of 12 patients was comprised of two with amyotrophic lateral sclerosis, two with Alzheimer's disease, two with cervical spondylosis, one with SCD, one with Parkinson's disease, one with progressive supranuclear palsy, one with spinal cord infarction, one with epilepsy and one with conversion hysteria. The demographic features of the patients are also summarized in Table 1. The ages at onset and LP, and CSF/serum albumin ratio were significantly higher in OS-multiple sclerosis than in C-multiple sclerosis ($P = 0.017$, 0.028 and 0.028, respectively).

CSF sample collection

At least 5 ml of CSF was obtained from all patients by non-traumatic LP. Twenty-three CSF samples (all at the relapse phase) from 20 OS-multiple sclerosis patients, 22 CSF samples (all at the relapse phase) from 20 C-multiple sclerosis patients, and 19 CSF samples from 19 control patients were obtained for extracellular cytokine analysis. Neither multiple sclerosis nor control patients had any ongoing or recent infection at the time of LP. Intracellular IFN-γ and IL-4 analyses were performed using 11 CSF samples (10 at the relapse phase and one at the remission phase) from 11 OS-multiple sclerosis patients, 13 CSF samples (seven at the relapse phase, three at the remission phase and three at the progressive phase) from 13 C-multiple sclerosis patients, and 12 CSF samples from 12 control patients. CSF samples were immediately centrifuged at 800 r.p.m./min at 4°C for 5 min, and the supernatants stored at −70°C until analysis. The cell counts, total protein amounts, CSF/serum albumin ratio and IgG index used for the study are shown in Table 1.

Multiplexed fluorescent bead-based immunoassay

CSF supernatants were collected and analysed simultaneously for 16 different cytokines and chemokines, namely IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IFN-γ, TNF-α, G-CSF, MCP-1 and MIP-1β, using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions (Kellar *et al*., 2001; de Jager *et al*., 2003). Briefly, 50 µl of each CSF supernatant and various concentrations of each cytokine standard (Bio-Rad) were added to 50 µl of antibody-conjugated beads (Bio-Rad) in a 96-well filter plate (Millipore, Billerica, MA). After a 30 min incubation, the plate was washed and 25 µl of a biotinylated antibody solution (Bio-Rad) was added to each well, followed by another 30 min incubation. The plate was then washed and 50 µl of streptavidin-conjugated phycoerythrin (PE; Bio-Rad) was added to each well and incubated for 10 min. Following a final wash, the contents of each well were resuspended in 125 µl of assay buffer (Bio-Rad) and analysed using a Bio-Plex Array Reader (Bio-Rad). The cytokine concentrations were calculated by reference to a standard curve for each cytokine derived using various concentrations of the cytokine standards (0.2, 0.78, 3.13, 12.5, 50, 200, 800 and 3200 pg/ml) assayed in the same manner as the CSF samples. The same lots of monoclonal antibodies for Bio-Plex Cytokine Assay System were used throughout the experiments, and the inter- and intra-assay variability was reported to be <10% (manufacturer's instructions). The detection limit of each cytokine was determined by the recovery of the corresponding cytokine standard, and the lowest values showing >50% recovery were set as the lower detection limits. The lower detection limit for each cytokine was as follows: 0.2 pg/ml for IL-2, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IFN-γ and TNF-α, 0.78 pg/ml for IL-1β and IL-6, and 3.13 pg/ml for G-CSF, MCP-1 and MIP-1β. All samples were analysed undiluted in duplicate.

Intracellular cytokine analysis by flow cytometry

Each CSF supernatant was carefully removed and the cell sediment was suspended in RPMI 1640 (Nipro, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD; Lot # 3217341S), followed by incubation with 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO), 1.0 µg/ml ionomycin (Sigma) and 10 µg/ml brefeldin A (BFA; Sigma) in a 24-well plate at 37°C for 4 h under 5% CO_2. After washing with phosphate-buffered saline containing 0.1% bovine serum albumin (0.1% BSA–PBS), cells were stained with perCP-conjugated anti-CD4 monoclonal antibodies (Immunotech, Marseille, France) and incubated on ice in the dark for 15 min. Following another wash with 0.1% BSA–PBS, fluorescence-activated cell sorting (FACS) permeabilizing solution (Becton Dickinson, San Jose, CA) was added and the cells were placed in the dark for 10 min. After two washes with 0.1% BSA–PBS, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ (Immunotech) and PE-conjugated anti-IL-4 (Immunotech) antibodies for intracellular cytokine analysis, or with mouse IgG2a–FITC (Immunotech) and IgG1–PE (Immunotech) as controls, respectively. After a 30 min incubation on ice in the dark, the percentages of intracellular IFN-γ- and IL-4-producing cells were immediately analysed by flow cytometry using an Epics XL System II (Coulter, Hialeah, FL). Analysis gates were first set on lymphocytes according to the forward and side scatter properties and then on $CD4^+$ lymphocytes. Cases with a $CD4^+$ cell count of <500 were discarded from the analysis to increase the reliability.

Neuroimaging of the spinal cord

For spinal cord MRI, T2-weighted (SE 2500–4900/113–116) and T1-weighted (SE 400–500/11–12) images were obtained in the sagittal and axial planes. For the contrast-enhanced study, MRI was initiated 2–3 min after intravenous administration of gadolinium-pentetic acid (0.1 mmol/kg), using the T1-weighted sequences in the sagittal and axial planes. Lengths of spinal cord lesions were expressed in cm. Longitudinally extensive spinal cord lesions were defined as those extending over three vertebral spine lengths, which are considered to be exceptional in multiple sclerosis (McDonald *et al*., 2001). Spinal cord MRIs taken at the time of relapse when the CSF samples were drawn (within 30 days of the onset of acute or subacute exacerbation) were evaluated independently by two examiners, of whom one (F.M.) was an experienced neuroradiologist and was blinded to the diagnosis. Spinal cord MRIs at the relapse were available for 34 of 40 multiple sclerosis patients (17 OS-multiple sclerosis and 17 C-multiple sclerosis patients) and, of these, 30 had spinal cord symptomatology (17 OS-multiple sclerosis and 13 C-multiple sclerosis).

Histopathological analysis of infiltrating cells in autopsied spinal cord specimens of OS-multiple sclerosis and C-multiple sclerosis

For the neuropathological analysis, spinal cord specimens obtained at autopsy from six OS-multiple sclerosis and two C-multiple sclerosis cases were used. Each autopsied specimen was fixed in 10% buffered formalin for several weeks, and then embedded in paraffin. Sections were either stained with haematoxylin and eosin, or immunostained using rabbit polyclonal antibodies against myeloperoxidase (1 : 500; NeoMarkers, Fremont, CA) or myelin basic protein (1 : 200; DAKO, Denmark) or mouse monoclonal antibodies against phosphorylated neurofilaments (1 : 200; DAKO; clone 2F11), macrophages (1 : 500; DAKO; clone KP1), T-cell antigen (1 : 500; DAKO; clone UCHL1) or B-cell antigen (1 : 500; DAKO; clone L26). Autoclave or microwave pre-treatment was performed to retrieve the antigens for immunostaining. The number of infiltrating cells, including neutrophils, macrophages, and T and B cells, was averaged for four separate fields (each field ×200) in the lesion in the same cross-section of the spinal cord.

Statistical analysis

Fisher's exact probability test was employed for comparisons of the detection rates of cytokines and chemokines in each group. The non-parametric Mann-Whitney *U* test was employed for comparison of the cytokine and chemokine levels in each group. The difference between PBLs and CSF in each group was analysed by Wilcoxon signed-rank test. Statistical significance was set at $P < 0.05$. Spearman's rank correlation analysis was used for statistical analysis of correlations between various clinical parameters and CSF cytokine levels.

Results

Detection rates of each cytokine and chemokine in CSF supernatants

The detection rate of IL-10 was significantly higher in OS-multiple sclerosis and C-multiple sclerosis patients than in control patients (91.3 versus 36.8%, $P = 0.00027$ in OS-multiple sclerosis; 81.8 versus 36.8%, $P = 0.0046$ in C-multiple sclerosis), while that of IL-7 was significantly lower in OS-multiple sclerosis and C-multiple sclerosis patients than in control patients (60.9 versus 100%, $P = 0.002$ in OS-multiple sclerosis; 68.2 versus 100%, $P = 0.0098$ in C-multiple sclerosis). In addition, IL-17 was significantly higher in OS-multiple sclerosis patients than in control patients (73.9 versus 36.8%, $P = 0.028$), while TNF-α was significantly higher in C-multiple sclerosis patients than in control patients (77.3 versus 42.1%, $P = 0.029$). The detection rates of other cytokines did not differ significantly between control patients and the two multiple sclerosis subtypes. When the five C-multiple sclerosis and 10 OS-multiple sclerosis patients on immunomodulatory therapies were excluded, essentially the same results were obtained, except that the increased detection rate of TNF-α in C-multiple sclerosis lost significance. IL-2 was not used for further statistical analysis because of its extremely low detection rate (<20% in total) in CSF.

Comparison among diseases of cytokine and chemokine levels in CSF supernatants

The cytokine and chemokine levels in CSF supernatants from controls, OS-multiple sclerosis and C-multiple sclerosis patients analysed by the multiplexed fluorescent bead-based immunoassay are shown in Fig. 1. Among the 16 cytokines examined, IL-17, MIP-1β, IL-13 and IL-1β were only significantly increased in OS-multiple sclerosis patients compared with control patients. IL-8, IL-10 and TNF-α were significantly increased in both OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients. On the other hand, IL-7 and MCP-1 were significantly decreased in both OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients. When the cytokine levels were compared between the two multiple sclerosis subtypes, IL-17, IL-8 and IL-5 were significantly increased in OS-multiple sclerosis patients compared with C-multiple sclerosis patients.

Considering the possibly low reliability for the lower ranges of the cytokine/chemokine concentrations (<1 pg/ml), we set 1 pg/ml as the cut-off level and reanalysed the data. Even when this cut-off level was used, the increased IL-17, MIP-1β, IL-13 and IL-1β in OS-multiple sclerosis patients, increased TNF-α in C-multiple sclerosis patients, increased IL-8 in both OS-multiple sclerosis and C-multiple sclerosis patients, decreased IL-7 in C-multiple sclerosis patients and decreased MCP-1 in both OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients, and higher levels of IL-17, IL-8 and IL-5 in OS-multiple sclerosis patients than in C-multiple sclerosis patients were all still statistically significant. When the five C-multiple sclerosis and 10 OS-multiple sclerosis patients on immunomodulatory therapies were excluded, the increased IL-17 and MIP-1β in OS-multiple sclerosis patients, increased IL-8 in both OS-multiple sclerosis and C-multiple sclerosis patients, and decreased MCP-1 in C-multiple sclerosis patients compared with control patients, and higher levels of IL-17 in OS-multiple sclerosis patients than in C-multiple sclerosis patients still held statistical significance using this cut-off level. The statistical significances for the cytokine/chemokine changes are summarized in Table 2 according to cut-off level and the presence or absence of immunomodulatory therapies.

Comparison of the intracellular cytokine production in CD4$^+$ T cells between CSF and PBLs and among diseases

IFN-γ$^+$IL-4$^-$ CD4$^+$ T-cell percentages were significantly higher in CSF cells than in PBLs for all three groups (Fig. 2). In PBLs, there were no significant differences in the IFN-γ$^+$IL-4$^-$ CD4$^+$ T-cell percentages among the three groups, while in CSF cells, IFN-γ$^+$IL-4$^-$ CD4$^+$ T-cell percentages were significantly increased in both OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients. For the IFN-γ$^-$IL-4$^+$ CD4$^+$ T-cell percentages, CSF cells only showed a significantly lower value than PBLs in control patients. There was no significant change in the IFN-γ$^-$IL-4$^+$ T-cell percentages between PBLs and CSF cells in either OS-multiple sclerosis or C-multiple sclerosis patients, and in OS-multiple sclerosis patients, CSF cells even showed a higher IFN-γ$^-$IL-4$^+$ T-cell percentage than PBLs. In PBLs, IFN-γ$^-$IL-4$^+$ CD4$^+$ T-cell percentages were significantly decreased in OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients. In CSF cells, IFN-γ$^-$IL-4$^+$ CD4$^+$ T-cell percentages did not differ significantly between control and OS-multiple sclerosis patients, while C-multiple sclerosis patients had a significantly lower IFN-γ$^-$IL-4$^+$ CD4$^+$ T-cell percentage than OS-multiple sclerosis patients, and showed a lower value than control patients although the difference was not significant.

Consequently, the intracellular IFN-γ/IL-4 ratio in CD4$^+$ T cells was significantly higher in CSF cells than in PBLs in

Table 2 *Summary of cytokine/chemokine changes in CSF supernatants*

	Altered cytokine/chemokine	All samples	⩾1 pg/ml*	⩾1 pg/ml*, no immunomodulatory drugs
OS-multiple sclerosis versus control	IL-17	↑ $P = 0.0031$	↑ $P = 0.0038$	↑ $P = 0.0093$
	IL-8	↑ $P = 0.000009$	↑ $P = 0.000009$	↑ $P = 0.0014$
	MIP-1β	↑ $P = 0.0042$	↑ $P = 0.0042$	↑ $P = 0.024$
	IL-1β	↑ $P = 0.027$	↑ $P = 0.039$	NS
	IL-13	↑ $P = 0.027$	↑ $P = 0.022$	NS
	IL-10	↑ $P = 0.000051$	NS	NS
	TNF-α	↑ $P = 0.026$	NS	NS
	MCP-1	↓ $P = 0.04$	↓ $P = 0.04$	NS
	IL-7	↓ $P = 0.0048$	NS	NS
C-multiple sclerosis versus control	IL-8	↑ $P = 0.0012$	↑ $P = 0.0012$	↑ $P = 0.015$
	TNF-α	↑ $P = 0.0055$	↑ $P = 0.015$	NS
	IL-10	↑ $P = 0.00054$	NS	NS
	MCP-1	↓ $P = 0.034$	↓ $P = 0.034$	↓ $P = 0.024$
	IL-7	↓ $P = 0.000053$	↓ $P = 0.013$	NS
OS-multiple sclerosis versus C-multiple sclerosis	IL-17	↑ $P = 0.045$	↑ $P = 0.024$	↑ $P = 0.028$
	IL-8	↑ $P = 0.029$	↑ $P = 0.029$	NS
	IL-5	↑ $P = 0.0048$	↑ $P = 0.0051$	NS

NS = not significant; ↑ = elevated; ↓ = decreased. *The cut-off level was 1 pg/ml.

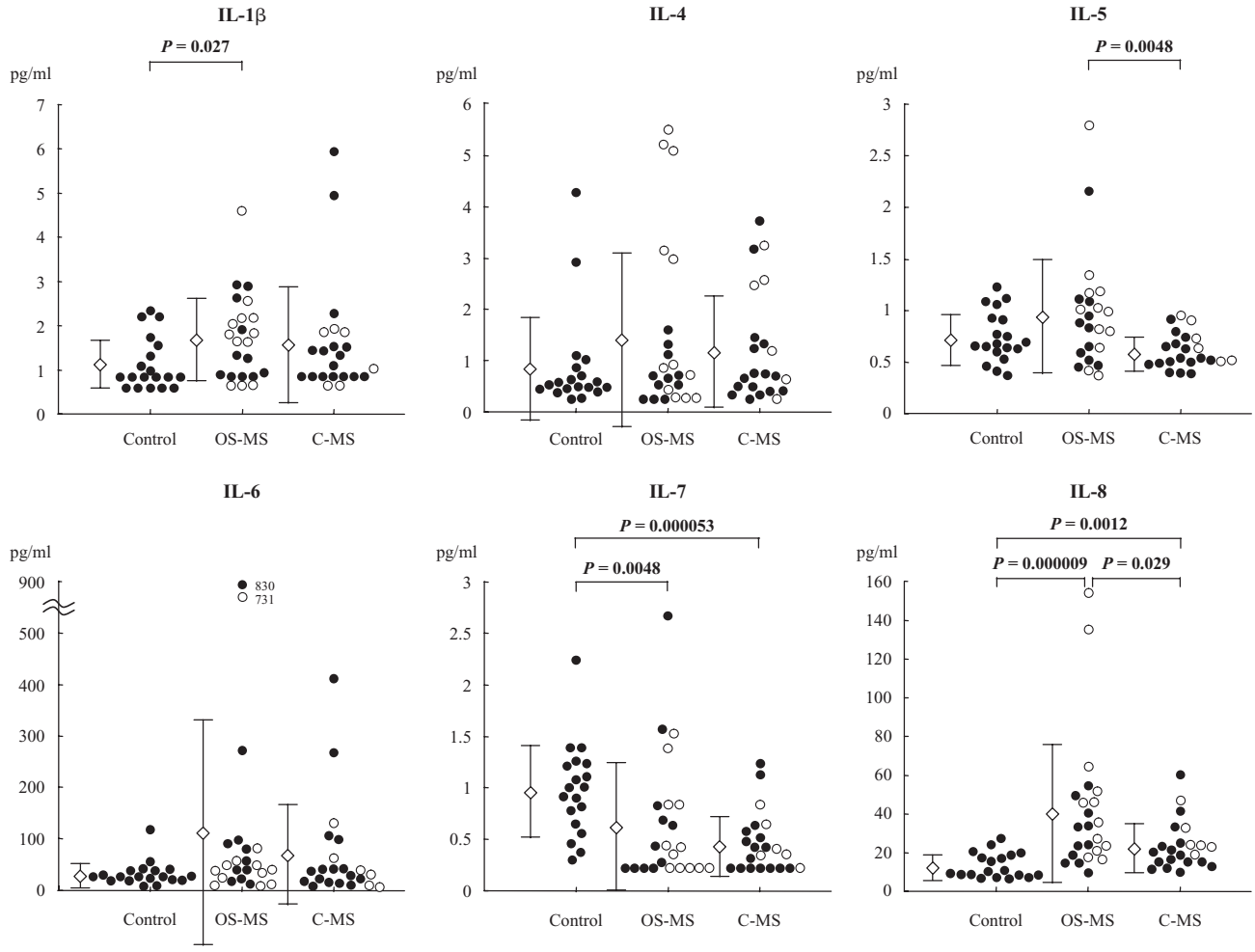

Fig. 1 Continued.

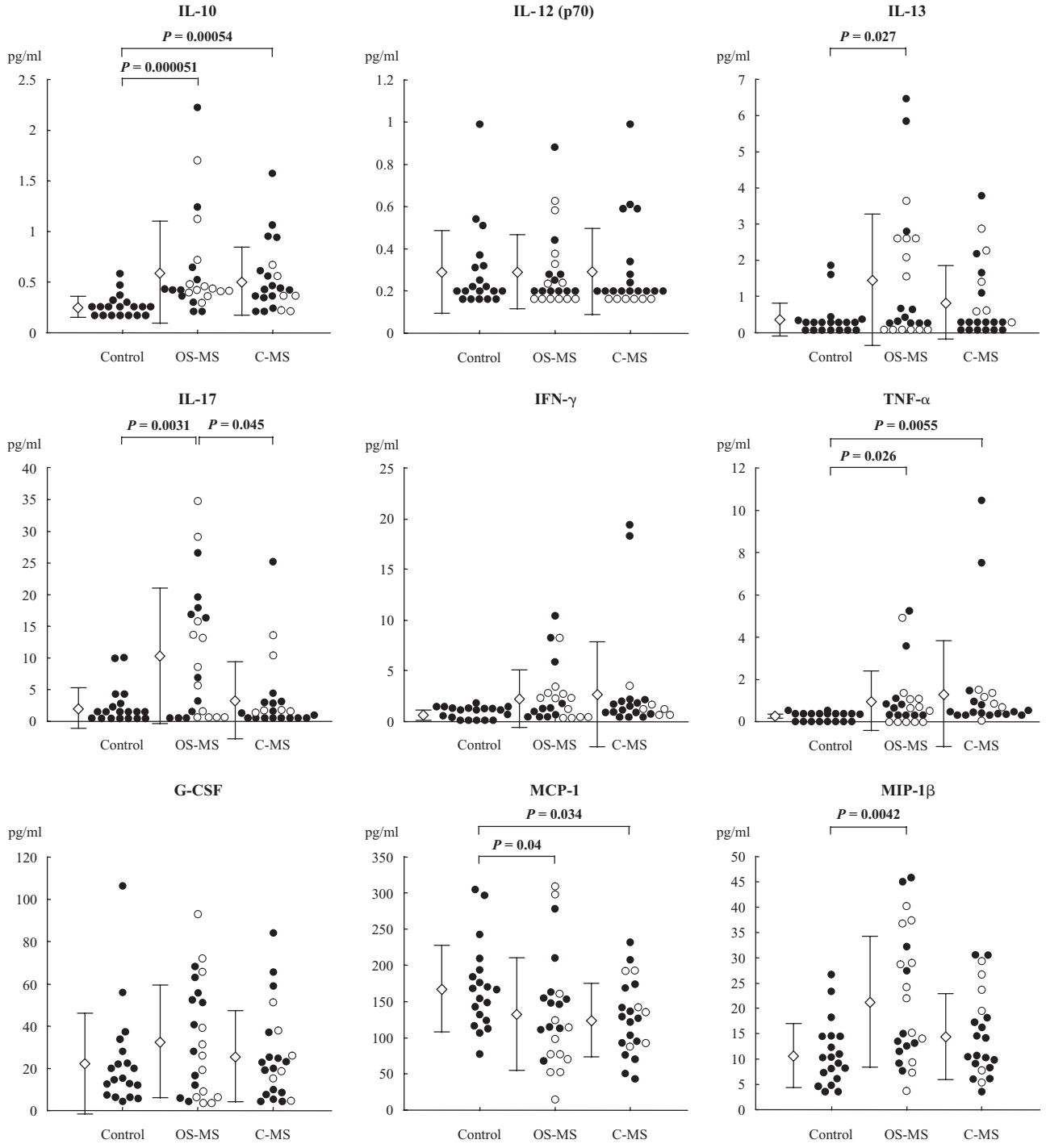

Fig. 1 Cytokine and chemokine levels in CSF supernatants from patients with SCD (controls), OS-multiple sclerosis and C-multiple sclerosis assessed by the multiplexed fluorescent bead-based immunoassay. There were 47 samples in total: 19 controls, 23 OS-multiple sclerosis and 22 C-multiple sclerosis. Multiple sclerosis samples were all obtained from patients at relapse. Open circles indicate patients who were under immunomodulatory therapies (12 OS-multiple sclerosis and six C-multiple sclerosis samples), while closed circles indicate those who were not (11 OS-multiple sclerosis and 16 C-multiple sclerosis samples). IL-2 is not shown due to its low detection frequency in CSF. Open diamonds and bars indicate the mean ± SD for each group.

both control and C-multiple sclerosis patients, while it did not differ significantly between CSF cells and PBLs in OS-multiple sclerosis patients. In PBLs, the ratio was significantly elevated in both OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients, whereas in CSF cells, the ratio was significantly increased only in C-multiple sclerosis and not OS-multiple sclerosis patients. IFN-γ^+IL-4^+ $CD4^+$ T-cell percentages were significantly

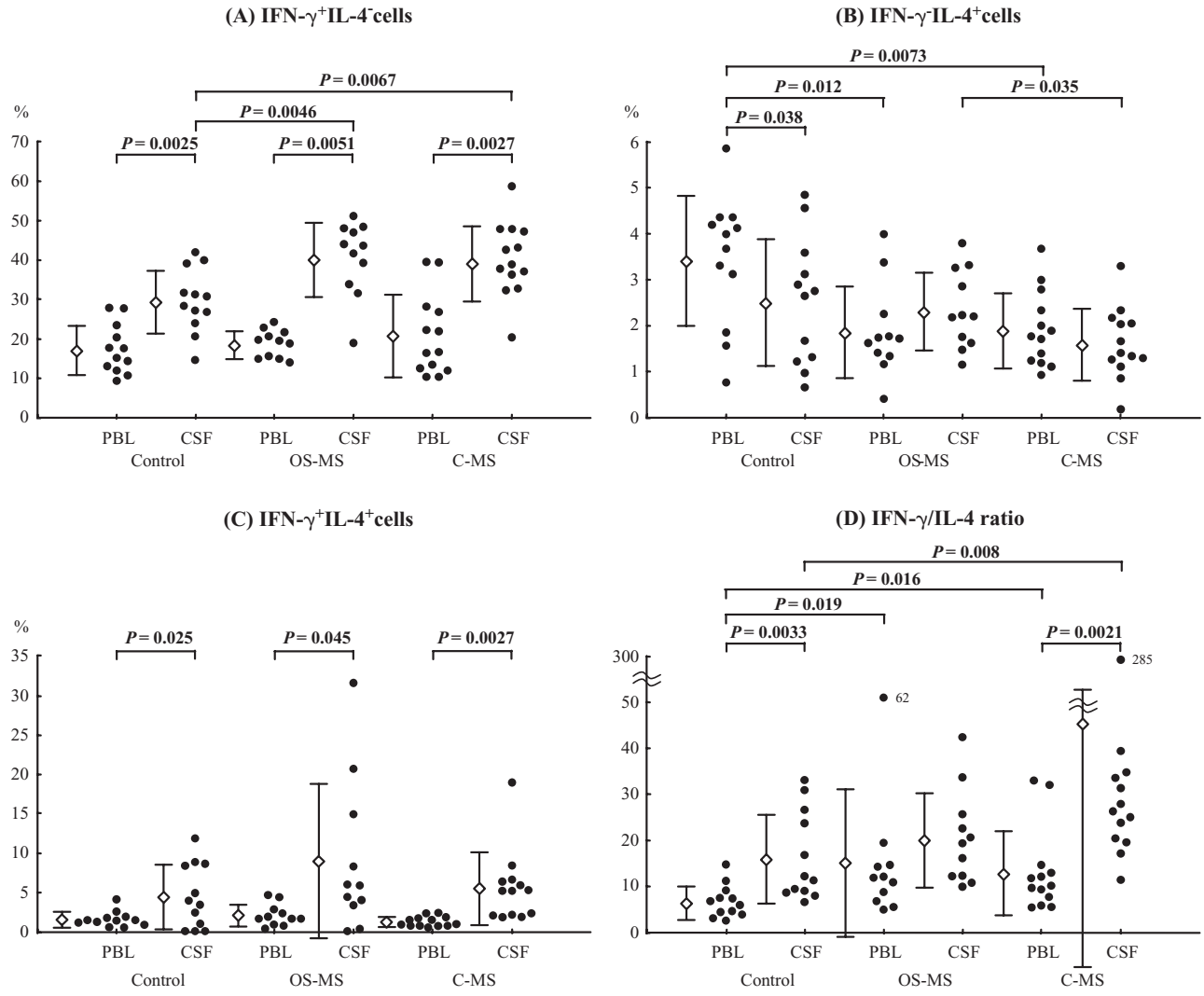

Fig. 2 Intracellular cytokine production patterns of PBLs and CSF CD4$^+$ T cells from OND (other non-inflammatory neurological diseases; control), OS-multiple sclerosis and C-multiple sclerosis patients. (**A**) IFN-γ^+IL-4$^-$ CD4$^+$ T-cell percentages. (**B**) IFN-γ^-IL-4$^+$ CD4$^+$ T-cell percentages. (**C**) IFN-γ^+IL-4$^+$ CD4$^+$ T-cell percentages. (**D**) Intracellular IFN-γ/IL-4 ratios in CD4$^+$ T cells. There were 36 samples in total: 12 controls, 11 OS-multiple sclerosis and 13 C-multiple sclerosis. Open diamonds and bars indicate the mean ± SD for each group.

higher in CSF cells than in PBLs in all three groups. IFN-γ^+IL-4$^+$ CD4$^+$ T-cell percentages did not differ significantly among control, OS-multiple sclerosis and C-multiple sclerosis patients in either PBLs or CSF cells.

Neuroimaging findings of spinal cord

Longitudinally extensive spinal cord lesions (Fig. 3) were found in 17 of 34 (50.0%) multiple sclerosis patients around the time their CSF was examined. The frequency of longitudinally extensive spinal cord lesions was higher in OS-multiple sclerosis than in C-multiple sclerosis (12 out of 17, 70.6 versus five out of 17, 29.4%, $P = 0.038$). In addition, the spinal cord lesions were also longer in OS-multiple sclerosis than in C-multiple sclerosis (5.5 ± 3.1 versus 2.7 ± 3.3 cm, $P = 0.021$).

Correlations between clinical parameters and cytokine levels in CSF supernatants in multiple sclerosis

Among the cytokines/chemokines elevated in multiple sclerosis CSF supernatants, only IL-8 showed a significant correlation with the EDSS score (Kurtzke, 1983) in multiple sclerosis (Fig. 4). Significant positive correlations with the CSF/serum albumin ratio as well as CSF protein concentration were found for IL-8 and IL-17. Moreover, lengths of spinal cord lesions on MRI significantly correlated with IL-8 and IL-17 levels. Multiple sclerosis patients with longitudinally extensive spinal cord lesions on MRI (>3 vertebral length) had significantly higher levels of IL-8 and IL-17 than those without such lesions [49.23 ± 38.36 versus 16.35 ± 6.08 pg/ml (mean ± SD), $P = 0.000039$ for IL-8; 13.35 ± 11.43 versus 2.70 ± 4.97 pg/ml (mean ± SD),

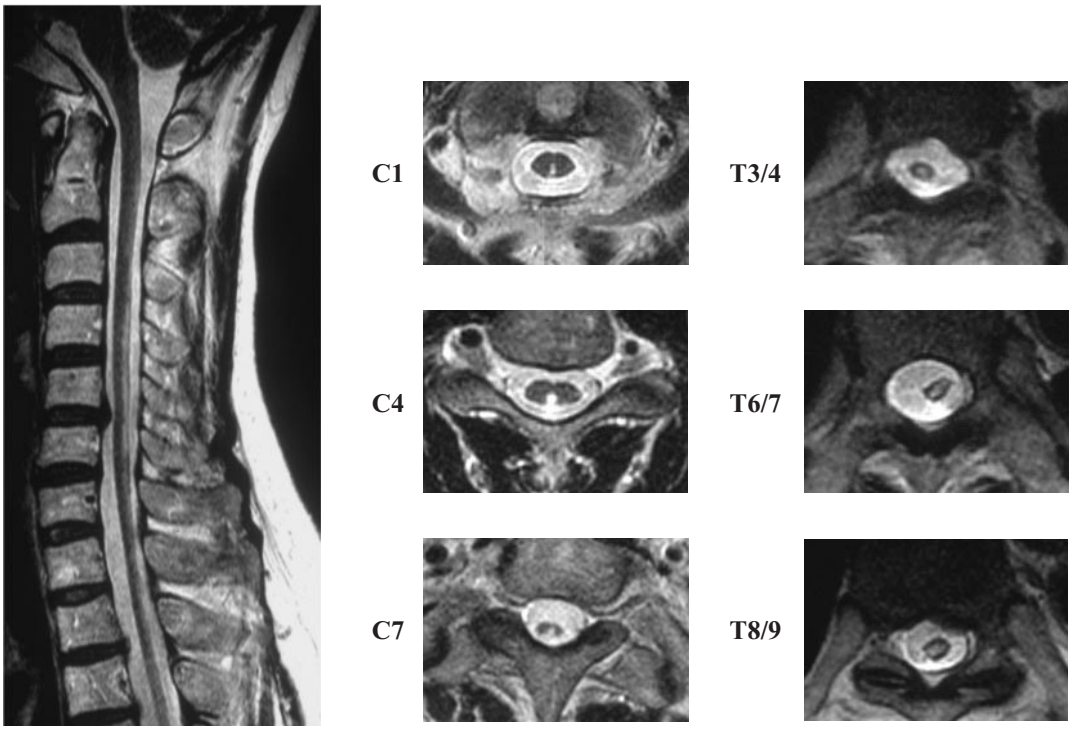

Fig. 3 Representative longitudinally extensive spinal cord MRI lesions on T2-weighted images from a 52-year-old patient with OS-multiple sclerosis at relapse.

$P = 0.0018$ for IL-17]. No other clinical parameters, such as age at onset, age at examination, disease duration, sex or clinical course, showed any significant correlation with the CSF supernatant cytokine/chemokine levels. Even when the five C-multiple sclerosis and 10 OS-multiple sclerosis patients on immunomodulatory therapies were excluded, essentially the same correlations were obtained.

Histopathological analysis of infiltrating cells in autopsied specimens of OS-multiple sclerosis and C-multiple sclerosis

We examined spinal cord specimens obtained at autopsy from six OS-multiple sclerosis and two C-multiple sclerosis cases. Of these, prominent CSF neutrophilia was noted in two of the five OS-multiple sclerosis cases whose CSF records were available. Their demographic features and neuropathological findings are summarized in Table 3. In the OS-multiple sclerosis cases, the spinal cord lesions extended over the white matter to the grey matter, and all the cases showed moderate to severe myelin and axonal damage, indicating so-called necrotic lesions. In the lesions, macrophage infiltration was universal in all cases, and was severe in three, moderate in one and mild in two. Numerous lymphocytes, mostly consisting of T lymphocytes, infiltrated either the perivascular area or parenchyma. In addition, three cases showed neutrophilic infiltration in both the grey and white matter of the spinal cord. Regarding the neutrophil infiltration, either focal accumulation or a diffuse scattered pattern was seen. In particular, focal neutrophil accumulation was noted around vessel walls or in parenchyma in two cases (Fig. 5). However, we found no eosinophil infiltration in any of the lesions by haematoxylin–eosin staining. Although the spinal cord lesions in one of the two C-multiple sclerosis cases showed severe necrosis with macrophage infiltration, infiltration of neutrophils was not evident in either of these C-multiple sclerosis cases.

Discussion

In the present study, although the numbers of CSF samples at relapse were limited due to the rarity of multiple sclerosis in Japanese and the popular use of immunomodulatory drugs, we successfully uncovered subtype-related CSF cytokine/chemokine changes in multiple sclerosis, since upregulation of the neutrophil-recruiting IL-17/IL-8 axis was characteristic for OS-multiple sclerosis and correlated with the development of longitudinally extensive spinal cord lesions. Furthermore, we have directly shown that in CSF cells, IFN-γ^+IL-4^- $CD4^+$ T cells are increased in both OS-multiple sclerosis and C-multiple sclerosis, while IFN-γ^-IL-4^+ $CD4^+$ T cells are significantly more abundant in OS-multiple sclerosis than in C-multiple sclerosis, even at relapse.

Cytokine analysis at the cellular level in CSF has been difficult because of the extreme fragility and limited numbers of CSF cells. In the current study, however, our method successfully measured the intracellular cytokine production

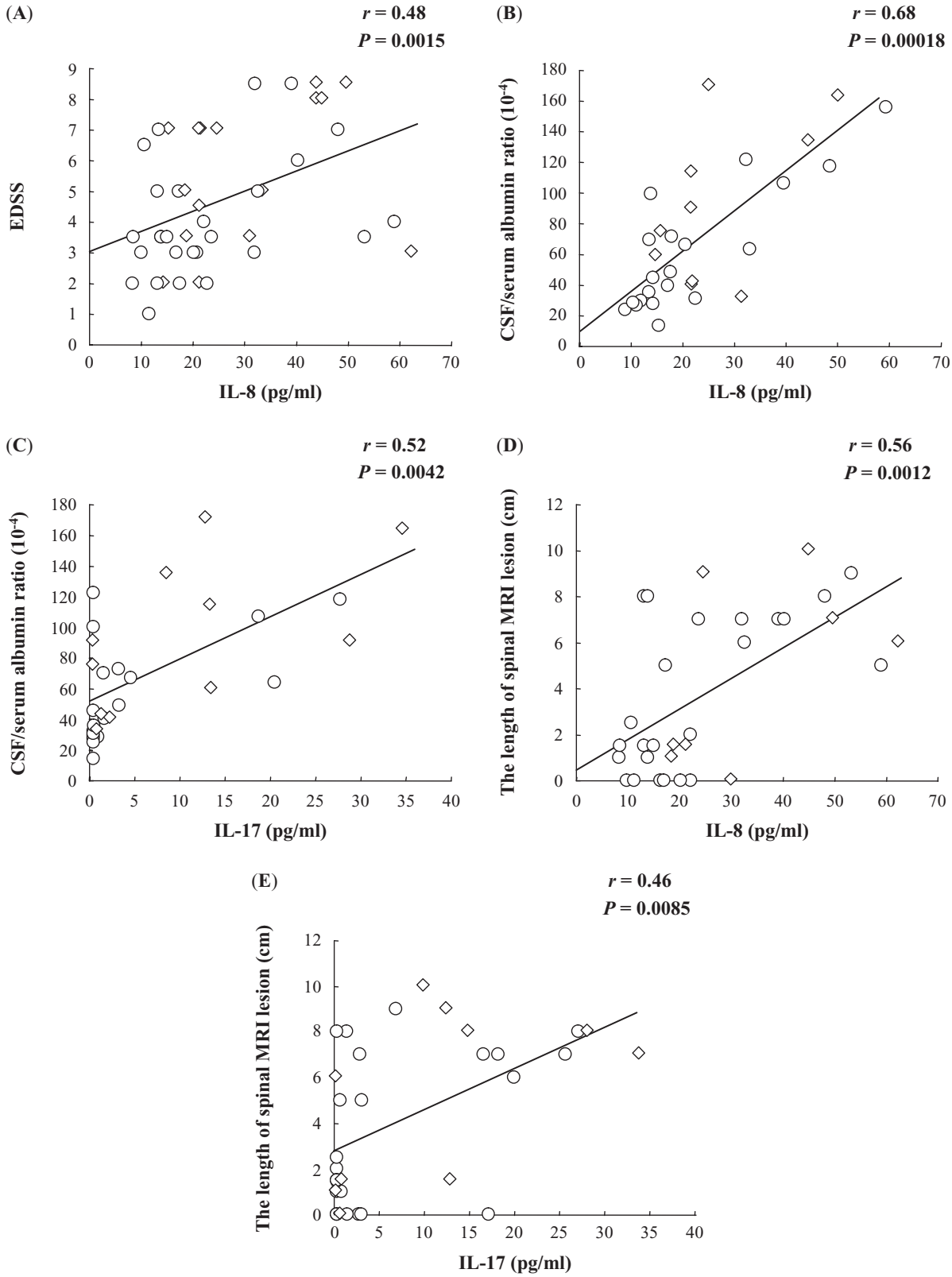

Fig. 4 Correlations between various clinical parameters and the cytokine/chemokine levels in CSF supernatants. (**A**) EDSS scores and IL-8 levels. (**B**) CSF/serum albumin ratio and IL-8 levels. The CSF protein concentration was also significantly correlated with IL-8 ($r = 0.56$, $P = 0.00026$, data not shown in the figure). (**C**) CSF/serum albumin ratio and IL-17 levels. The CSF protein concentration was also significantly correlated with IL-17 ($r = 0.53$, $P = 0.00054$, data not shown in the figure). (**D**) Length of spinal cord MRI lesions and IL-8 levels. (**E**) Length of spinal cord MRI lesions and IL-17 levels. Open diamonds indicate patients who were under immunomodulatory therapies, while open circles indicate those who were not.

Table 3 *Demographic features and neuropathological findings of spinal cords in eight multiple sclerosis autopsy cases*

Cases	OS-multiple sclerosis						C-multiple sclerosis	
	1	2	3	4	5	6	7	8
Age/sex	31/M	35/F	37/F	54/F	28/F	44/F	39/M	47/F
Duration of disease (years)	6	7	10	3	4	1	19	7
No. of relapses	8	5	>10	5	6	8	>10	Progressive
CSF								
Cell count (/μl)	3	/	136	54	78	2	8	5
Neutrophilia	–	/	+(67%)	–	+(60%)	–	–	–
Total protein amount (mg/dl)	39	/	256	113	240	23	104	77
Spinal cord pathology								
Tissue destruction	+++	++	+++	+++	+++	++	+	+++
Myelin loss	+++	+++	++	+++	+++	++	+++	++
Axonal loss	+++	+++	++	+++	+++	++	+	++
Neutrophil infiltration	F (+++), D (+)	None	D (+)	None	None	F (++), D (+)	None	None
Macrophage infiltration	+++	+	++	+++	+	+++	++	+
T-cell infiltration	+++	+	+++	++	++	++	+	++
B-cell infiltration	+	+	+	+	+	+	+	+

OS-multiple sclerosis = opticospinal form of multiple sclerosis; C-multiple sclerosis = conventional form of multiple sclerosis; / = no data available. Tissue damage (evaluated by haematoxylin–eosin staining): + mild, ++ moderate, +++ severe. Myelin damage (evaluated by myelin basic protein immunostaining): + mild, ++ moderate, +++ severe. Axonal damage (evaluated by phosphorylated neurofilament immunostaining): + mild, ++ moderate, +++ severe. Neutrophil infiltration (evaluated by myeloperoxidase immunostaining): F = focal accumulation (none 0, + <20 per field, ++ 20–40 per field, +++ >40 per field), D = diffuse scattered (none 0, + <10 per field, ++ >10 per field). Each field had a magnification of ×200. Macrophage infiltration: + slight infiltration, ++ moderate infiltration, +++ severe infiltration. T-cell infiltration: none 0, + <10 per field, ++ 10–30 per field, +++ >30 per field in the lesion. B-cell infiltration: none 0, + <10 per field, ++ 10–30 per field, +++ >30 per field in the lesion.

and revealed that, even in the absence of inflammation, CSF cells showed a significant Th1 shift compared with PBLs, which is consistent with the observation that Th1 cells bearing CXCR3, a specific chemokine receptor for CXCL10 (IP-10), are enriched in CSF cells compared with PBLs (Trebst and Ransohoff, 2001). However, such a Th1 shift was far more marked in C-multiple sclerosis patients at relapse than in controls. In C-multiple sclerosis patients, the significant Th1 shift in PBLs was due mainly to a marked decrease in IFN-γ^{-}IL-4^{+} CD4^{+} T cells (Th2 cells), while that in CSF cells was mainly attributable to a large increase in IFN-γ^{+}IL-4^{-} CD4^{+} T cells (Th1 cells) and partly to a decrease in IFN-γ^{-}IL-4^{+} CD4^{+} T cells. In contrast, in OS-multiple sclerosis patients, a significant Th1 shift compared with controls was only seen in PBLs (Ochi *et al*., 2001; present study), and not in CSF cells. The Th1 shift in PBLs was considered to be caused by a significant decrease in Th2 cells, while in the CSF cells, a significant increase in Th1 cells also occurred in OS-multiple sclerosis patients compared with controls, but at the same time Th2 cells were rather more increased in CSF than in PBLs. This latter increase partially cancelled out the increase in Th1 cells in CSF and caused a significant difference in the IFN-γ^{-}IL-4^{+} CD4^{+} T-cell percentages between the two multiple sclerosis subtypes. These findings suggest that the Th1/Th2 balance is differentially regulated in the peripheral blood and CSF compartments, and that OS-multiple sclerosis and C-multiple sclerosis have distinct systems of immune dysregulation.

A broad range of cytokine/chemokine concentrations was successfully measured in CSF supernatants in the present study. However, caution should be exercised with respect to the low ranges of sample concentrations (<1 pg/ml) until sufficient data for the various diseases have been obtained using multiplexed fluorescent bead-based immunoassays. Even when we set the cut-off level to 1 pg/ml and excluded patients on immunomodulatory therapies, increases of IL-17, IL-8 and MIP-1β in OS-multiple sclerosis and the increase of IL-8 and decrease of MCP-1 in C-multiple sclerosis in comparison with controls, and the difference of IL-17 between OS-multiple sclerosis and C-multiple sclerosis were all still significant. As we used SCD as controls, it is possible that SCD-related cytokine changes, if any, may have introduced a misleading element into our multiple sclerosis-related findings. However, as no inflammatory components have ever been reported in SCD CSF, we consider it appropriate for use as the control in this study, and the above-mentioned changes in multiple sclerosis to be relevant for each disease process.

In Western multiple sclerosis series, chemokines inducing Th1 cell mobilization, such as CXCL10 (IP-10) for CXCR3-bearing cells and RANTES for CCR5-bearing cells, have been shown to increase in the CSF at relapse, while chemokines for Th2 cells, such as MCP-1 for CCR2-bearing cells, decrease (Sørensen *et al*., 1999; Franciotta *et al*., 2001; Mahad *et al*., 2002; Scarpini *et al*., 2002). Furthermore, co-localization of CXCL10 and CXCR3 was noted in the

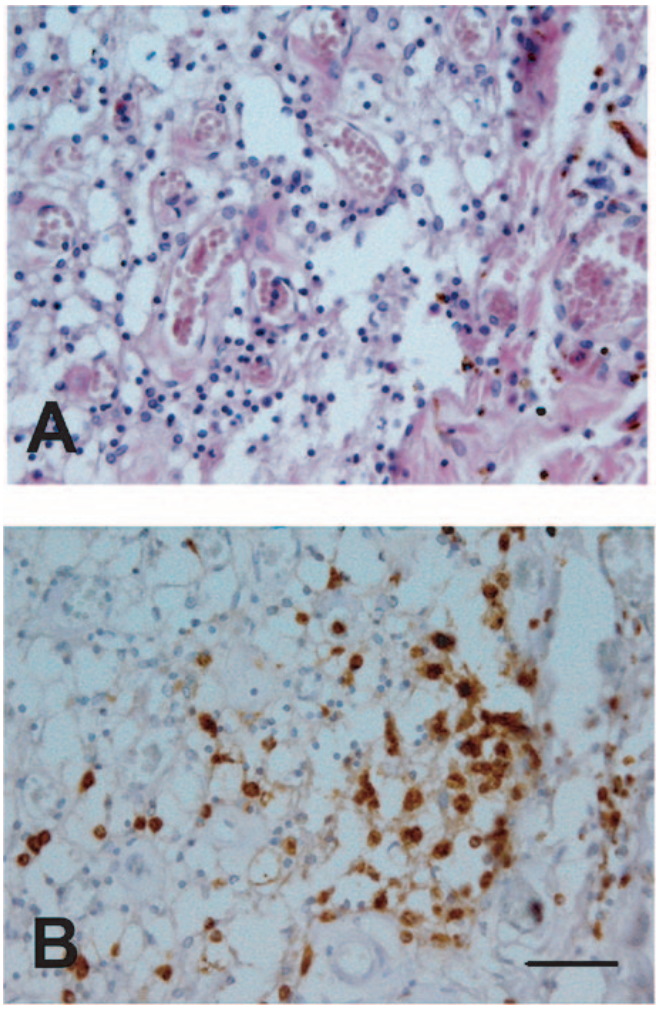

Fig. 5 Neuropathological findings of autopsied spinal cord specimens from OS-multiple sclerosis cases (case 1). The myelin and axon are severely damaged and vascular proliferation has occurred in the cornu laterale of the thoracic spinal cord. Numerous granulocytes containing myeloperoxidase (MPO)-positive neutrophils are accumulated and infiltrated in the lesion (**A**, haematoxylin–eosin stain; **B**, MPO immunostaining). The scale bar in **B** = 50 μm (also for **A**).

multiple sclerosis lesions (Sørensen *et al*., 2002). These observations are consistent with the Th1 shift found during the intracellular cytokine analysis of CSF cells in C-multiple sclerosis in the current study.

Among the cytokines and chemokines measured in the CSF supernatant, both IL-17 and IL-8 had a significant correlation with the CSF/serum albumin ratio and the length of the spinal cord lesions on MRI in multiple sclerosis, suggesting an involvement of both cytokines in the destruction of the blood–brain barrier and the formation of inflammatory spinal cord lesions. In the present CSF study, the increases in IL-17 and IL-8 were significantly greater in OS-multiple sclerosis patients than in C-multiple sclerosis patients. A previous report on IL-17 in Western multiple sclerosis series found no change at the protein level in CSF (Saruhan-Direskeneli *et al*., 2003), although all multiple sclerosis samples were below the detection limit, while IL-17 mRNA expression in CSF mononuclear cells was elevated in a fraction of multiple sclerosis patients, especially during clinical exacerbation (Matusevicius *et al*., 1999), and gene microarray analysis of multiple sclerosis plaques revealed an increased level of IL-17 transcripts (Lock *et al*., 2002). Our findings extend the latter observations at the mRNA level and suggest that the IL-17 response is much more prominent in OS-multiple sclerosis than in C-multiple sclerosis at the protein level. IL-17 is produced by activated memory Th1 cells (Aarvak *et al*., 1999) and induces various downstream cytokines and chemokines, such as IL-8, IL-6, G-CSF and prostaglandin E_2 (Fossiez *et al*., 1996; Dudler *et al*., 2000; Hwang *et al*., 2004). IL-17 causes neutrophil recruitment mainly through the release of IL-8, a CXC chemokine for neutrophils, and induces neutrophil activation, i.e. increases in myeloperoxidase and elastase activity (Laan *et al*., 1999; Hoshino *et al*., 2000; Linden and Adachi, 2002; Miyamoto *et al*., 2003; Witowski *et al*., 2004). Upregulation of IL-17 and IL-8 has been reported to cause severe destruction of tissues by neutrophilic inflammation in Th1 diseases, such as rheumatoid arthritis (Kotake *et al*., 1999; Ziolkowska *et al*., 2000; Miossec, 2003), as well as Th2 diseases, such as bronchial asthma (Linden, 2001; Molet *et al*., 2001). CSF neutrophilia and the heavy infiltration of neutrophils seen in the necrotic spinal cord lesions of OS-multiple sclerosis may well be related to the increases in IL-17 and IL-8 in CSF. The observation that only IL-8 is significantly correlated with the EDSS score further underscores a critical role for IL-8-induced neutrophil recruitment in the destruction of the spinal cord tissues in Japanese patients with multiple sclerosis. Since longitudinally extensive spinal cord lesions are more frequently encountered in OS-multiple sclerosis than in C-multiple sclerosis, and represent one of the two main determining factors for irreversible disability in OS-multiple sclerosis, intrathecal activation of the IL-17/IL-8 axis is considered to be crucial in OS-multiple sclerosis. Interestingly, although the IL-8 levels have not been reported to differ in either unstimulated PBLs or CSF from multiple sclerosis patients and controls in a Western population series (Comabella *et al*., 2002; Jalonen *et al*., 2002; Kleine *et al*., 2003), IL-8 expression in PBLs is markedly downregulated in IFN-β responders, but not in non-responders (Stürzebecher *et al*., 2003), indicating a potentially important role for IL-8 in multiple sclerosis, even in Westerners. In our C-multiple sclerosis patients, IL-8 was significantly elevated in CSF, whereas IL-17 was not increased. Since IL-8 is also driven by TNF-α (Hoffmann *et al*., 2002), the augmented TNF-α, but not IL-17, may potentiate IL-8 production in C-multiple sclerosis. Since surges of TNF-α are hardly detectable due to its extremely short half-life (30 min) (Li *et al*., 2001), it is reasonable that IL-8 rather than TNF-α is found to be correlated with the EDSS score.

It is interesting to note that the downregulation of Th2 cells in CSF in C-multiple sclerosis at relapse was not

found in OS-multiple sclerosis, and Th2 cells were rather increased in CSF at relapse in comparison with PBLs. Th2 polarized cells directed against myelin proteins are encephalitogenic in immunocompromised animals (Lafaille *et al*., 1997) and exacerbate experimental allergic encephalomyelitis (EAE) in non-human primates (Genain and Hauser, 1996). Moreover, in certain animal strains with Th2-prone genetic backgrounds, myelin oligodendrocyte glycoprotein (MOG)-induced EAE shows severe and selective involvement of the optic nerves and spinal cord (Storch *et al*., 1998; Stefferl *et al*., 1999). In these models, the accumulation of numerous neutrophils is a dominant feature (Lafaille *et al*., 1997; Storch *et al*., 1998; Stefferl *et al*., 1999). We previously reported that MOG-autoreactive T cells were more frequently established than those reactive to myelin basic protein or proteolipid protein epitopes in OS-multiple sclerosis patients (Minohara *et al*., 2001). Thus, in OS-multiple sclerosis, intrathecal activation of the IL-17/IL-8 axis by memory Th1 cells specific for myelin proteins such as MOG may contribute to the neutrophilic recruitment and destruction of the tissues under Th2-prone genetic backgrounds or even together with myelin protein-specific Th2 cells. Therefore, Th2-related genetic backgrounds as well as Th2 cell reactivity to myelin proteins could be future targets for studies on OS-multiple sclerosis.

IL-17 expression is induced by IL-23, a product of activated dendritic cells and macrophages/microglial cells (Becher *et al*., 2003; Cua *et al*., 2003), while IL-12 (p70), a disulphide-linked heterodimer of p35 and p40, has only marginal effects on IL-17 production (Aggarwal *et al*., 2003), yet both IL-23 and IL-12 (p70) share a common p40 subunit. Furthermore, IL-23, but not IL-12 (p70), has been shown to be a critical cytokine for autoimmune inflammation of the brain in an EAE model using knockout mice for each of their subunits (Cua *et al*., 2003). Previous reports have described that IL-12 (p40) was increased in multiple sclerosis patients with gadolinium-enhanced lesions on MRI (Fassbender *et al*., 1998), whereas IL-12 (p70) was only detectable in CSF in ~10% of multiple sclerosis patients (Drulovic *et al*., 1997; Fassbender *et al*., 1998). Since we also found no increase in IL-12 (p70) in CSF from our multiple sclerosis patients, we consider that a further study on IL-23 in the CSF compartment is urgently required.

We found that IL-5 levels were significantly higher in OS-multiple sclerosis than C-multiple sclerosis patients. In the latter, the IL-5 level was possibly depressed in some cases, reflecting an intrathecal down-modulation of Th2 cells in the acute stage. Instead, some OS-multiple sclerosis patients showed an increase in IL-5 in the CSF, although the increase was not statistically significant in comparison with control patients as a whole. Although we could not find any eosinophil infiltration in OS-multiple sclerosis spinal cord lesions, degranulated eosinophils are hard to detect by haematoxylin–eosin staining. Thus, immunostaining of activated eosinophil products is required to determine eosinophil involvement in OS-multiple sclerosis.

Lucchinetti *et al*. (2002) reported that in nine autopsied cases of Devic's NMO, eight relapsing and one monophasic, 56% had prominent infiltration of neutrophils and eosinophils into the spinal cord lesions, and marked deposition of immunoglobulins and complements were seen in all cases. A distinction between and the identities of relapsing NMO and OS-multiple sclerosis have long been discussed (Cree *et al*., 2002) and, in the recent study by Lennon *et al*. (2004), newly identified IgG autoantibody (NMO-IgG) was detected in both NMO and Japanese OS-multiple sclerosis patients. The considerable overlap between the two conditions suggests common pathomechanisms are operative. Our finding of marked increases of IL-17 and IL-8 in CSF may be relevant to neutrophil infiltration, while an IL-5 increase may relate to eosinophil infiltration. Moreover, a relative increase of Th2 cells in CSF compared with PBLs may correspond to the involvement of humoral immunity in relapsing NMO (Lucchinetti *et al*., 2002; Lennon *et al*., 2004). Further investigation into the deposition of immunoglobulins and complement proteins as well as activated eosinophil products in OS-multiple sclerosis may shed light on the contribution of the Th2 cell-mediated effector arm in this condition, and clarify the disease entities of relapsing NMO and OS-multiple sclerosis.

In summary, we successfully identified OS-multiple sclerosis-related CSF cytokine/chemokine changes, which may be useful both for monitoring disease activity and for developing future subtype-specific therapies, such as pharmacological blocking of neutrophil activation in OS-multiple sclerosis.

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