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ORIGINAL COMMUNICATION

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# Differences in systemic and central nervous system cellular immunity relevant to relapsing-remitting multiple sclerosis

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**Abstract** In order to elucidate the differences between systemic and central nervous system (CNS) immunity that are relevant to exacerbations of multiple sclerosis (MS), paired peripheral blood and cerebrospinal fluid (CSF) samples obtained from 36 non-treated patients with relapsing-remitting MS (RRMS) were simultaneously examined using flow cytometry to determine the percentages of functional lymphocyte subsets, as well as enzyme-linked immunosorbent assays for measuring soluble immune mediators. Active RRMS patients (n = 27) were characterized by an increase in $CD4^+CXCR3^+$ Th1 cells in blood as compared with inactive patients (n = 9), and this parameter was inversely correlated with plasma levels of IL-10 and IL-12p70. In contrast, an increase in the percentage of $CD4^+CD25^+$ cells and a decrease in the percentage of $CD8^+CD11a^{high}$ cells were features of CSF samples from those with active RRMS. Further, CSF $CD4^+CD25^+$ cells had a close association with leukocyte counts as well as albumin and CXCL10 levels in the CSF, and, thus, could be useful as a measure for inflammatory reactions in the CNS. On the other hand, $CD8^+CD11a^{high}$ cells may function as immunoregulatory cells, as their percentage in the CSF showed a positive correlation with CSF levels of the anti-inflammatory cytokine IL-4. These findings suggest that MS relapses occur in a combination with altered cell-mediated immunity that differs between the peripheral blood and CSF compartments, while measurement of lymphocyte subsets may be helpful for monitoring disease status.

**Key words** multiple sclerosis · cerebrospinal fluid · inflammation · $CD4^+CXCR3^+$ cell · $CD4^+CD25^+$ cell

## Introduction

Multiple sclerosis (MS) is an immune-mediated chronic inflammatory disease of the central nervous system (CNS), in which myelin protein antigens are assumed to be targeted [23]. In relapsing-remitting MS (RRMS) patients, active disease status can be judged from an acute worsening of neurological findings and/or the emergence of gadolinium (Gd)-enhanced lesions on magnetic resonance (MR) images [23]. However, immunological measurements useful for the assessment of disease activity in individual patients have yet to be established in a clinical setting [28], though many studies have been conducted for that purpose. For example, using flow cytometric analyses of surface antigens expressed by peripheral blood lymphocytes, increases in $CD4^+CD26^+$ activated/memory helper T cells [13], $CD4^+CD29^+$ helper inducer T cells [25, 34], and T helper type 1 (Th1) cells [21, 34], as well as decreases in $CD4^+CD45RA^+$ naive T cells [2, 6] and $CD8^+$ regulatory T cells [3], have been found to be associated with active

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RRMS. On the other hand, studies of cerebrospinal fluid (CSF) lymphocytes obtained during exacerbation of the disease have revealed an increase in Th1 cells [21], as well as in $CD4^+CD25^+$ and $CD4^+CD29^+$ cells [35]. In addition, decreases in $CD8^+$ lymphocyte subsets have also been reported [27, 34].

Another method for assessing immunological status is to measure the levels of soluble immune effector molecules such as cytokines [24] and chemokines [32], as well as mediators of inflammation, including matrix metalloproteinases (MMP) [14] and nitric oxide (NO) metabolites [4], which are known to be involved in the pathogenesis of MS. However, the relationships between the results of those measurements and those of flow cytometric analyses remain unknown. Further, no conclusion has been drawn as to whether blood or CSF samples are to be tested as a representative measure of immunological events leading to MS relapse [28]. In this regard, the major point of debate concerning the pathogenesis of MS has been whether it is a disorder originating from systemic immune abnormalities [9], or that caused by an aberrant immunity that is unique to the CNS [1].

The present study was performed in order to elucidate the differences, if any, between systemic and CNS cell-mediated immunity in relation to soluble immune mediators. We simultaneously measured a number of the above-mentioned immunological parameters in paired blood and CSF samples, as this type of approach may be helpful for defining the pivotal etiological factors in RRMS.

## Methods

### Patients

Thirty-six patients (24 women, 12 men) with RRMS, who were diagnosed according to revised diagnostic criteria [20], were enrolled in the study after giving informed consent. None showed a secondary progressive course or were receiving any immunomodulatory treatment. The patients were examined during an active (n = 27) or inactive (n = 9) stage of the disease, with active patients defined by the appearance of new neurological symptoms and/or the emergence of Gd-enhanced lesions on MR images, while inactive patients showed a neurologically stable state for more than 3 months as well as no Gd-enhanced lesions. We were also able to re-examine blood lymphocyte subsets in some of the patients during the course of the disease, at which time we collected and analyzed follow-up data in terms of the usefulness of serial blood sampling for monitoring disease activity. The study design was approved by our institutional review body.

### Flow cytometry

Blood mononuclear cells (MNCs) were isolated by Ficoll-Paque density gradient centrifugation, then further processed for staining and flow cytometry, as described previously [34]. CSF samples obtained by lumbar puncture were spun down at low speed at 4 °C [35].

The following monoclonal antibodies (MoAbs) were used for flow cytometry. Fluorescein isothiocyanate (FITC)-labeled T3 (CD3) and B1 (CD20) MoAbs (Beckman Coulter, Fullerton, CA) were used for identifying mature T cells and B cells, respectively. Combinations of FITC-labeled T4 (CD4, Beckman Coulter) MoAb along with phycoerythrin (PE)-labeled anti-IL-2 receptor (CD25, BD, San Jose, CA), Ta1 (CD26, Beckman Coulter), 4B4 (CD29, Beckman Coulter), or 2H4 (CD45RA, Beckman Coulter) MoAb, were also employed. FITC-labeled T8 (CD8, Beckman Coulter) and PE-labeled anti-CD11a (Beckman Coulter) MoAbs were used for separating $CD11a^{high}$ cytotoxic and $CD11a^-$ suppressor T cells. For defining Th1 cells, a combination of PE-labeled Leu3a (CD4) (BD) and FITC-labeled anti-CXCR3 (Dako Japan, Kyoto) MoAbs was used, while, for Th2 cells, a combination of PE-labeled Leu3a and FITC-labeled anti-CCR4 (provided by Dr. Matsushima) MoAbs was employed. In addition, CXCR3-positive CD8 cells were stained with PE-labeled Leu2a (CD8, BD) and FITC-labeled anti-CXCR3.

A 50-µl cell suspension aliquot was used for each staining, incubated with MoAbs on ice for 45 minutes and washed once. Analysis for positive green (FITC) and red (PE) fluorescence was performed using an Epics XL laser flow cytometer (Beckman Coulter).

### CSF measurements

A small portion of the CSF samples were used for enumerating cells in a Fuchs-Rosenthal counting chamber. After collecting CSF cells by centrifugation of the remaining CSF sample, a portion of the supernatant was also measured for protein, albumin, and IgG levels. Markers of humoral immunity, i. e., IgG index and intra-blood-brain barrier (BBB) IgG production, were also calculated by the following formulae: $(IgG_{csf}/IgG_{serum})/(albumin_{csf}/albumin_{serum})$ for IgG index, and $[(IgG_{csf} - IgG_{serum}/369) - (albumin_{csf} - albumin_{serum}/230) \times (IgG_{serum}/albumin_{serum})(0.43)] \times 5$ for intra-BBB IgG production [31].

### Soluble immune mediators

Three to four milliliters of plasma and the CSF supernatants were kept frozen at –80°C for measuring levels of the proinflammatory cytokines IL-2, IL-12p70, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), as well as the anti-inflammatory cytokines IL-4, IL-10, and transforming growth factor-β1 (TGF-β1), using an enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's instructions (Immunotech, Marseille, France). In addition, the chemokines CCL2 and CCL5 were measured using ELISA kits purchased from Immunotech, while CCL3 and CXCL10 were measured using kits purchased from TECHNE (Minneapolis, MN) and HyCult biotechnology (Uden, the Netherlands), respectively. Furthermore, levels of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1), as well as nitrite and nitrate, were also measured using ELISA kits purchased from Amersham Biosciences (Tokyo, Japan), Fuji Chemical Industries (Takaoka, Japan), and BioDynamics Laboratory (Columbus, OH), respectively.

### Statistical analysis

As a first step, the results of flow cytometry for blood and CSF lymphocytes were compared between active and inactive MS patients, using unpaired t and Mann-Whitney U tests, in order to elucidate the important parameters of cellular immunity able to discriminate between the 2 groups. If relevant lymphocyte subsets were found in the blood or CSF, then, as a second step, the relationship of each subset to the results for soluble immune mediators measured in plasma or CSF samples, respectively, was analyzed using Pearson's correlation coefficient as well as nonparametric Spearman's rank correlation coefficient tests. For assessing the relevance of any fluctuation in flow cytometric data during the course of the disease, longitudinal results of a particular lymphocyte subset were analyzed using two-factor analysis of variance (ANOVA). When a longitudinal change in a parameter was significant, the results obtained at each time point were further compared using a paired t test.

## Results

Blood samples obtained from active RRMS patients showed a significantly higher percentage of $CD4^+CXCR3^+$ cells ($p = 0.0468$) as compared with inactive patients, using a Mann-Whitney U test (Table 1). In addition, the mean percentage of cells (14.7 %) in active patients was significantly higher ($p = 0.0161$ by Mann-Whitney U test) than the mean (10.2 %; standard deviation, 4.4) of 18 healthy volunteers tested (unpublished data). Further, the percentage of this cell population showed a significant negative correlation with plasma levels of IL-10 (Fig. 1A) and IL-12p70 (Fig. 1B), when analyzed using Pearson's method, whereas no correlation was found between the same parameters in the CSF samples.

In contrast, a significant increase in the percentage of $CD4^+CD25^+$ cells ($p = 0.0053$) as well as a significant decrease in the percentage of $CD8^+CD11a^{high}$ cells ($p = 0.0174$) was a feature of CSF samples from active RRMS patients, when analyzed by unpaired t and Mann-Whitney U tests, respectively (Table 2). However, the absolute numbers of both of these 2 cell populations were significantly increased in active MS CSF samples as compared to inactive, because the CSF cell counts in active MS patients were significantly higher than those in inactive patients (Table 2). Representative fluorescence patterns for $CD4^+CD25^+$ and $CD8^+CD11a^{high}$ cells are shown in Fig. 2. Nonspecific fluorescence-positive cells for CD25 antigen among $CD4^+$ cells, as well as those for CD11a antigen among $CD8^+$ cells, were always less than 0.1 %, when checked using a control antibody (data not shown). As for their relationship to soluble immune mediators, the percentage of CSF $CD4^+CD25^+$ cells was positively correlated with CSF cell count (Fig. 3A), as well as CSF levels of albumin (Fig. 3B) and CXCL10 (Fig. 3C), whereas the absolute numbers showed a significant correlation with only the former 2 parameters ($p < 0.0001$ and $p = 0.0001$, respectively), when analyzed by Pearson's method. On the other hand, the percentage of CSF $CD8^+CD11a^{high}$ cells was inversely correlated with CSF cell number (Fig. 4A) and CSF TIMP-1 levels (Fig. 4C), and positively correlated with IL-4 levels in the CSF, as shown in Fig. 4B. Interestingly, the absolute numbers of CSF $CD8^+CD11a^{high}$ cells did not show any correlation with TIMP-1 or IL-4 levels, and the correlations found in the percentages of $CD4^+CD25^+$ and $CD8^+CD11a^{high}$ cells in the CSF did not exist in the blood samples.

Among the 3 lymphocyte subsets found relevant to MS activity in the present study, the percentages of $CD4^+CXCR3^+$ and $CD4^+CD25^+$ cells in the blood were simultaneously re-examined during follow-up examinations in 5 active MS patients, including during a subsequent episode of MS relapse that occurred independently of the former and the following inactive stages. Two-factor ANOVA revealed that the percentage of blood $CD4^+CXCR3^+$ cells significantly changed in association with disease activity ($p = 0.0117$), while the percentages determined during the original and subsequent active stages were significantly decreased in the following inactive stage ($p = 0.0287$ and $p = 0.0109$, respectively, by paired t test). On the other hand, the percentage of blood $CD4^+CD25^+$ cells did not show such change at the same time points and the data were rela-

**Table 1** Circulating Lymphocyte Subsets in Active and Inactive MS Patients

| Phenotype | Subset | Active MS n = 27 | Inactive MS n = 9 | p value |
|---|---|---|---|---|
| $CD3^+$ (%) | mature T | 66.0±16.5 | 63.5±11.0 | NS |
| $CD20^+$ (%) | B cell | 5.2±5.6 | 4.7±2.4 | NS |
| $CD4^+$ (%) | Th | 45.1±11.6 | 56.2±10.2 | NS |
| $CD4^+CD25^+$ (%) | IL-2R$^+$ Th | 2.7±1.4 | 2.1±1.1 | NS |
| $CD4^+CD26^+$ (%) | memory Th | 26.9±11.4 | 28.1±9.5 | NS |
| $CD4^+CD29^{high}$ (%) | Th inducer | 23.1±6.6 | 19.0±6.2 | NS |
| $CD4^+CD45RA^+$ (%) | naive Th | 19.5±9.4 | 19.1±9.7 | NS |
| $CD4^+CCR4^+$ (%) | Th2 | 5.0±2.7 | 5.4±2.4 | NS |
| $CD4^+CXCR3^+$ (%) | Th1 | 14.7±6.3 | 10.2±2.4 | 0.0468* |
| $CD8^+$ (%) | Ts/CTL | 20.2±10.9 | 21.7±7.1 | NS |
| $CD8^+CD11a^{high}$ (%) | CTL | 8.4±7.9 | 9.8±5.9 | NS |
| $CD8^+CD11a^-$ (%) | Ts | 5.4±5.2 | 4.1±2.5 | NS |
| $CD8^+CXCR3^+$ (%) | | 15.0±5.4 | 17.1±9.5 | NS |

Values are expressed as mean ± standard deviation
p values were determined by an unpaired t or * Mann-Whitney U test
*Th* helper T; *Th1* T helper type 1; *Th2* T helper type 2; *IL-2R* IL-2 receptor; *Ts* suppressor T cell; *CTL* cytotoxic T lymphocyte; *NS* not significant

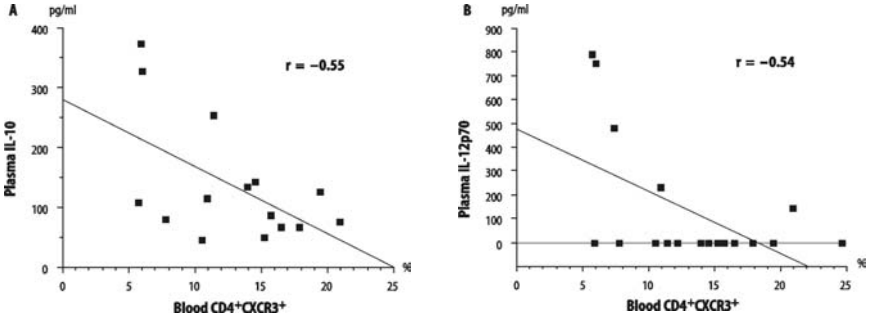



**Fig. 1** Relationships between the percentage of $CD4^+CXCR3^+$ cells in blood, and plasma levels of IL-10 and IL-12p70. The percentage of $CD4^+CXCR3^+$ cells was inversely correlated with levels of IL-10 (**A**, $p = 0.032$, $r = -0.55$) and IL-12p70 (**B**, $p = 0.0183$, $r = -0.54$)

**Table 2** CNS Cellular Immunity Distinguishing Active and Inactive MS Stages

| Phenotype | Active MS n = 27 | Inactive MS n = 9 | p value |
|---|---|---|---|
| **Cell number (/ml)** | **7510 ± 12000** | **1420 ± 1120** | **0.0077*** |
| $CD3^+$ (%) | 89.5 ± 6.8 | 87.2 ± 7.8 | NS |
| $CD20^+$ (%) | 1.2 ± 1.4 | 3.4 ± 4.1 | NS |
| $CD4^+$ (%) | 67.2 ± 8.9 | 56.2 ± 10.2 | 0.0046 |
| $CD4^+CD25^+$ (%) | 5.3 ± 3.0 | 2.1 ± 1.2 | 0.0053 |
| **No. of $CD4^+CD25^+$ (/ml)** | **650 ± 1520** | **40 ± 50** | **0.0017*** |
| $CD4^+CD26^+$ (%) | 39.0 ± 8.1 | 36.5 ± 11.2 | NS |
| $CD4^+CD29^{high}$ (%) | 63.0 ± 9.4 | 56.4 ± 9.0 | NS |
| $CD4^+CCR4^+$ (%) | 2.7 ± 2.3 | 2.2 ± 1.6 | NS |
| $CD4^+CXCR3^+$ (%) | 49.0 ± 8.5 | 51.8 ± 7.4 | NS |
| $CD8^+$ (%) | 22.6 ± 9.0 | 31.7 ± 12.7 | 0.0275 |
| $CD8^+CD11a^{high}$ (%) | 15.7 ± 7.2 | 22.6 ± 4.7 | 0.0174* |
| **No. of $CD8^+CD11a^{high}$ (/ml)** | **1140 ± 1070** | **390 ± 280** | **0.0297*** |
| $CD8^+CD11a^-$ (%) | 3.9 ± 2.6 | 3.2 ± 2.2 | NS |
| $CD8^+CXCR3^+$ (%) | 24.4 ± 8.5 | 31.6 ± 6.7 | NS |

Values are expressed as mean ± standard deviation
p values were determined by an unpaired t or * Mann-Whitney U test
*NS* not significant

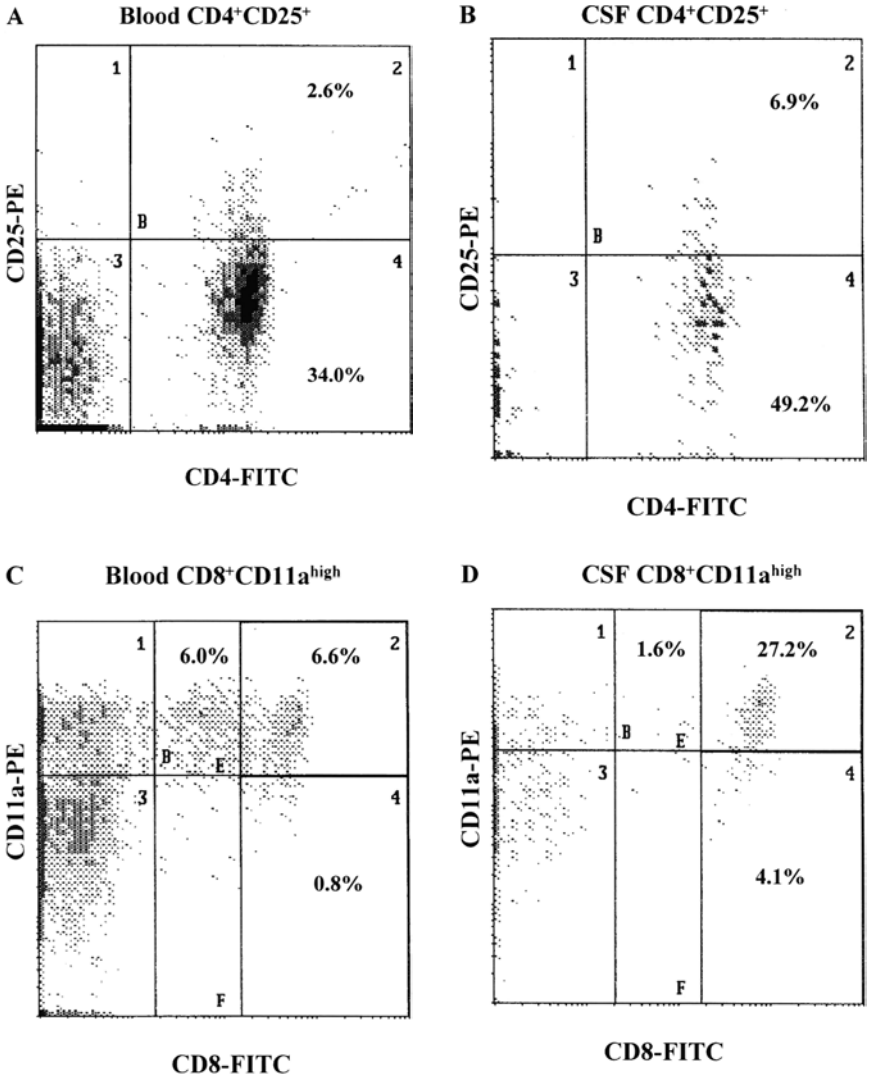
tively consistent. These results are shown in Fig. 5 and suggest that our data obtained by flow cytometry demonstrated good reproducibility, while measurements of blood $CD4^+CXCR3^+$ cells may be useful for monitoring disease activity.

## Discussion

The present results demonstrated that circulating $CD4^+CXCR3^+$ cells were modestly, though significantly, increased in active RRMS patients when compared with inactive patients, and that their percentage changed in parallel with disease activity. $CD4^+CXCR3^+$ cells in the blood are known to mainly consist of Th1 cells [15], and are correlated with the number of Gd-enhanced lesions [34] as well as the change in annual T2 lesion load [5] on MR images of MS patients. Further, our novel finding of a negative correlation between the percentage of circulating $CD4^+CXCR3^+$ Th1 cells and the plasma levels of Th2-related cytokine IL-10, along with a report showing decreased IL-10 mRNA levels in association with MS relapse [33], suggest substantial roles for such cells in the pathogenesis of MS [23]. On the other hand, their inverse relationship to IL-12p70 levels found in the present study seems to be paradoxical, as IL-12 is critical for the development of Th1 responses [12] and IL-12p40 mRNA levels have been shown to be increased during active stages of MS [33]. However, our finding might be attributed to the consumption of this biologically active form (p70) of IL-12 in the systemic circulation due to the persistent Th1 immune deviation. Interestingly, the same discrepancy between the results in the p40 subunit and the heterodimer p70 measurements for IL-12 was also found in MS CSF samples in another study [7], and thus needs further clarification.

In contrast to $CD4^+CXCR3^+$ cells in the blood, the percentage of such cells in the CSF was nearly equivalent in both active and inactive MS patients, which could be due to the fact that the majority of $CD3^+$ T cells infiltrating the CNS express CXCR3 without regard to the presence or absence of ongoing inflammatory processes [16]. Indeed, in the present study, the proportions of CXCR3-bearing cells among $CD4^+$ and $CD8^+$ cells were similar in the active and inactive MS groups (data not shown). Therefore, as a representative marker of cell-mediated immunity in the CNS of RRMS patients, the $CD4^+CD25^+$ cell population in the CSF seems to be more appropriate, as its increase distinguished active patients, and was associated with elevated white cell counts and albumin levels in the CSF.

The CD25 molecule is an $\alpha$ chain of the IL-2 receptor that is expressed upon antigenic stimulation [10] and, further, myelin antigen-reactive T cell clones have been shown to be present among CD25-positive cells in the CSF of MS patients [11]. In addition, since elevated CSF albumin concentrations are known to reflect a breakdown of the BBB [28], the present finding is consistent with our previous one that the number of CSF $CD4^+CD25^+$ cells is correlated with that of Gd-enhanced lesions [35]. Further, we found that the percentage of this cell population showed a close relationship to CXCL10 levels in the CSF. CSF levels of CXCL10 have been reported to be elevated during MS relapse [8, 29], and correlated with CSF $CD4^+CXCR3^+$ Th1 cells [18], CSF leukocyte count [29], as well as levels of CSF MMP-9 and intra-BBB IgG synthesis [30]. Thus, CXCL10 in the CSF is assumed to have a role in the maintenance of inflam-



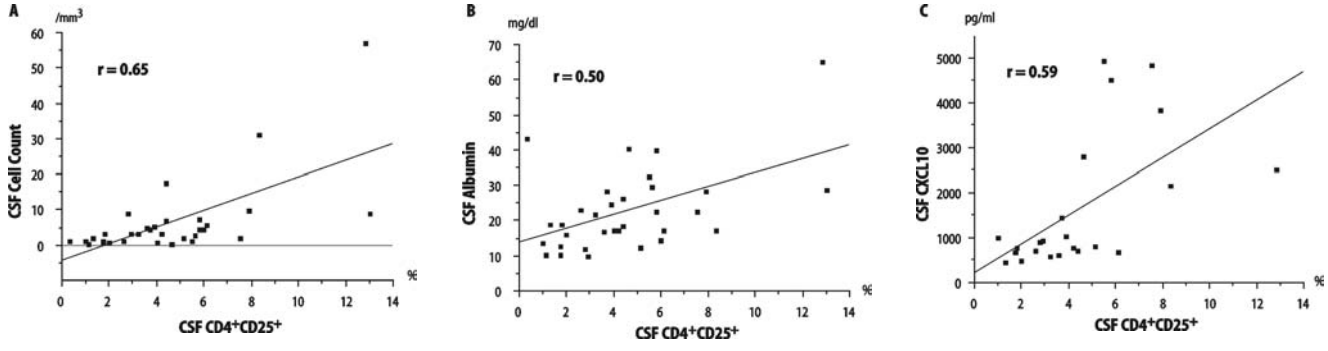




**Fig. 2** Representative fluorescence patterns for $CD4^+CD25^+$ and $CD8^+CD11a^{high}$ cells obtained from a patient with active MS. $CD4^+CD25^+$ cells from blood (2.6 %) and CSF (6.9 %) samples are shown in the upper right quadrant in panels **A and B**, respectively, while $CD8^+CD11a^{high}$ cells in blood (6.6 %) and CSF (27.2 %) are shown in the upper right compartment in panels **C and D**, respectively

**Fig. 3** Relationships between the percentage of CSF $CD4^+CD25^+$ cells and other parameters in the CSF. CSF $CD4^+CD25^+$ cells were positively correlated with CSF cell count (**A**, $p < 0.0001$, $r = 0.65$), as well as CSF levels of albumin (**B**, $p = 0.003$, $r = 0.50$) and CXCL10 (**C**, $p = 0.0027$, $r = 0.59$)

mation in the CNS of MS patients [30]. Taken together, these results show that the percentage of $CD4^+CD25^+$ cells in the CSF may serve as a measure of inflammatory reactions occurring in the CNS. However, it has been reported that $CD4^+CD25^+$ cells in the blood comprise immunoregulatory cells [26]. Therefore, their measure-

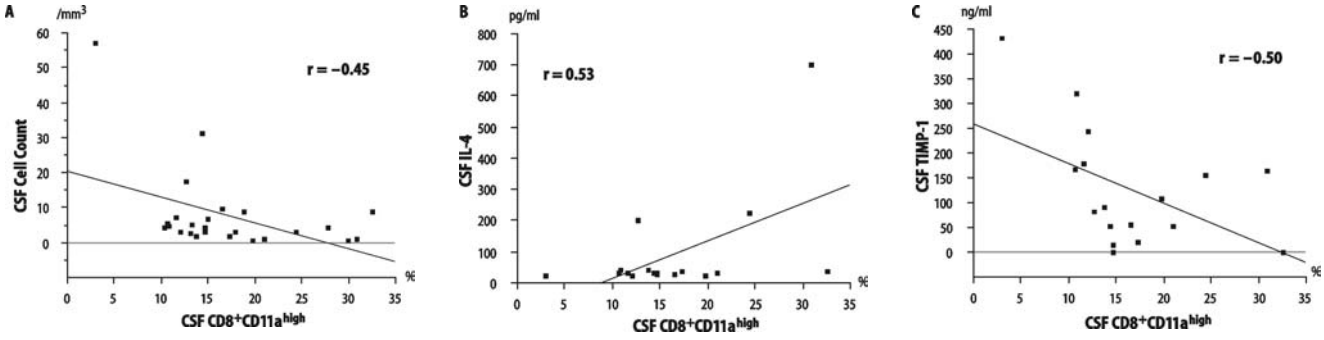
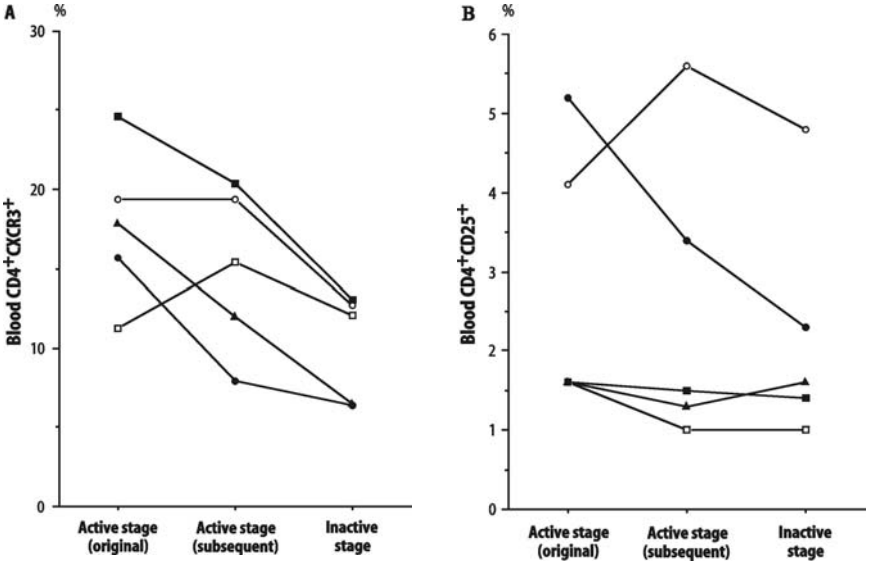



**Fig. 4** Relationships between the percentage of CSF $CD8^+CD11a^{high}$ cells and other parameters in the CSF. CSF $CD8^+CD11a^{high}$ cells were negatively correlated with CSF cell count (**A**, p = 0.0242, r = –0.45) and CSF TIMP-1 levels (**C**, p = 0.04, r = –0.50), and positively correlated with IL-4 levels in the CSF (**B**, p = 0.0263, r = 0.53)

**Fig. 5** Longitudinal changes in $CD4^+CXCR3^+$ and $CD4^+CD25^+$ cells in blood samples from 5 active MS patients. The percentages of $CD4^+CXCR3^+$ (**A**) and $CD4^+CD25^+$ (**B**) cells were determined during the original active stage, the subsequent relapse, and later during the following inactive stage within a singular time sequence. Each symbol represents data from an individual patient




ment in CSF samples obtained from patients with other inflammatory CNS disorders, such as acute encephalitis, would help to characterize their nature.

With respect to CSF $CD8^+CD11a^{high}$ cells, which have been designated as killer effector cells [22], our present results showing a decreased percentage despite an increased number of those cells during active stages of MS requires careful interpretation. Another study reported a significant decrease in the percentage of the $CD8^+$ subset with a $CD11b^+$ phenotype in the CSF during acute relapse [27]; thus, relative decreases in $CD8^+$ subsets are likely a constant feature of active MS CSF samples. In addition, we previously found that the percentage of $CD8^+CD11a^{high}$ cells in the blood was negatively correlated with the proportion of IL-2-producing $CD4^+$ (Th1) cells stimulated *in vitro* [19], suggesting that the balance between functionally opposing lymphocyte subsets, as represented by their percentages, could affect the final outcome of immune reactions. Provided that $CD8^+CD11a^{high}$ cells exert a similar function within the CNS, a decreased percentage, though not an elevated number, of those cells in the CSF is likely to be associated with low levels of the Th2-related anti-inflammatory cytokine IL-4 during active MS stages, as seen in the present study. Likewise, the inverse correlation found between the percentage of CSF $CD8^+CD11a^{high}$ cells and CSF TIMP-1 levels suggests that TIMP-1 production, which is known to become up-regulated in opposition to persistently elevated CSF levels of MMP-9 [17], may become attenuated with infiltration of $CD8^+CD11a^{high}$ cells into the CNS.

In conclusion, acute exacerbations of RRMS were associated with, at least in part, a combination of altered cellular immunity that differed between peripheral blood and CSF samples, and did not only mirror events occurring in the other compartment. A long-term follow-up study is necessary to determine whether such immunologic factors could serve as a measure for monitoring disease activity.

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