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Increased CD8+ central memory T cells in patients with multiple sclerosis

Guang-Zhi Liu¹, Li-Bo Fang², Peter Hjelmström³ and Xu-Guang Gao¹

A T-cell-mediated autoimmune process against central nervous system myelin is believed to underlie the pathogenesis of multiple sclerosis (MS). Formation of immunological memory is based on the differentiation of naïve T cells to memory T cells after exposure to antigens and specific cytokines. The aim of this study was to analyse peripheral blood mononuclear cells in patients with MS for different T-cell subsets including naïve and memory T cells. Flow cytometry and enzyme-linked immunosorbent assay were used to analyse memory T-cell subsets and plasma concentration of interleukin-15 (IL-15) in peripheral blood of MS patients, patients with other neurological disorders and healthy controls. MS patients had a skewed distribution of T cells with an increased level of CD8+/CCR7+/CD45RA- central memory T cells (TCM) compared to healthy controls. In addition, MS patients showed significantly higher levels of plasma IL-15 than healthy controls did. Upregulated CD8+ TCM in MS patients may reflect a persistent chronic inflammatory response that may have been induced during early stages of the disease. This derangement may be important for maintaining chronic inflammation in MS.
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Key words: CCR7; interleukin-15; memory T cell; multiple sclerosis; peripheral blood

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS), causing a variable degree of axonal damage. Although its causes remain unclear, it is believed that an autoreactive T-cell-mediated autoimmune process against CNS myelin underlies its pathogenesis [1–3]. So far, various studies have reported that myelin-reactive lymphocytes exhibit a memory phenotype in MS patients [4–7].

Recently, our understanding of different subtypes of T cells has been substantially improved [8–14]. T cells are heterogeneous and comprise distinct populations that can be distinguished based on surface markers and effector functions such as cytokine secretion and cytotoxicity. Among these, expression of chemokine receptor 7 (CCR7), which controls homing of lymphocytes to secondary lymphoid organs, was reported to divide human

memory T cells into two functionally distinct subsets, CCR7+/CD45RA- central memory T cells (TCM) and CCR7-/CD45RA- effector memory T cells (TEM) [14]. In short, naïve T cells, which migrate to secondary lymphoid organs in search of antigens, express the CCR7 and the membrane-bound signalling protein CD45RA. After exposure to antigens and cytokines such as interleukin-2 (IL-2), IL-7 and IL-15, the naïve T cells differentiate into memory T cells. These can be divided into TCM, TEM and, for the CD8+ population, CCR7-/CD45RA+ terminal TEM [15,16]. For the CD8+ population, the cytokine IL-15 appears to be particularly important for proliferation and, most likely also survival, of memory cells (reviewed in Ref. [17]) and we therefore focused our analysis on this cytokine.

In rheumatoid arthritis (RA) patients, the affected joint synovial tissue has an increased proportion of naïve T cells expressing CD45RA [18].

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Another recent study showed an increase of CCR7⁻/CD45RA⁺/CD8⁺ TEM cells expressing perforin and/or granzyme B in patients with systemic lupus erythematosus (SLE) [19]. In contrast to these findings, a study on patients with autoimmune thyroid disease showed a decrease in CCR7⁺ naïve and TCM in peripheral blood [20]. In patients with inflammatory neurological diseases, including MS, an expansion of both CD4⁺ and CD8⁺ T-cell subsets with a memory phenotype was seen in the cerebrospinal fluid (CSF) compared to peripheral blood of the patients [21]. In peripheral blood of MS patients, a higher frequency of CD4⁺ TEM after specific antigen-driven stimulation has been reported [22]. Two different studies have shown that most CSF T cells consist of CCR7⁺ TCM whereas T cells in parenchymal MS lesions comprised CCR7⁻ TEM [23,24]. Nevertheless, there is still a great need for further studies of the distribution of T-cell subtypes in MS and the possible role of skewed T-lymphocyte homeostasis in the inflammatory pathogenesis. Therefore, we have analysed peripheral blood mononuclear cells (PBMC) for different memory T-cell subsets and the regulatory cytokine IL-15 in patients with this disease.

Materials and methods

Patients and controls

Nineteen patients with clinically definite MS (6 men and 13 women; mean age 49.8 ± 12.6 years) were included in this study. Ten of the patients had relapsing–remitting MS (RRMS) and nine suffered from secondary progressive MS (SPMS). None of the patients were treated with immunosuppressants, including corticosteroids, for at least six months before the time of sample collection. The Expanded Disability Status Scale (EDSS) score was used to reflect the disease severity at the time of blood sampling [25]. In addition, five of these RRMS patients receiving treatment with interferon- β 1a (IFN- β 1a, Rebif) were chosen for serial study. Twenty patients with stroke (18 patients) and Parkinson disease (2 patients) (8 men and 12 women; mean age 52.4 ± 6.9 years) were enrolled as other neurological disease (OND) controls. Twenty-two healthy volunteers were included as normal controls (9 men and 13 women; mean age 40 ± 12 years). All participating subjects gave their informed consent before the start of the study. Blood leukocyte counts, including differential analysis, were determined in both the patients and healthy controls at the Department of Clinical Chemistry using routine methods.

Sample collection

Heparinized blood specimens were collected between 9 and 12 a.m. For serial study, blood specimens of the patients receiving treatment with IFN- β were collected twice after two and four weeks of the therapy. After centrifugation, plasma was stored at -70°C in small aliquots and thawed just before further use.

Flow cytometry

PBMC were isolated by density gradient centrifugation from heparinized blood samples using a standard protocol. The PBMC of the patients and control subjects were characterized for the expression of chemokine receptors by three-colour direct immunofluorescence and flow cytometry using a FACScan (Becton Dickinson, San Jose, CA, USA). The cells were washed with PBS (0.5% BSA, pH 7.2) and resuspended in PBS at a final concentration of 4×10^6 cells/mL. The cells used for staining with antibody were first Fc-blocked by treatment with 1 μg of normal mouse IgG (Caltag Laboratories, CA, USA) per 10^5 cells for 15 min at room temperature. The following antibodies were added with the Fc-blocked 1×10^5 cells in a 25- μL cell suspension according to the directions of the manufacturer: 1) IgG1 FITC (Becton Dickinson [BD], San Jose, CA, USA), IgG2a PE (R&D Systems, Oxford, UK), CD4 PerCP (BD); 2) CD45RA FITC (BD), CCR7 FITC (R&D Systems, Oxford, UK), CD4 PerCP; 3) IgG1 FITC, IgG2a PE, CD8 PerCP (BD); 4) CD45RA FITC, CCR7 PE (R&D Systems, Oxford, UK), CD8 PerCP (BD). After incubation for 30 min at 4°C , the cells were washed twice with PBS, fixed with 1% paraformaldehyde in PBS and analysed on the FACScan using CellQuest software (BD).

Interleukin-15

Plasma was collected at the same time as samples for flow cytometry from the patients. The samples were centrifuged at 2500 rpm for 10 min and stored at -70°C for later analysis with a commercial enzyme-linked immunosorbent assay (ELISA) (detection limit 2 pg/mL) according to the instructions from the manufacturer (R&D Systems Inc., Minneapolis, MN, USA).

Statistics

The data are presented as mean \pm standard deviation of the mean value (T-cell subsets) or median with range (IL-15). Data with a normal distribution were analysed with one-way analysis of variance

(ANOVA) with Student-Newman-Keul's post-hoc test or Pearson's correlation test. Data with a non-normal distribution were analysed with Kruskal-Wallis ANOVA or Spearman's correlation test. *P*-values less than 0.05 were considered to be statistically significant.

Results

T-cell subsets

MS patients had an increased proportion of CD8+ TCM (CCR7+/CD45RA-) and a decreased proportion of CD8+ TEM (CCR7-/CD45RA+) compared to healthy controls on the FACScan (*P* < 0.05). There was, however, no difference in the levels of CD8+ T cells between the three groups. Although there was a slight decrease in CD4+ naïve T cells (CCR7+/CD45RA+) and an increase in CD4+ TCM in MS patients, no significant differences were found between the three groups (*P* > 0.05, Table 1). Moreover, five MS patients who were treated with IFN-β1a showed a tendency of continuous decrease in CD4+ TCM and CD8+ TCM cells after two and four weeks of treatment (Figure 2C and G), while the remaining cell subsets presented slight or irregular changes (Figure 1A, B, D-F). As a control we found that total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophils in both patients and healthy controls were within the normal ranges of the Hospital Laboratory (data not shown).

Plasma IL-15 levels

The plasma IL-15 levels were calculated using a standard curve constructed with known concentrations of this protein. There was an increase in plasma IL-15 levels of MS patients (median = 36.01) compared to the levels of healthy controls

(median = 9.53, *P* < 0.05). No significant difference was found between MS and OND patients (median = 15.55, Figure 2). In addition, five MS patients who were treated with IFN-β1a showed a continuous decrease in plasma IL-15 levels after two and four weeks of treatment (Figure 3).

Correlation analysis

Each of the memory T-cell subsets or plasma IL-15 levels were not significantly correlated with EDSS in MS patients, except a marginally significant correlation for CD4+ naïve T cells (*r* = 0.5171, *P* = 0.0484). Plasma IL-15 levels also showed no significant correlation with each of the T-cell subsets.

Discussion

Recent studies have provided strong evidence that although CD4+ helper type 1 T cells may have an important role, a variety of other immune cells, including B cells, CD8+ T cells, NK T cells and CD4+/CD25+ regulatory T cells, seem to be involved in disease pathogenesis by inducing or controlling the immune response in MS [1-3]. In particular, there is increasing evidence that auto-reactive CD8+ T cells play an important role in the pathogenesis of MS and its animal models (reviewed in Ref. [26]). For instance, activated CD8+ T cells predominate over CD4+ T cells in the infiltrate of the CNS of MS patients, particularly in regions of active demyelination [27].

Our study shows that MS patients seem to have a skewed distribution of T-cell subsets (particularly CD8+), with an increase in TCM compared to healthy controls. On the other hand, total CD8+ cells and naïve CD8+ T cells in MS patients showed a trend to decrease compared to healthy controls. These findings indicate that the increase of CD8+ TCM occurs due to an increased differentiation of

Table 1 Subsets of T cell in peripheral blood of patients with MS, OND and healthy controls (HC)

	MS (n = 19) %	OND (n = 20) %	HC (n = 22) %	<i>P</i> -value (ANOVA)
CD4+				
Total CD4+	54.69 ± 11.66	51.44 ± 9.55	49.74 ± 7.33	0.3553
Naïve	36.43 ± 14.86	38.03 ± 12.33	42.16 ± 12.23	0.2572
Central memory	49.75 ± 13.46	44.31 ± 11.12	44.22 ± 12.76	0.2903
Effector memory	11.83 ± 6.89	13.58 ± 6.17	9.95 ± 4.86	0.1527
CD8+				
Total CD8+	23.12 ± 9.61	28.18 ± 7.45	28 ± 5.1	0.0645
Naïve	35.52 ± 20.23	33.44 ± 14.66	37.08 ± 14.96	0.7498
Central memory	18.91 ± 9.73*	13.28 ± 5.12	12.49 ± 6.39	0.0141
Effector memory	21.01 ± 12.06	18.25 ± 8.01	18.53 ± 6.96	0.5871
Terminal effector	25.09 ± 14.58*	35.03 ± 17.29	38.67 ± 18.74	0.0405

**P* < 0.05 for post-hoc comparison with healthy controls.

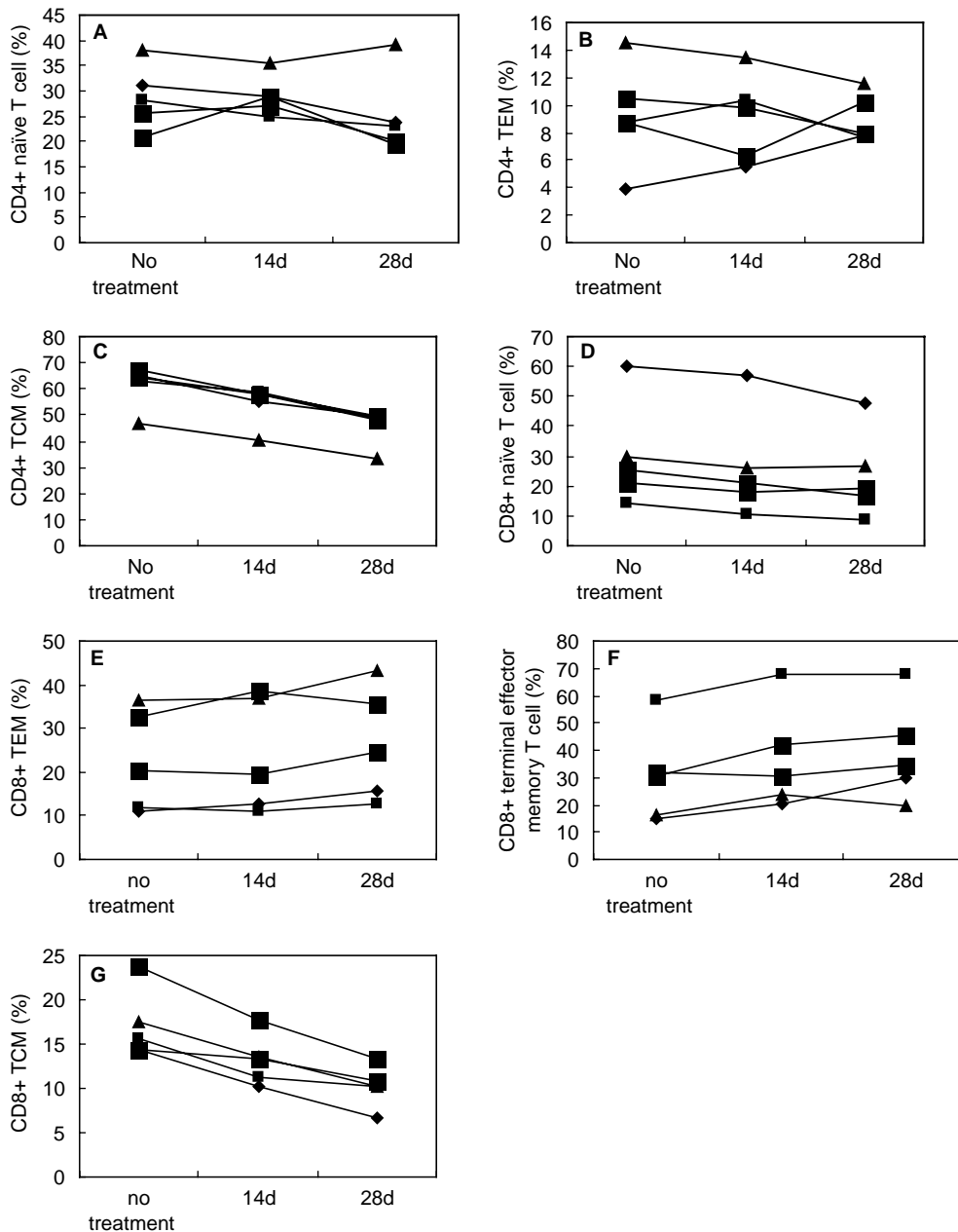


Figure 1 Serial study of (A–G) memory T-cell subsets in peripheral blood of five patients with MS before treatment and after two (14 days) and four weeks (28 days) of treatment with IFN- β 1a. 14 d = 14 days; 28 d = 28 days.

naïve cells, rather than due to an isolated expansion of the central memory cell pool. In addition, higher levels of plasma IL-15 in MS patients further substantiate this finding, as IL-15 has been identified as being of importance for the proliferation and survival of CD8+ memory T cells [28]. Interestingly, no differences between T-cell subsets could be found between MS patients and patients with OND. Eighteen of the OND patients were hospitalized due to atherosclerotic stroke and this category

of patients has previously in several studies been found to have an increase in TCM [29].

The exact lineage relationship among these memory T-cell subsets remains unresolved. Several models have been proposed: 1) TCM provide a perpetual source of TEM [30]; 2) TCM and TEM represent two mostly distinct lineages using distinct TCR repertoires [8]; 3) TEM slowly convert to TCM over time [31]. Recent studies seem to favour the last model in which TEM and TCM follow a linear

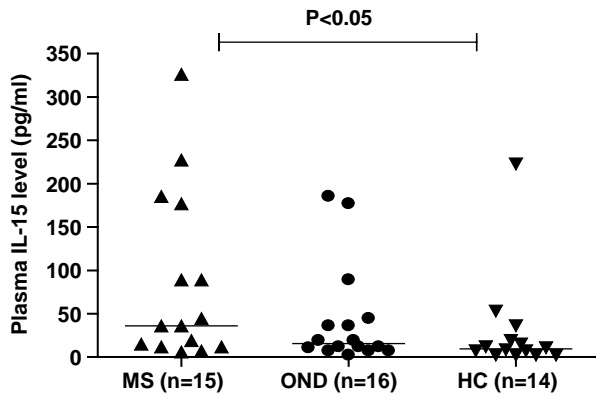


Figure 2 Comparison of plasma IL-15 levels between patients with MS, OND and healthy controls (HC). Horizontal lines indicate median values.

differentiation pathway from naïve T cells to effector T cells to TEM to TCM [32,33]. Indeed, our results showed a skewed differentiation from naïve T cells to TCM in peripheral blood, particularly CD8+ T cells. Interestingly, we also observed an increased proportion of CD4+ TCM, instead of CD8+ TCM, in CSF of eight RRMS patients compared to eight OND (migraine, subacute combined degeneration) patients (data not shown). Together with the previous studies that showed a predominant distribution of CD4+ TCM in CSF and CCR7- TEM in parenchymal MS lesions [21,23,24], our study further substantiates the existence of an abnormal T-cell differentiation and distribution that occurs in the different compartments of MS.

The exact role of CD8+ TCM in MS remains unknown. We can only speculate about what induces the increase in the TCM in MS and what function these cells have in the development of

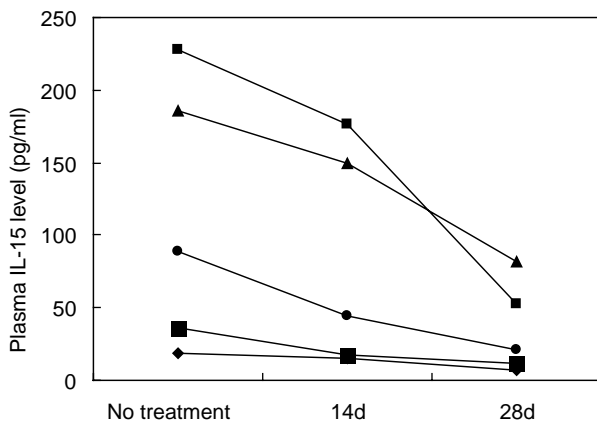


Figure 3 Serial study of plasma IL-15 levels in five patients with MS before treatment and after two (14 days) and four weeks (28 days) of treatment with IFN-β1a. 14 d = 14 days; 28 d = 28 days.

the disease. An upregulation of specific cytokines, such as IL-15, during episodes of acute inflammation in blood and CSF of MS patients [34–38], which drive the differentiation of naïve cells to TCM and maintain the survival of the TCM [16,28], is a possible theory. Indeed, the MS patients in our study showed a significant increase in plasma IL-15 levels compared to healthy controls, indicating that IL-15 may contribute to facilitate the differentiation or survival of CD8+ TCM as TCM physiologically circulates through the bloodstream, tissue compartments and lymphoid organs [39]. On the other hand, other factors such as IL-7, viral infection [40] and HLA [41] should certainly also be considered, as we did not find a significant correlation between plasma IL-15 levels and the memory T-cell subsets. The skewed distribution of CD8+ TCM cells may be a mechanism that contributes to the maintenance of MS. A recent study showed that myelin basic protein (MBP)-reactive CD8+ T-cell lines from MS patients were characteristic of the CD45RO+ memory T-cell subset and produced tumour necrosis factor-α and IFN-γ. Furthermore, these T-cell lines exhibited specific cytotoxicity toward autologous target cells pulsed with MBP-derived peptides in the context of major histocompatibility complex (MHC) class I molecules, supporting the potential role of CD8+ memory T cells in the proceeding injury of oligodendrocytes expressing both MHC class I molecules and MBP [42]. Unexpectedly, we did not find a significant correlation between CD8+ TCM and disease severity (EDSS) of the MS patients, although the TCM cells tended to decrease following treatment. Further serial studies are however needed with non-treated MS patients to confirm this finding. Our results, however, indicate the positive regulatory and complex role that the CD8+ TCM cells may play in the disease pathogenesis. The immunologic derangement of CD8+ TCM could as well be a general phenomenon in chronic inflammatory disorders, as upregulation of the memory T cells has been described also in other inflammatory conditions [21,43].

In conclusion, this study shows that CD8+ TCM cells are upregulated in MS patients. This may reflect a persistent chronic inflammatory response that may have been induced during early stages of the disease. We speculate that this derangement may be important for maintaining the chronic inflammation. Further studies on the influence of different aetiologies of MS, and other inflammatory diseases, on the distribution of the TCM are needed to gain more insight into the pathophysiological significance of this T-cell subset in human MS patients.

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