

Cytokines and adhesion molecules in multiple sclerosis patients treated with interferon- β 1b

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Abstract

Multiple sclerosis (MS), an inflammatory, demyelinating disease of the central nervous system (CNS), is thought to be caused by a T cell-mediated attack on CNS myelin and axons. Recombinant interferon (IFN)- β is an established treatment of multiple sclerosis, and is known to reduce the number of disease relapses and the development of irreversible symptoms and signs of disease. The mechanism of action of IFN- β treatment is, however, not completely understood. Previous studies have suggested major effects on mononuclear cell cytokine production and T cell migration, but results have been inconsistent. We found decreases in CD4 and CD8 T cell expression of the CD49d/VLA-4 molecule, increases in plasma concentrations of soluble vascular cell adhesion molecule (sVCAM-1), and increases in plasma concentrations of tumor necrosis factor and interleukin (IL)-12 p40 chain in patients with MS who were initiated on de novo treatment with IFN- β 1b. We found only minor associations between the different changes induced by IFN- β 1b-treatment. Our findings are consistent with changes in T cell expression of CD49d/VLA-4 and induction of sVCAM-1 as important effects of treatment with IFN- β 1b in multiple sclerosis, whereas the role of changes in TNF and IL-12 p40 chain concentrations is more difficult to interpret.

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1. Introduction

A T cell-mediated immune-inflammatory attack on central nervous system (CNS) myelin is thought to be essential in the pathogenesis of multiple sclerosis (MS) [1]. Treatment with interferon (IFN)- β attenuates clinical and magnetic resonance imaging (MRI) disease activity in multiple sclerosis (MS) [2]. The mechanism of action is not understood in detail, but changes in T cell activation and regulation, cytokine production and leukocyte migration have been reported in MS patients treated with IFN- β [3].

It has been suggested that IFN- β induces a shift in cytokine production by myelin-reactive CD4 T cells from disease-promoting Th1 cytokines such as tumor necrosis factor (TNF, also known as TNF- α or TNFSF2), lymphotoxin (LT)- α (also known as TNF- β or TNFSF1), interleukin (IL)-12, and IFN- γ to the production of Th2 cytokines such as IL-4, IL-5 and IL-13, and the production of IL-10 which could have beneficial effects in MS [3]. However, data on cytokine production in MS patients treated with IFN- β are confusing and not clearly consistent with a Th1 to Th2 cytokine shift. Indeed, IFN- β can substitute for IL-12 in the induction of Th1 immune responses in human T cells, and IFN- β induces some Th1-associated responses in MS patients [4–6].

The suppression of inflammatory disease activity on gadolinium-enhanced MRI of the brain can be observed

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within the first month of treatment with IFN- β [7,8]. Treatment also results in a decrease in cerebrospinal fluid leukocyte counts [9]. Several effects of IFN- β on T cells may result in a lower potential for migration across the blood–brain barrier. IFN- β treatment inhibits the expression of chemokine receptors involved in the chemoattraction of T cells across the blood–brain barrier, and inhibits the production of matrix metalloproteinase (MMP)-9 needed for the degradation of extracellular matrix proteins during T cell migration [3,10]. IFN- β treatment also results in decreased T cell expression of the integrin CD49d/CD29 (α 4/ β 1 integrin or very late antigen (VLA)-4) which has an important role in T cell migration in MS [11,12]. Finally, treatment results in an increase in the plasma concentration of a soluble form of the VLA-4 ligand vascular cell adhesion molecule (sVCAM)-1 [13]. sVCAM-1 has been reported to block the function of the VLA-4 molecule at physiological concentrations, and its production may be induced in brain endothelial cells by IFN- γ and TNF [14].

Although IFN- β -induced changes in cytokines and adhesion molecules have been addressed in numerous previous studies, the results are inconsistent, and few studies have analyzed the relationship between treatment-induced changes in expression of different molecules. In order to address these issues, we studied a panel of plasma cytokines, soluble intercellular adhesion molecule (sICAM)-1, sVCAM-1, and T cell expression of VLA-4 in a series of patients initiated on de novo therapy with IFN- β 1b. The molecules under study were chosen because they should allow the identification of major changes in cytokines believed to be involved in the pathogenesis of MS, as well as changes in adhesion molecules thought to be of pivotal importance in leukocyte migration to the CNS. Accordingly, the study should allow the identification of changes in systemic immune activation that are related to the clinical efficacy of IFN- β treatment.

2. Materials and methods

2.1. Patients and controls

Ten patients with MS (6 women, 4 men; median age 41 years, range 34–54 years) and 10 healthy control subjects (6 women, 4 men; median age 32 years, range 25–37 years) were included in the study. The patients all had clinically definite MS, relapsing-remitting disease type, and had suffered at least two attacks in the 2 years prior to the initiation of treatment [15]. All patients were in clinical remission at study entry, none of the patients had experienced an acute attack or received glucocorticoid treatment within 4 weeks, and none had been treated with IFN- β or other immunomodulating drugs prior to study entry. Their median disease duration was

7 years (range 3–14 years) and their median Kurtzke EDSS score was 3.5 (range 2.0–5.0) [16]. The patients were consecutively enrolled in the study when starting treatment with recombinant IFN- β 1b (Betaferon, Schering AG, Berlin, FRG), 8 million IU by subcutaneous injection every other day. None of the patients discontinued therapy, and none of the patients suffered an attack during the study period. The study was conducted in accordance with the Declaration of Helsinki, was approved by the regional scientific ethics committee, and informed written consent was obtained from all participants.

2.2. Sample preparation

Venous blood samples were obtained between 9 and 12 a.m. at baseline, after 1 month, and after 3 months of treatment. Blood drawn in EDTA tubes was immediately cooled on ice, and plasma aliquots were stored at -80°C within 2 h and were not thawed until analysis. Peripheral blood mononuclear cells (MNC) for flow cytometry studies were obtained by density gradient centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway), washed and resuspended in phosphate buffered saline with sodium azide (FACS PBS) at a final concentration of 1 million cells per ml. Commercial enzyme-linked immunosorbent assays (ELISA) kits were used according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN) to measure plasma concentrations of the following analytes (lower limit of the assay standard curve is given for all analytes): TNF (0.5 pg/ml), LT- α (31 pg/ml), IFN- γ (31 pg/ml), IL-2 (31 pg/ml), IL-4 (0.25 pg/ml), IL-10 (0.78 pg/ml), IL-12 p40 (31 pg/ml), IL-12 p70 (0.78 pg/ml), soluble intercellular adhesion molecule (sICAM)-1 (45 ng/ml) and sVCAM-1 (200 ng/ml).

2.3. Flow cytometry analysis

For flow cytometry 100,000 blood mononuclear cells were stained with a combination of phycoerythrin-conjugated anti-CD49d antibody or isotype control antibody, peridinin chlorophyll protein (PerCP)-conjugated anti-CD8 antibody, and allophycocyanin (APC)-conjugated anti-CD4 antibody (all from Becton Dickinson Immunocytometry Systems, San Jose, CA), washed and fixed with 1% paraformaldehyde before analysis on a FACS CaliburTM flow cytometer equipped with CellQuestTM 3.1 software (Becton Dickinson). T cell subsets (CD4 or CD8) were identified in dot plots using logical gates based on the PerCP and APC fluorescence and the light scatter properties of individual cells. Subsequently, T cell expression of the specific molecules was analyzed in histogram plots of PE fluorescence intensity. Statistical markers were set at the fluorescence intensity level expressed by 1% of cells stained with isotype control

antibody, and cells showing specific antibody fluorescence intensity above this level were considered positive.

2.4. Statistics

Statistical analysis of differences between patients and controls was by the Mann–Whitney U-test. Friedman's test for several related samples (non-parametric ANOVA) was used to compare differences over the study period in patients initiated on IFN- β treatment, and Wilcoxon's matched-pair rank test was applied to determine differences between baseline, 1 month and 3 month follow-up samples when the Friedman test indicated that significant changes occurred during treatment. The relationship between different variables was analyzed by Spearman rank correlation analysis. A 5% significance level was employed.

3. Results

3.1. T cell CD49d/VLA-4 expression

At baseline the percentage of CD4 T cells that expressed CD49d/VLA-4 was somewhat, but not significantly, higher in MS patients than in controls (Fig. 1; $p=0.08$), and the percentage of CD49d/VLA-4 positive CD8 T cells was higher in MS patients than in controls (Fig. 1; $p=0.04$). The percentage of CD4 T cells ($p=0.001$) and CD8 T cells ($p=0.007$) expressing CD49d/VLA-4 changed significantly from baseline values during treatment with IFN- β (Fig. 1; Friedman test). After 1 month the percentage of CD4 T cells expressing CD49d/VLA-4 had decreased almost significantly from baseline values ($p=0.06$), and after 3 months values were significantly lower than at baseline ($p=0.007$) and in healthy controls ($p=0.006$). In CD8 T cells the percentage of cells expressing CD49d/VLA-4 were lower than at baseline after 1 month ($p=0.02$) and 3 months ($p=0.005$). The percentage of CD49d/VLA-4 positive CD8 T cells in IFN- β treated MS patients was slightly lower than in healthy controls after 3 months ($p=0.1$), but there was no difference between healthy controls and MS patients after 1 month of treatment with IFN- β .

3.2. Plasma sVCAM-1 and sICAM-1 studies

Plasma concentrations of sVCAM-1 did not differ in untreated MS patients and healthy controls (Fig. 2). The plasma concentration of sVCAM-1 changed significantly during treatment with IFN- β as assessed by the Friedman test (Fig. 2; $p=0.002$). After 1 month and 3 months the plasma concentrations of sVCAM-1 were significantly higher than at baseline ($p=0.005$ and $p=0.02$, respectively) and than plasma concentrations of sVCAM-1 in healthy controls ($p=0.004$ and $p=0.02$,

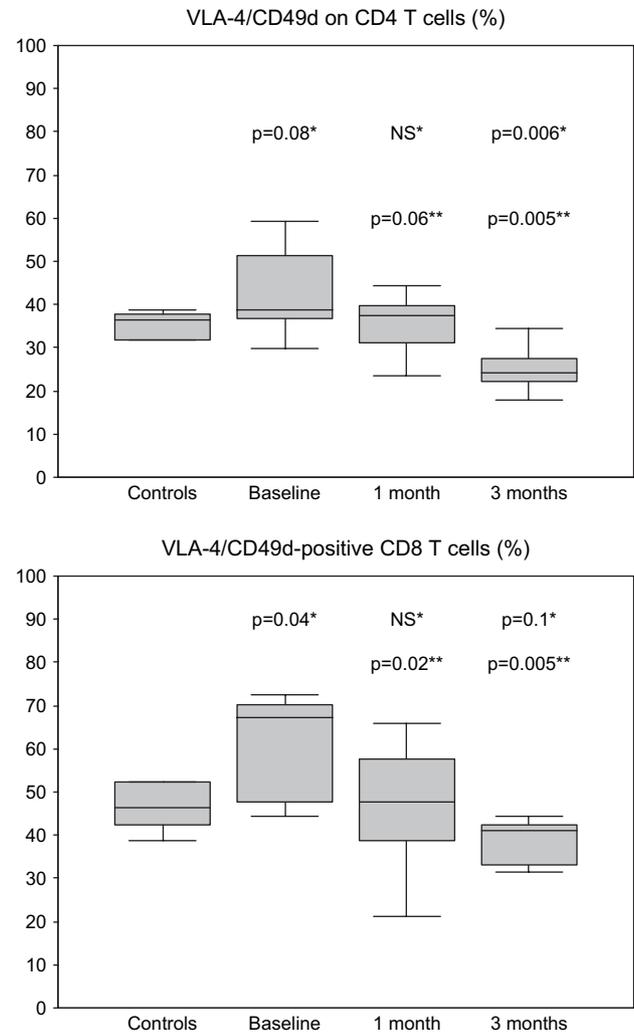


Fig. 1. The percentage of peripheral blood CD4 and CD8 T cells expressing CD49d/VLA-4 was measured by flow cytometry in healthy controls and patients with multiple sclerosis at baseline, and after 1 and 3 months of treatment with interferon- β . Box-and-whisker plots show median, inter-quartile range, and range of observations. The percentage of CD4 T cells ($p=0.001$) and CD8 T cells ($p=0.007$) expressing CD49d/VLA-4 changed significantly from baseline values during treatment with IFN- β (Friedman test). p -values for comparisons between controls and patients* (Mann–Whitney test) and changes from baseline values in patients** (Wilcoxon test) are given in the figure. A 5% significance level was employed. NS, not significant.

respectively). Before treatment we found higher plasma concentrations of sICAM-1 in MS patients than in healthy control subjects ($p=0.04$). Plasma concentrations of sICAM-1 remained elevated during treatment with IFN- β (1 month, $p=0.02$; 3 months, $p=0.04$) with no significant changes compared to baseline concentrations (data sICAM-1 not shown).

3.3. Plasma cytokine studies

Plasma concentrations of IL-12 p40 and TNF did not differ in untreated MS patients and healthy controls

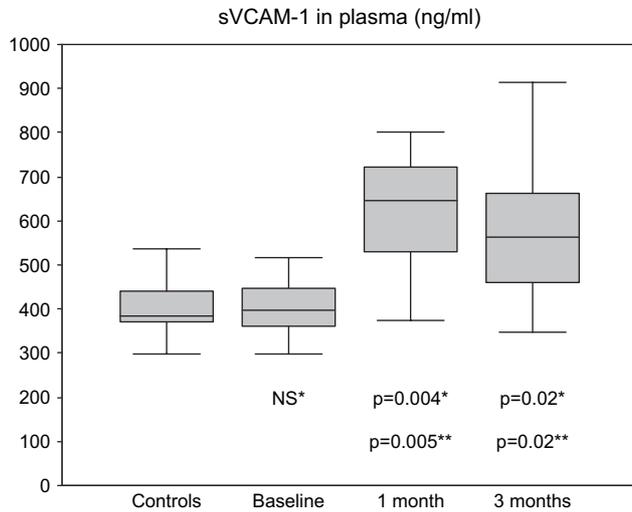


Fig. 2. Plasma concentrations of soluble vascular cell adhesion molecule (sVCAM)-1 were measured by ELISA in healthy controls and patients with multiple sclerosis at baseline and after 1 and 3 months of treatment with interferon-1b. Box-and-whisker plots show median, inter-quartile range, and range of observations. The plasma concentration of sVCAM-1 changed significantly during treatment with IFN-β1b ($p=0.002$, Friedman test). p -values for comparisons between controls and patients* (Mann–Whitney test) and changes from baseline values in patients** (Wilcoxon test) are given in the figure. A 5% significance level was employed. NS, not significant.

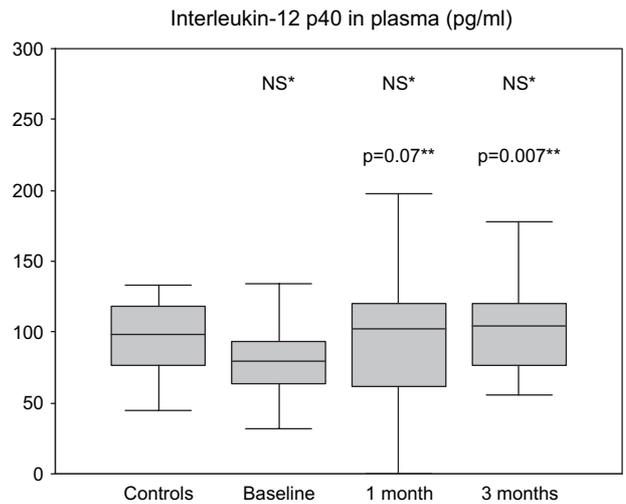
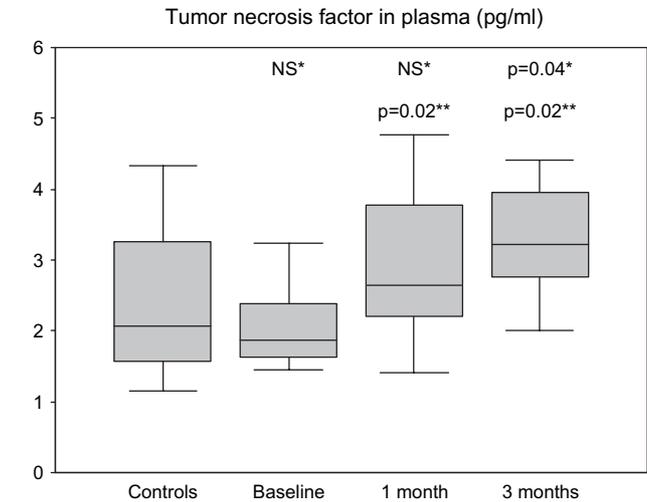


Fig. 3. Plasma concentrations of tumor necrosis factor and interleukin-12 p40 were measured by ELISA in healthy controls and patients with multiple sclerosis at baseline and after 1 and 3 months of treatment with interferon-1b. Box-and-whisker plots show median, inter-quartile range, and range of observations. After treatment with IFN-β1b the plasma concentrations of IL-12 p40 ($p=0.007$) and TNF ($p=0.02$) changed significantly (Friedman test). p -values for comparisons between controls and patients* (Mann–Whitney test) and changes from baseline values in patients** (Wilcoxon test) are given in the figure. A 5% significance level was employed. NS, not significant.

(Fig. 3). IL-2, IL-12 p70, IFN-γ, and LT-α were not detected, and IL-4 and IL-10 were detected only in a minority of samples, with no clear differences between patients and controls (data not shown). After treatment with IFN-β1b we observed significant changes in the plasma concentrations of IL-12 p40 ($p=0.007$) and TNF ($p=0.02$) as assessed by the Friedman test (Fig. 3). Plasma concentrations of IL-12 p40 had increased slightly but not significantly compared to baseline levels after 1 month ($p=0.07$), and significantly compared to baseline levels after 3 months of treatment with IFN-β1b ($p=0.007$). However, there were no significant differences between IL-12 p40 concentrations in healthy controls and treated MS patients at any time point. Plasma concentrations of TNF had increased significantly compared to baseline levels after 1 and 3 months of treatment with IFN-β1b (both $p=0.02$), and after 3 months of treatment MS patients had significantly higher plasma concentrations of TNF than did healthy controls ($p=0.04$).

3.4. Correlations between adhesion molecules and cytokines

The relationship between T cell expression of CD49d/VLA-4 and plasma concentrations of sVCAM-1, sICAM-1, TNF, and IL-12 p40 was studied by Spearman rank correlation analysis. At baseline the plasma concentrations of sVCAM-1 and TNF correlated

significantly (Spearman’s $\rho=0.65$, $p=0.04$), but these variables did not correlate during treatment with IFN-β1b. After 1 month of treatment with IFN-β1b the percentage of CD4 T cells expressing CD49d/VLA-4 correlated with the plasma concentration of IL-12 p40 (Spearman’s $\rho=0.64$, $p<0.05$), and the percentage of CD8 T cells expressing CD49d/VLA-4 correlated with the plasma concentration of sICAM-1 (Spearman’s $\rho=0.82$, $p=0.004$). After 3 months of treatment with IFN-β1b we observed a negative correlation between the plasma concentration of TNF and the percentage of CD8 T cells expressing CD49d/VLA-4 (Spearman’s $\rho=-0.69$, $p=0.03$). Apart from these correlations, we

observed no significant correlations between the variables under study at baseline or during IFN- β 1b therapy. Neither did we observe any significant correlations in the healthy controls.

4. Discussion

We found that treatment of MS patients with IFN- β 1b results in lower expression of CD49d/VLA-4 on CD4 and CD8 T cells, increases in the plasma concentration of sVCAM-1, and increases in the plasma concentrations of TNF and IL-12 p40. The effects on T cell expression of CD49d/VLA-4 and plasma concentrations of sVCAM-1 are in agreement with previous studies [11–13,17–19]. We found no change in the plasma concentration of sICAM-1 during treatment with IFN- β 1b, which is in agreement with other studies finding either no effect or a transient increase in sICAM-1 concentrations in MS patients treated with IFN- β [13,19–21]. It was previously reported that sVCAM-1 can down-regulate leukocyte expression of CD49d/VLA-4 *in vitro* [11]. We did not observe any correlation between plasma concentrations of CD49d/VLA-4 and sVCAM-1, and plasma concentrations of sVCAM-1 were maximally increased already after 1 month of treatment, whereas T cell expression of CD49d/VLA-4 decreased further from 1 month to 3 months of treatment, suggesting that there is no simple relationship between increased plasma concentrations of sVCAM-1 and decreased T cell expression of CD49d/VLA-4.

It may be argued that the number of patients included in the present study is too low to allow for meaningful statistical analysis. However, MRI studies have shown clear and consistent effects of IFN- β treatment on brain inflammation within the first weeks of treatment in studies of low numbers of patients [7,8]. The results of the present study are also in agreement with other studies on changes in sVCAM-1 and VLA-4 expression, and we therefore consider it unlikely that the findings are spurious. Indeed, a small patient sample would be expected to allow for the identification of major treatment effects whereas it is only smaller effects that may be overlooked (i.e., increased risk of type II error for measures with a lower standardized difference). In contrast, the risk of a false-positive result (type I error) is not affected by sample size.

We found no change in the plasma concentrations of most of the cytokines under study. Indeed, most cytokines were undetectable or detected only in minority of patients. This is consistent with the paracrine effects of most cytokines, which are mainly produced within lymphoid organs and inflamed tissues. We did, however, find some increase in the plasma concentrations of IL-12

p40 during treatment with IFN- β 1b, but changes were modest and at no time-point did the concentration of IL-12 p40 in plasma differ between patients and healthy controls. Two previous studies found increases in IL-12 p70 and IL-12 p40 plasma concentrations in IFN- β -treated MS patients [22,23], whereas no changes were observed in two other studies [24,25]. We observed a correlation between the plasma concentration of IL-12 p40 and the percentage of CD4 T cells expressing CD49d/VLA-4 after 1 month of treatment with IFN- β 1b, which suggests at least some association between the production of IL-12 p40 and a T cell phenotype associated with disease activity in MS [26]. The biological effects of an increase in IL-12 p40 are, however, difficult to predict since IL-12 p40 is a component of several different cytokines. Together with IL-12 p35 it forms the bioactive form IL-12 p70 which is pivotal in the development of Th1 immune responses [27]. The increase in IL-12 p40 immunoreactivity observed after treatment with IFN- β 1b in the present study is unlikely to reflect the induction of bioactive IL-12 as we found no IL-12 p70 immunoreactivity in any patient or control subject. IL-12 p40 itself may bind as an antagonist to the IL-12 receptor, and IL-12 p40 treatment has been reported to suppress the development of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [28]. Finally, IL-12 p40 may heterodimerize with the IL-23 p19 chain to form bioactive IL-23, a recently discovered cytokine with strong Th1-promoting and macrophage-activating effects, which plays a key role in EAE development [29,30].

We also observed an increase in plasma concentrations of TNF during IFN- β 1b treatment. Previous studies of TNF in MS patients treated with IFN- β have given highly variable results. Some studies have reported increased plasma concentrations and increased TNF mRNA levels in blood mononuclear cells at least transiently during treatment [22,31,32], others have reported decreased TNF expression during treatment [18,33,34]. Although TNF is generally assumed to play a detrimental role in the pathogenesis of MS, the role of TNF in MS patients treated with IFN- β may be more complex. Thus, in a recent study it was suggested that patients who did not respond to treatment with IFN- β did show a down-regulation of TNF mRNA in blood mononuclear cells, whereas there was no change in TNF mRNA in patients who responded to treatment [35]. Furthermore, clinical trials of TNF blockade using anti-TNF antibodies (Infliximab) or TNF-receptor fusion proteins (Lenercept) showed a paradoxical increase in disease activity in treated patients [36,37].

In general, we observed only few significant correlations between plasma concentrations, cytokines, and CD49d/VLA-4 expression on T cells. Studies in the EAE model have provided compelling evidence that in addition to disease promoting effects, TNF is also

involved in the control of pathogenic T cell activation [38]. We observed a negative correlation between the plasma concentration of TNF and CD8 T cell expression of CD49d/VLA-4 which might reflect a negative effect of TNF on T cell activation, but this correlation was observed only after 3 months of treatment with IFN- β 1b. In addition to direct effects on T cells, TNF can induce the expression of sVCAM-1 in human brain endothelial cells. sVCAM-1 may block the interaction of circulating T cells with VCAM-1, and the induction of sVCAM-1 has been associated with a decrease in MRI lesions in MS patients treated with IFN- β [13,14]. We observed a correlation between the plasma concentration of TNF and sVCAM-1 at baseline, but this was not observed in patients treated with IFN- β 1b. This could reflect an interaction between TNF and IFN- β 1b, which may act synergistically in the induction of sVCAM-1 in endothelial cells [14], or it might reflect a direct role of T cells in the induction of VCAM expression as suggested by in vitro studies [39]. We also observed a correlation between the plasma concentration of sICAM-1 and the percentage of CD8 T cells expressing CD49d/VLA-4 after 1 month of treatment but not at baseline or after 3 months of treatment. This transient correlation is difficult to interpret in mechanistic terms, especially since we observed significant changes in CD49d/VLA-4 expression but not in sICAM-1 concentrations during treatment with IFN- β .

In conclusion we found some increases in plasma concentrations of TNF and IL-12 p40 that could have either beneficial or detrimental effects depending on the precise context. Furthermore, we found a major effect of IFN- β 1b treatment on T cell expression of CD49d/VLA-4 and plasma concentrations of sVCAM-1. Increased plasma concentrations of sVCAM-1 are likely to be a major effect of treatment and correlates with an effect of treatment on MRI [13]. Furthermore, a beneficial role of a lower percentage of CD49d/VLA-4 expressing T cells is supported by studies showing lower disease activity in MS patients treated with an anti-CD49d/VLA-4 antibody [40,41]. We found little association between the different effects of treatment, suggesting that these are differentially regulated in MS patients treated with IFN- β 1b.

Acknowledgements

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