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CD4⁺ memory T cells with high CD26 surface expression are enriched for Th1 markers and correlate with clinical severity of multiple sclerosis[☆]

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Abstract

An aberrant immune activation is believed to be important in the pathogenesis of multiple sclerosis (MS). Expression of CD4⁺ T lymphocyte surface molecules indicative of immune activation and effector functions has been correlated with disease severity and activity. CD4⁺ CD45R0⁺ CD26^{high} memory T lymphocytes contained the high levels of markers of Th1, activation, and effector functions and cell counts of this subset correlated with MS disease severity. This subset had lower expression of PD-1, CCR4, and L-selectin in MS than in controls. These changes were only partially normalised by treatment with interferon- β . We point to this subset as a putative target for immunological monitoring of MS disease activity and of treatment efficacy.

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Keywords: Multiple sclerosis; T-lymphocyte subsets; Antigens, CD4; Antigens, CD26; PD-1; Interferon-beta; Biological markers

1. Introduction

Multiple sclerosis (MS) is an inflammatory CNS disease with myelin degradation, axonal damage, and ensuing neurological symptoms and disability. CD4⁺ T helper type 1 (Th1) cells, similar to the pathogenic T cells in the animal model Experimental Autoimmune Encephalomyelitis (EAE), are important in MS pathophysiology, but other cell-types are also involved (Sospedra and Martin, 2005). Interferon (IFN)- β is a standard therapy for the relapsing–remitting form of MS (RRMS), reducing the relapse rate by one-third and slowing disease progression. Although the mechanism of action is unclear, alterations in antigen presentation, leukocyte activa-

tion, proliferation, apoptosis, and migration are probably involved.

CD4⁺ T helper cells comprise multiple subsets, representing different differentiation stages and activation levels. A major distinction is between naïve (predominantly CD45R0–CCR7+ CD62L+ (L-selectin)) and antigen-experienced memory CD4⁺ T cells (predominantly CD45R0+), which can be further divided into central memory cells (T_{CM}, CCR7+) which home to secondary lymphoid organs, and effector memory cells (T_{EM}, CCR7–) which home to sites of inflammation (Sallusto et al., 2004).

Activated T cells migrate into the CNS by means of specific adhesion molecules and chemokine receptors (Ransohoff et al., 2003). CD4⁺ Th1 cells express the chemokine receptors CCR5 and CXCR3 (Loetscher et al., 1998; Bonecchi et al., 1998), and also T cells in active MS lesions express these chemokine receptors (Sorensen et al., 1999; Balashov et al., 1999). CD4⁺ Th1 cells from patients with clinically active MS also express high levels of the adhesion molecule Very Late Antigen (VLA)-4 (CD29/CD49d) (Barrau et al., 2000), which

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is crucial in CNS transmigration, and treatment with the anti-VLA-4 antibody natalizumab is highly efficacious in patients with relapsing–remitting MS (Polman et al., 2006).

The cytokine interleukin (IL)-12 is crucial for the development of Th1 cells, and the IL-12 receptor (IL-12R) β 2 (CD212) is expressed on Th1 cells (Murphy and Reiner, 2002). Th1 cells also express the chemokine receptor CXCR6 whereas the chemokine receptor CCR4 is expressed by Th2 cells (Bonecchi et al., 1998; Kim et al., 2001).

Costimulatory signals delivered through the CD28 molecule are important for the priming of naïve T cells, whereas memory T cells are less dependent on costimulatory signals, and the majority of human cytotoxic CD4⁺ T cells do not express CD28 (Amyes et al., 2005). Other molecules of the CD28 family such as cytotoxic T cell antigen (CTLA)-4 and programmed death (PD)-1 inhibit T cell activation (Greenwald et al., 2005). Immunoregulatory signals are also provided by the Fas/Fas ligand (FasL) system, which can induce apoptosis in T cells (Siegel et al., 2000). FasL is, however, also expressed on Th1 cells (Roberts et al., 2003; Hahn and Erb, 1999).

CD26 (dipeptidyl peptidase IV) is involved in the activation of T cells, and is expressed on antigen-reactive memory T cells (Hafler et al., 1986; Morimoto and Schlossman, 1998). Expression of CD26 has been correlated with clinical and MRI disease activity measures in MS, and CD26^{high} T cells express Th1 cytokines and CCR5, suggesting that this subset could comprise activated Th1 effector cells, a subset of particular interest in MS pathogenesis (Bleul et al., 1997; Hafler et al., 1985; Khoury et al., 2000; Constantinescu et al., 1995; Jensen et al., 2004). Indeed, myelin basic protein (MBP)-specific T cells derived from MS patients express high levels of CD26, and specific inhibitors of the enzymatic activity of CD26 suppress the activation of the MBP-specific T cells (Reinhold et al., 1998). In vivo inhibition of CD26 delays and attenuates disease severity in EAE (Steinbrecher et al., 2001). We therefore set out to characterise the immunological phenotype of memory CD4⁺ CD26^{high} cells by measuring the expression of surface markers linked to specific CD4⁺ T-cell functions, as we hypothesised that the numbers and the phenotype of the CD4⁺ CD45R0⁺ CD26^{high} cells would correlate with MS disease severity. We also wanted to assess whether IFN- β therapy in MS patients causes changes in phenotypes or cell counts in this particular subset.

2. Materials and methods

2.1. Patients and healthy controls

The study comprised a group of 15 patients with RRMS initiating treatment with IFN- β (median age 31 years, 80% women) and 14 healthy controls (median age 34 years, 60% women). In the MS patients the median disease duration was 4 years and median score on the Kurtzke Expanded Disability Status Scale (EDSS) was 2.0. Disease severity was calculated

using the MS Severity Score that takes into account both EDSS score and disease duration (Roxburgh et al., 2005); median score was 5.87 (scale range: 0–10). No patients had received previous immunomodulatory treatment, or had been treated with methylprednisolone for at least 4 weeks prior to inclusion. All patients were relapse-free at the time of blood collection. Blood samples were drawn from patients before initiation of IFN- β therapy (IFN- β 1a), and after 3 months of treatment. All blood samples were collected between 1 and 7 days after an IFN- β injection. The study was approved by the regional scientific ethics committee and conducted according to the Helsinki declaration.

2.2. Blood mononuclear cell preparation and flow cytometry

Blood was collected in Na-heparin tubes and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) within 30 min and washed twice in ice cold PBS with 1% human serum albumin and 0.1% NaN₃ (FACS PBS). 100–200,000 freshly isolated PBMCs suspended in 50 μ L FACS PBS were incubated on ice for 30 min in the dark with fluorechrome-conjugated monoclonal antibodies, washed twice with FACS PBS, and fixed in 1% paraformaldehyde. Fixed cells were kept in the dark until analysis on a four-colour FACSCalibur flow cytometer within 24 h. The following antibodies were used: CD4 APC (clone MT310; Dako, Glostrup, Denmark); CD45R0 PerCP (clone UCHL1, custom conjugation; BD Pharmingen, San Diego, CA); and CD26 FITC (clone BA5; Dako). PE-conjugated antibodies: CCR4 (clone 1G1; BD Pharmingen); CCR5 (clone 2D7; BD Pharmingen); CCR7 (clone 150503; R&D Systems, Abingdon, UK); CXCR3 (clone 1C6; BD Pharmingen), CXCR6 (clone 56811; R&D Systems); CD28 (clone CD28.1; Dako);

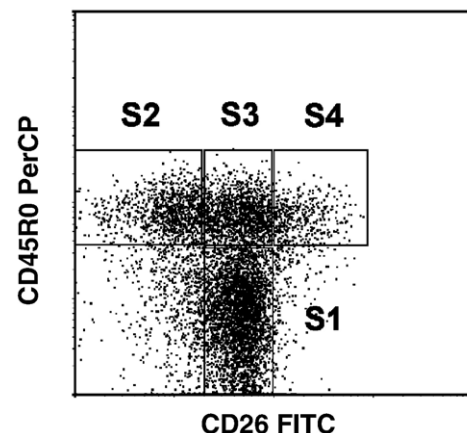


Fig. 1. Major CD4⁺ T cell subsets defined by surface CD45R0 and CD26 expression. Representative flow cytometry dot-plot gated for CD4⁺ T lymphocytes. Regions designate four major subsets selected by their differential expression of CD45R0 and CD26. S1: CD4⁺ CD45R0[–] CD26^{intermediate}; S2: CD4⁺ CD45R0⁺ CD26^{low}; S3: CD4⁺ CD45R0⁺ CD26^{intermediate}; S4: CD4⁺ CD45R0⁺ CD26^{high}.

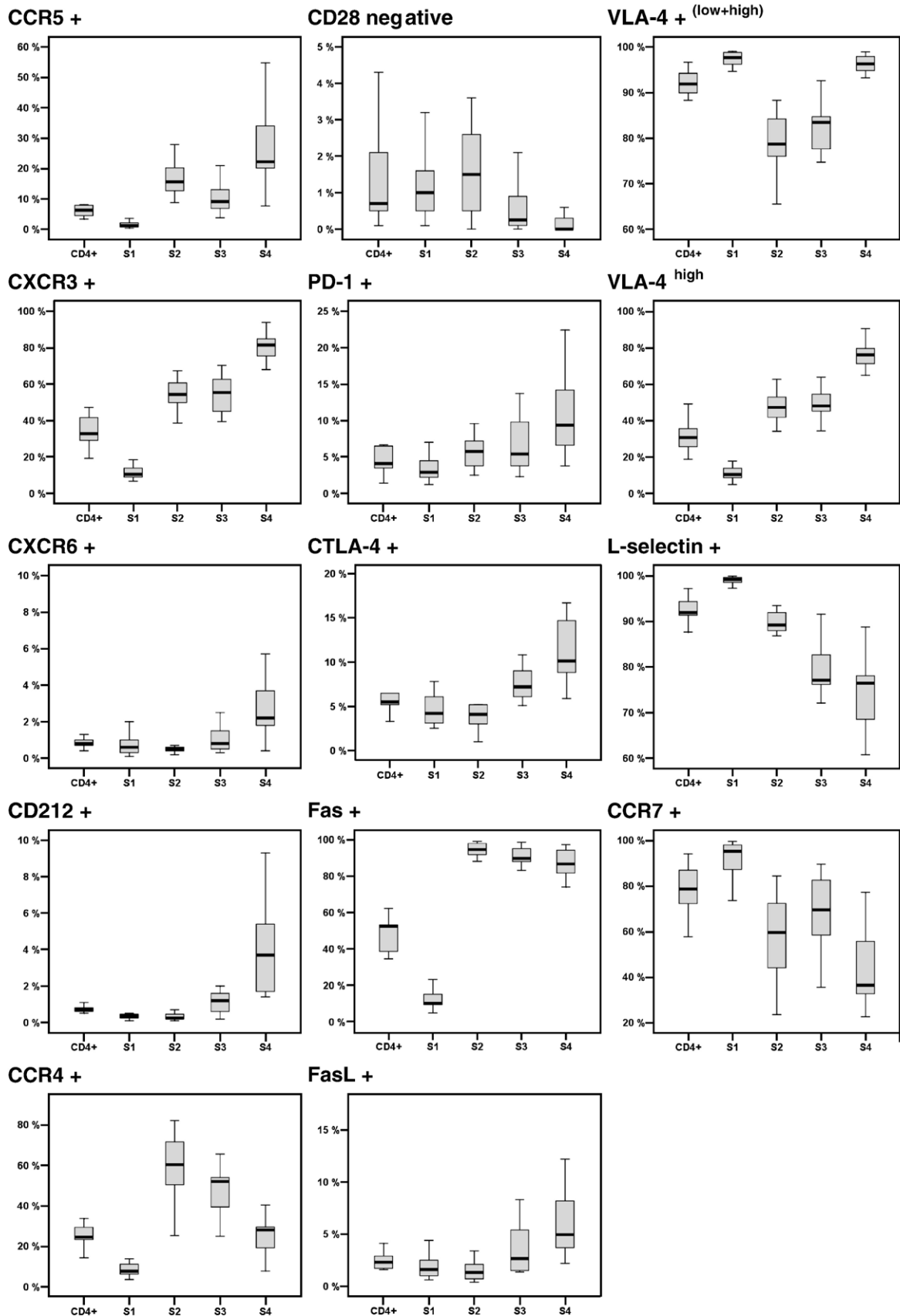


Fig. 2. Surface molecule expression in CD4+ T cells and subsets from healthy controls. **CD4+**: Total CD4+ T lymphocytes; **S1**: CD4+ CD45R0- CD26^{intermediate}; **S2**: CD4+ CD45R0+ CD26^{low}; **S3**: CD4+ CD45R0+ CD26^{intermediate}; **S4**: CD4+ CD45R0+ CD26^{high}. Please note differing scales.

VLA-4 (clone 9F10; BD Pharmingen); CD62L (clone DREG-56; BD Pharmingen); CD212 (clone 2B6; BD Pharmingen); Fas (clone DX2; Dako); FasL (clone Alf-2.1a; Ancell, MN, USA); CTLA-4 (clone BNI3; BD Pharmingen), and PD-1 (clone MIH4; eBioscience, CA, USA). Antibodies were titrated prior to analysis. Markers were set at 1% positive staining by isotype matched control antibodies.

2.3. ELISA measurements

Plasma concentrations of soluble Fas (sFas) and soluble Fas Ligand (sFasL) were measured in plasma samples stored at -70°C after sequential centrifugation at $1000\times g$ and $10,000\times g$. Concentrations were measured using commercial ELISAs (Quantikine, R&D Systems).

2.4. Statistical analyses

When data followed a normal distribution, the *t*-test for unpaired or paired data was used. Otherwise, the non-parametric Mann–Whitney test was used for unpaired data, the Wilcoxon Signed Rank Test was used for paired data, and the Friedman test was used for comparing multiple related data. Spearman's rho or Pearson's *r* was used for correlation analyses.

3. Results

3.1. CD26-defined subsets of CD4+ T cells in healthy controls

When CD4+ T cells from healthy controls were stained with CD26 and CD45R0 antibodies, they could be divided into four major subsets (Fig. 1): a CD45R0– CD26^{intermediate} population (Subset 1, median 48%); a CD45R0+ CD26^{low} population (Subset 2, median 11%); a CD45R0+ CD26^{intermediate} population (Subset 3, median 24%); and a CD45R0+ CD26^{high} population (Subset 4, median 6%). Surface expression of all the molecules under study differed significantly between the four CD26 and CD45R0-defined CD4+ T cell subsets (Fig. 2: all $p < 0.001$). Significant differences in the expression of all studied molecules were also found when the analysis was restricted to CD4+ CD45R0+ memory cells (all $p < 0.001$). However, the pattern of expression of the molecules differed markedly between the four cell subsets. Subset 1: The putative naïve subset of CD4+ T cells, the CD45R0-cells, was characterised by expression of CCR7 (median 95%), CD62L (median 99%), and VLA-4^{low} (median 98%) but was almost lacking cells expressing CCR5, CXCR3, CXCR6, CD212, CCR4, Fas, FasL, PD-1, and VLA-4^{high} (Fig. 2). Subset 4: The CD4+ T cell subset of special interest in this study, the

Table 1

Percentages of surface molecule-positive cells in healthy controls, untreated MS patients, and MS patients after 3 months of IFN- β therapy (IFN- β MS)

		Healthy controls (<i>N</i> =14)	Untreated MS (<i>N</i> =15)	IFN- β MS (<i>N</i> =15)	<i>p</i>
CCR5	CD4+	6 (4–8)	5 (2–7)	8 (4–14)	<0.05 ^b
	CD4+ CD45R0+ CD26 ^{hi}	22 (19–34)	14 (5–29)	29 (18–39)	<0.01 ^b
CXCR3	CD4+	33 (29–42)	32 (29–42)	29 (25–43)	N.S.
	CD4+ CD45R0+ CD26 ^{hi}	82 (76–85)	78 (70–85)	78 (71–83)	N.S.
CXCR6	CD4+	1 (1–1)	1 (1–1)	1 (1–1)	N.S.
	CD4+ CD45R0+ CD26 ^{hi}	2 (2–4)	2 (2–3)	3 (2–4)	N.S.
CD212	CD4+	1 (1–1)	1 (1–1)	1 (1–1)	N.S.
	CD4+ CD45R0+ CD26 ^{hi}	4 (2–6)	3 (2–3)	3 (2–4)	N.S.
CCR4	CD4+	25 (23–31)	20 (12–28)	27 (17–31)	<0.05 ^{a,b}
	CD4+ CD45R0+ CD26 ^{hi}	28 (19–30)	14 (12–24)	26 (20–29)	<0.05 ^{a,b}
CD28 negative	CD4+	1 (1–2)	1 (1–10)	1 (0–9)	N.S.
	CD4+ CD45R0+ CD26 ^{hi}	0 (0–0)	0 (0–0)	0 (0–0)	N.S.
PD-1	CD4+	4 (3–7)	4 (3–6)	4 (3–4)	N.S.
	CD4+ CD45R0+ CD26 ^{hi}	9 (7–14)	6 (5–8)	6 (6–7)	<0.01 ^a
CTLA-4	CD4+	6 (5–8)	6 (5–6)	5 (4–7)	N.S.
	CD4+ CD45R0+ CD26 ^{hi}	10 (8–16)	8 (7–11)	9 (6–12)	N.S.
Fas	CD4+	53 (39–54)	42 (33–54)	52 (37–64)	<0.01 ^b
	CD4+ CD45R0+ CD26 ^{hi}	87 (81–95)	83 (75–88)	89 (81–95)	<0.05 ^b
Fas-Ligand	CD4+	2 (2–3)	3 (3–3)	2 (2–3)	<0.01 ^b
	CD4+ CD45R0+ CD26 ^{hi}	5 (4–9)	5 (4–6)	4 (3–6)	N.S.
VLA-4 ^{low+high}	CD4+	92 (89–94)	92 (89–93)	91 (90–93)	N.S.
	CD4+ CD45R0+ CD26 ^{hi}	96 (95–98)	95 (93–97)	95 (92–97)	N.S.
VLA-4 ^{high}	CD4+	31 (25–37)	29 (24–40)	28 (19–41)	N.S.
	CD4+ CD45R0+ CD26 ^{hi}	76 (71–80)	76 (70–84)	74 (66–85)	N.S.
CD62L	CD4+	92 (91–95)	92 (91–93)	91 (89–94)	N.S.
	CD4+ CD45R0+ CD26 ^{hi}	76 (68–79)	67 (64–74)	67 (64–80)	<0.05 ^a
CCR7	CD4+	79 (70–87)	74 (63–82)	79 (75–85)	<0.05 ^b
	CD4+ CD45R0+ CD26 ^{hi}	36 (32–56)	28 (23–43)	45 (38–48)	<0.01 ^b

Data are given as medians and interquartile range.

^aUntreated MS versus healthy controls. ^bIFN- β treated MS versus untreated MS. N.S.: No statistically significant differences.

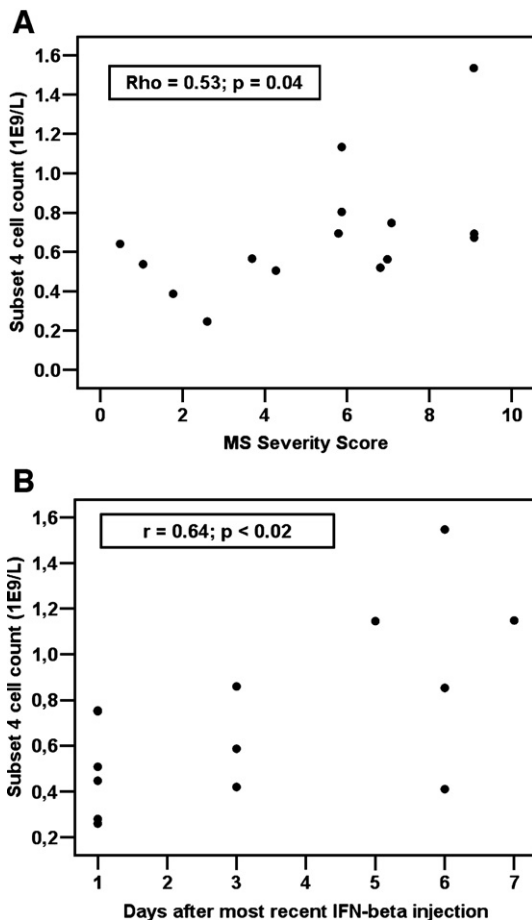


Fig. 3. (A) Correlation between MS Severity Score and circulating CD4+ CD45R0+ CD26^{high} T lymphocyte counts (subset 4) in untreated MS patients. (B) Correlation between the number of days since the last IFN- β injection and circulating CD4+ CD45R0+ CD26^{high} T lymphocyte counts in MS patients treated with IFN- β for 3 months.

CD45R0+ CD26^{high} cells, had the highest percentage of cells expressing markers of Th1 effector functions (CCR5, CXCR3, CXCR6, CD212, FasL, and VLA-4^{high}) compared with any of the other three subsets (Table 1, Fig. 2). This subset also contained lower numbers of cells expressing CCR7 (median 36%) and CD62L (median 76%), indicating more T_{EM} cells than T_{CM} cells. CD28 expression was abundant in all subsets. The largest proportion of CD28⁻ cells was found among CD45R0+ CD26^{low} (median 2%) cells whereas only 0.2% of CD45R0+ CD26^{high} cells were CD28⁻. A high percentage of CD45R0+ CD26^{high} CD4+ T cells expressed CTLA4 on the cell surface. CTLA4 is expressed at low levels on the surface of activated T cells, consistent with the CD45R0+ CD26^{high} subset containing a large fraction of recently *in vivo* activated cells.

3.2. Comparison of surface markers on CD4+ CD45R0+ CD26^{high} cells in healthy controls and untreated MS patients

When comparing the expression of surface markers in the CD4+ CD45R0+ CD26^{high} subset in healthy controls to that of

untreated MS patients, we found lower expression of CD62L ($p=0.03$), PD-1 ($p=0.007$), and CCR4 ($p=0.02$) in MS patients (Table 1). Of these markers, only the lower CCR4 expression was paralleled in total CD4+ T cells ($p=0.02$).

3.3. Correlation of CD4+ CD45R0+ CD26^{high} cell counts with MS disease severity

We found a positive correlation between the numbers of circulating CD4+ CD45R0+ CD26^{high} T cells and the MSSS score ($\rho=0.53$, $p=0.04$, Fig. 3A). None of the other CD4+ T cell subsets under study showed correlation with MSSS (data not shown).

3.4. Changes in surface molecule expression during interferon- β therapy

Three months of IFN- β therapy resulted in a moderate increase in the fractions of CD4+ CD45R0+ CD26^{high} T cells expressing Fas, CCR4, CCR5, and CCR7 (Table 1). The increased expression of these markers was not restricted to the CD4+ CD45R0+ CD26^{high} T cells but was generally more prominent in this subset compared to other subsets. Conversely, expression of FasL decreased slightly in total CD4+ T cells while CD4+ CD45R0+ CD26^{high} T cells showed no change of FasL (Table 1). There was a positive correlation between the number of days after an IFN- β injection and the CD4+ CD45R0+ CD26^{high} count (Pearson's $r=0.64$; $p=0.015$, Fig. 3B), suggesting a short-lasting suppression of CD4+ CD45R0+ CD26^{high} counts after an IFN- β injection. Similar correlations between the interval from IFN- β injection and blood sampling were found in all putative memory (CD45R0+) subset counts (subsets 2–4) but not in the naïve (CD45R0⁻) subset count (subset 1, data not shown).

3.5. Plasma concentrations of soluble Fas and FasL

The mean plasma concentration of sFasL in untreated MS patients was significantly lower than in healthy controls (Table 2; $p=0.02$). The mean sFas level was also somewhat lower in MS patients compared to healthy controls, although this difference was not statistically significant (Table 2; $p=0.11$). After 3 months of IFN- β therapy, both sFas and sFasL plasma concentrations increased significantly (Table 2). There was no correlation between the percentage of CD4+ T cells expressing Fas or FasL and the plasma concentration of sFas or sFasL, respectively (data not shown).

4. Discussion

Memory T lymphocyte biology is complex, and several attempts have been made to define functionally meaningful subsets based on the expression of surface markers. Current models discriminate memory T cell subsets by their

Table 2

Mean concentrations of soluble Fas and FasL in plasma from healthy controls, untreated MS patients, and MS patients after 3 months of IFN- β therapy (IFN- β MS)

	Healthy controls	Untreated MS	IFN- β MS	<i>p</i> (untreated MS vs. healthy controls)	<i>p</i> (IFN- β MS vs. untreated MS)
sFas	3786; 1677	3185; 652	3796; 812	0.11	<0.001
sFasL	102; 28	83; 19	107; 40	0.02	<0.002

Data are given as mean concentrations (pg/mL) and standard deviations.

expression of chemokine receptors, activation markers, or co-stimulatory molecules (Amyes et al., 2005; Hamann et al., 1997; Sallusto et al., 1999; Appay et al., 2002). We analysed CD26 expression by CD4⁺ T cells as this molecule has been linked to clinical disease activity in MS. We found that the CD4⁺ CD45R0⁺ CD26^{high} subset showed a correlation with clinical MS disease severity and it was also the subset that contained the largest fraction of cells positive for markers of Th1 effector functions, activation, and CNS-transmigratory potential (as assessed by expression of CCR5, CXCR3, CXCR6, CD62L, CD212, FasL, and VLA-4^{high}). Thus, this subset was enriched for surface markers that have previously been linked to MS disease activity or severity: CCR5 (Misu et al., 2001), CXCR3 (Misu et al., 2001; Mahad et al., 2003; Sindern et al., 2002; Nakajima et al., 2004), and VLA-4^{high} (Barrau et al., 2000). Conversely, surface molecules favouring homing to secondary lymphoid tissues (CCR7 and CD62L) and those indicative of Th2 functions (CCR4) were under-expressed in this subset. Furthermore, the percentage of CD4⁺ CD45R0⁺ CD26^{high} T cells that expressed CD62L was lower in patients with MS than in controls, suggesting *in vivo* activation of this putative T effector memory cell subset in MS patients. We observed no difference in the percentage of CD4⁺ T cells in the CD26 and CD45R0-defined subsets between patients and control subjects, but this would not necessarily be expected since none of the patients suffered from a relapse at baseline. CD26 expression is highest in patients with a relapse, and decreases significantly during remission (Jensen et al., 2004).

The molecule PD-1 is related to the costimulatory molecule CD28 but has inhibitory effects on T cell activation rather than costimulatory effects (Greenwald et al., 2005). We found that although PD-1 was mainly expressed on CD4⁺ CD45R0⁺ CD26^{high} T cells, a lower percentage of these cells expressed PD-1 in patients with MS than in controls. PD-1 expression has not previously been studied in MS, but in EAE PD-1 knockout animals have more severe disease than wild type animals, supporting a role of PD-1 in regulating pathogenic T cell activation (Salama et al., 2003). Furthermore, the reduced PD-1 expression in MS patients was not countered by an increased CTLA-4 surface expression. However, we found that CD4⁺ CD45R0⁺ CD26^{high} T cells was the subset where the highest percentage of cells expressed CTLA4 on the cell surface. This is

consistent with the notion that this CD4⁺ T cell subset contains cells that were recently activated *in vivo*, as well as with the known central role of this molecule in the regulation of T cell activation (Linsley et al., 1996; Teft et al., 2006). We found no change in the expression of PD-1 after treatment with IFN- β . This implies that the immunoregulatory effects of the PD-1 ligand PD-L1, which belongs to the B7 family and whose expression is increased on antigen presenting cells by treatment with IFN- β , could be blunted in MS (Schreiner et al., 2004). The effect of the lower expression of PD-1 must, however, be assessed in functional assays since a recent study found that the effect of the two PD-1 ligands PD-L1 and PD-L2 may differ markedly in different EAE models (Zhu et al., 2006). Although other inhibitory CD28 family members exist, these findings suggest that CD4⁺ CD45R0⁺ CD26^{high} T cells in patients with MS may have a reduced potential for negative regulation by B7 family members.

CD4⁺ CD45R0⁺ CD26^{high} cell counts were transiently reduced following an IFN- β injection. Treatment with IFN- β increased the CD4⁺ T cell expression of Fas, CCR4, CCR5, and CCR7, and the increases were more pronounced in, but not restricted to, the CD45R0⁺ CD26^{high} subset. The effect of treatment with IFN- β on chemokine receptors on CD4⁺ T cells is discussed in more detail in a different paper (Krakauer et al., 2006). Although statistically significant, the observed decrease in CD4⁺ T cell expression of FasL during IFN- β therapy was minor and the biological significance questionable. The levels of sFasL in untreated MS patients were reduced, and IFN- β therapy resulted in increased plasma sFas and sFasL concentrations. The implications of the combined changes in Fas and FasL expression are difficult to interpret as Fas and FasL expressed on the cell surface, sFas, and sFasL form a complex network of apoptosis-inducing and proliferative signals (Siegel et al., 2000). However, a defect in T cell apoptosis is present in MS and an effect of treatment with IFN- β on apoptosis has been reported. The observed changes in Fas and FasL expression may reflect this phenomenon (Kaser et al., 1999; Macchi et al., 1999; Comi et al., 2000; Sharief et al., 2002; Gniadek et al., 2003; Bilinska et al., 2003; Prieto et al., 2006).

In conclusion, our study verified that the number of circulating CD4⁺ CD45R0⁺ CD26^{high} T cells correlated with disease severity in MS. CD4⁺ CD45R0⁺ CD26^{high} T cells expressed surface molecules in a pattern consistent with the hypothesis that these cells represent Th1 cells with CNS-homing potential in MS. Furthermore, this T cell subset showed evidence of activation (lower expression of CD62L) and less potential for down-regulation (lower expression of PD-1) in untreated patients with MS, and this did not change after treatment with IFN- β . A possible CD26-inhibiting/down-regulating effect of IFN- β should be further investigated. Additional studies that address the antigen-specificity of the CD4⁺ CD45R0⁺ CD26^{high} T cells and the relationship between activation of CD4⁺ CD45R0⁺ CD26^{high} T cells, relapses, and disease progression in MS, are also needed.

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