

Regulatory T cells fail to suppress CD4⁺ T-bet⁺ T cells in relapsing multiple sclerosis patients

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS).¹ Important steps in the pathogenesis of MS are the generation of autoaggressive brain-specific T cells and their invasion of the target organ.² According to the current concept, the autoaggressive T cells are activated in the peripheral immune organs (e.g. by cross-reacting with microbial antigen) before they are capable of trans-migrating across the blood–brain barrier.^{2,3} Within the brain, the autoaggressive T cells are re-activated and trigger inflammation, resulting in myelin and neuronal damage.⁴

The differentiation of T helper (Th) cells into the different subsets is determined by several factors, including

Summary

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system and a defect in the regulatory T-cell subset seems to be involved in the pathogenesis of the disease. Foxp3 is a transcription factor that is selectively expressed in CD4⁺ CD25⁺ regulatory T cells and is required for their development and function. T-bet is a key transcription factor for the development of T helper 1 (Th1) cells. We found that both the percentage of circulating CD4⁺ CD25⁺ Foxp3⁺ cells and Foxp3 expression were lower in relapsing–remitting (RR) MS patients during relapses than during remission. Otherwise, the percentage of CD4⁺ T-bet⁺ T cells and T-bet expression in CD4⁺ T cells were higher in relapsing than in remitting RRMS patients. CD4⁺ CD25⁺ T cells both from relapsing and from remitting RRMS patients showed significantly less capacity than corresponding cells from healthy subjects to suppress autologous CD4⁺ CD25⁻ T-cell proliferation, despite a similar Foxp3 expression level. CD4⁺ CD25⁺ T cells from healthy subjects and patients in remission clearly reduced T-bet mean fluorescence intensity (MFI) in CD4⁺ CD25⁻ T cells up to a ratio of 1:10, whereas CD4⁺ CD25⁺ T cells from patients in relapse were able to reduce T-bet expression only at a high ratio. Our data indicate that the increased number of regulatory T (T-reg) cells and the increased Foxp3 expression in circulating CD4⁺ CD25⁺ T cells may contribute to the maintenance of tolerance in the remission phase of MS. Moreover, the inhibitory capacity of CD4⁺ CD25⁺ T cells seems to be impaired in relapsing patients under inflammatory conditions, as shown by the high levels of T-bet expression in CD4⁺ T cells.

Keywords: Foxp3; multiple sclerosis; relapse; remission; T-bet

cytokines, strength of T-cell receptor (TCR) signal, dose and form of antigens, antigen-presenting cells, co-stimulator molecules engagement and genetic background. However, the main regulation of T-cell differentiation and cytokine pathways seems to be provided by cytoplasmic and nuclear transcription factors.⁵ T-bet has been identified as a key transcription factor for the development of Th1 cells and the induction of interferon- γ (IFN- γ) production.⁶ T-bet is induced during T-cell activation by the IFN- γ -signal transducer and activator of transcription 1 (STAT1) signalling pathway⁷ and once expressed amplifies the production of IFN- γ .⁸ Mice lacking T-bet are protected from the development of experimental autoimmune encephalomyelitis (EAE)⁹ and *in vivo* administration of T-bet-specific antisense oligonucleotide

inhibits EAE.¹⁰ CD4⁺ Th1 cells producing IFN- γ were previously thought to mediate EAE, but new data have shown that interleukin (IL)-17-producing cells play a crucial role in the pathogenesis of the disease.¹¹ A recent study showed that T-bet is also necessary for the expression of IL-23 receptor (IL-23R) and the survival of Th17 cells.¹² Foxp3 is a transcription factor that is expressed in CD4⁺ CD25⁺ regulatory T (T-reg) cells,¹³ and it is required for T-reg cell development and function.^{14,15} Ectopic expression of Foxp3 is sufficient to confer suppressive activity and to induce a T-reg cell phenotype in conventional CD4⁺ CD25⁻ T cells repressing the production of cytokines and up-regulating the expression of CD25, glucocorticoid-induced tumour necrosis factor receptor (GITR) and cytotoxic T-lymphocyte antigen-4 (CTLA-4).¹⁶ Mutations in Foxp3 result in autoimmune lymphoproliferative diseases in both humans and mouse.^{17,18} CD4⁺ CD25⁺ Foxp3⁺ T-reg cells limit inflammation and tissue damage in disease models of organ-specific autoimmune diseases such as EAE,¹⁹ and a decreased expression of Foxp3 protein^{20,21} and mRNA^{20,22} was found in CD4⁺ CD25⁺ T cells from relapsing remitting MS (RRMS) patients but not in secondary progressive MS patients.²²

In our previous study we found a strong up-regulation of T-bet in circulating CD4⁺, CD8⁺ T cells and monocytes from the peripheral blood of RRMS patients in relapse compared with patients in remission and controls.²³ In the present study we evaluated the number of circulating CD4⁺ T-bet⁺ cells and the mean T-bet expression in CD4⁺ lymphocytes and the number of circulating T-reg cells and the mean Foxp3 expression in CD4⁺ CD25⁺ T cells from RRMS patients in different phases of disease and from healthy subjects. We also studied the capacity of T-reg cells to suppress CD4⁺ CD25⁻ cell proliferation and T-bet expression.

Materials and methods

Patients

Patients with RRMS defined by McDonald's criteria¹ and healthy subjects were included in our study. No patients had received immunosuppressive drugs or disease-modifying agents. No corticosteroid treatment was used for 6 months before inclusion in the study and patients in relapse were bled before starting high-dose corticosteroid treatment. Clinical examination and brain and spinal cord magnetic resonance imaging (MRI) were performed in all patients before starting the enrolment. Patients were considered in relapse (relapsing patients) when they showed an episode of new neurological disturbance lasting at least 24 hr and MRI activity [≥ 1 gadolinium-diethylenetriaminepenta-acetic acid (Gd-DTPA)-enhancing lesion]. Patients were considered in remission (remitting

patients) when neither new neurological symptoms nor MRI activity was registered. Disability degree was assessed using the Expanded Disability Status Scale (EDSS). MRI data were acquired using a high-resolution 1.5 Tesla system (5-mm slice thickness). Scanning sessions included acquisition of proton-density [echo time (TE) 20/repetition time (TR) 2500], T2-weighted (TE 80/TR 2500) and T1-weighted (TE 17/TR 600) images. The T1-weighted images were acquired before and 10 min after an intravenous injection of Gd-DTPA (0.1 mmol/kg). This study was approved by the ethic committee of the Catholic University (Rome, Italy), and all the participants gave written informed consent before enrolment.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by density gradient centrifugation (2500 g, 30 min) over a Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden). PBMC were then harvested by pipetting cells from the Ficoll/serum interface and washed twice.

Flow cytometry

For the detection of Foxp3 expression, PBMC were analyzed by three-colour intracellular flow cytometry using anti-CD4-phycoerythrin-cyanine 5 (PC5) conjugate (Beckman Coulter, Miami, FL), anti-CD25-fluorescein isothiocyanate (FITC) conjugate (Beckman Coulter) and anti-Foxp3-phycoerythrin (PE) conjugate monoclonal antibody (mAb) (clone 236A/E7; eBioscience, San Diego, CA). In particular, isolated PBMC were washed once in culture medium (Dulbecco) containing fetal calf serum (FCS) and once in phosphate-buffered saline (PBS) and incubated with both anti-CD4-PC5 and anti-CD25-FITC. After fixation, cells were permeabilized using a commercially available permeabilization/wash kit (BD Biosciences/Pharmingen, Franklin Lakes, NJ). Upon permeabilization, 5×10^5 cells were resuspended in 100 μ l of PBS and incubated for 30 min with the anti-Foxp3-PE conjugate. Cells were washed again with cold PBS and resuspended in PBS for flow cytometry (EPICS XLTM; Beckman Coulter). For the detection of T-bet expression, a double-labelling procedure was performed using anti-CD4-PC5 staining, followed by fixation, permeabilization and incubation with anti-T-bet (4B10)-PE (Santa Cruz Biotechnology, Santa Cruz, CA). Either Foxp3 or T-bet mean fluorescence intensity (MFI) and the percentage of CD4⁺ CD25⁺ Foxp3⁺ or CD4⁺ T-bet⁺ cells were evaluated using two different flow cytometry protocols. Each analysis was performed using at least 50 000 cells that were gated in the region of the lymphocyte population, as determined by light

scatter properties (forward scatter versus side scatter). In order to analyse the expression of transcription factors in lymphocytes (CD4⁺ T cells), cells were gated in both the lymphocyte and CD4⁺ regions. Quadrants of dot-plots were set using appropriate isotype controls for each intracellular and extracellular antibody. Appropriate fluorochrome-conjugated isotype-matched mAbs (Beckman Coulter) were used as a control for background staining in each flow acquisition. In these assays, careful colour compensation was performed before cell analysis.

Separation of CD4⁺ CD25⁺, CD4⁺ CD25⁻ T cells and monocytes

PBMC CD4⁺ T cells were purified by positive selection with magnetic beads conjugated with anti-CD4 (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ CD25⁺ T cells were purified from sorted CD4⁺ T cells by positive selection with magnetic beads conjugated with anti-CD25 (Miltenyi Biotec). The purity of separated CD4⁺ CD25⁺, CD4⁺ CD25⁻ T cells, evaluated by flow cytometry, was 90–95%. For monocyte separation, CD4⁺ T cell-depleted PBMC were incubated in RPMI-1640 containing 2*n*-glutamine and 5% AB⁺ human serum in 96-well plates for 1 hr. Supernatants with non-adherent cells were collected while the remaining adherent monocytes were used as antigen-presenting cells (APC) for the suppressive assay.

Detection of Foxp3 expression by western blotting

For immunoblotting, isolated cell populations (CD4⁺ CD25⁺, CD4⁺ CD25⁻) were directly lysed in radioimmunoprecipitation assay (RIPA) buffer, separated by electrophoresis through 12% sodium dodecyl sulphate–polyacrylamide gels and then transferred to nitrocellulose membrane. Filters were blocked for 4 hr in Tris-buffered saline containing 0.1% Tween 20 plus 5% freeze-dried milk, then incubated with polyclonal goat anti-(human Foxp3), overnight at 4°C in the same buffer. Immunoreactive bands were detected by chemiluminescence using an enhanced chemiluminescence (ECL) kit.

Immunocytochemistry

For detection of Foxp3, the bead-sorted CD4⁺ CD25⁺ cells were fixed with 4% paraformaldehyde for 10 min, rinsed in PBS, permeabilized using a commercially available permeabilization buffer (BD Biosciences/PharMingen) and then incubated with purified anti-(human Foxp3) (eBioscience) diluted 1:200. After incubation with the appropriate FITC-labelled secondary antibody, anti-(mouse IgG) (Jackson ImmunoResearch Laboratories West Grove, PA), cells were examined using a fluorescence microscope.

Suppression assay

To examine the inhibition rate of purified CD4⁺ CD25⁺ T cells, co-cultures were established in 96-well U-bottom plates. CD4⁺ CD25⁻ T cells and CD4⁺ CD25⁺ T cells (5×10^4 cells/well) were cultured in RPMI-1640 supplemented with 5% AB⁺ human serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml of streptomycin, in different suppressor/responder ratios (1:1, 1:2, 1:5, 1:10 and 0:1). Therefore, every well contained 3×10^5 APC responder and suppressor cells (bead-selected CD4⁺ CD25⁻) at different ratios and 2 µg/ml of anti-CD3 (PharMingen; San Diego, CA). For the carboxy fluorescein diacetate succinimidyl ester (CFSE) suppression assay, freshly harvested CD4⁺ CD25⁻ T cells were resuspended in PBS and then labelled with CFSE (CellTrace; Invitrogen Life Technologies, Carlsbad, CA). CFSE-labelled CD4⁺ CD25⁻ responder cells were then washed and co-cultured with CD4⁺ CD25⁺ T cells at different ratios. After 5 days of stimulation with anti-CD3, cells were harvested and the CFSE signal of gated lymphocytes was analysed by flow cytometry. In particular, the suppressive capacity (percentage) of CD4⁺ CD25⁺ T cells towards responder cells in co-culture (at each mixed cell ratio) was calculated relative to the maximal proliferation of responder cells alone after anti-CD3 stimulation [$100 \times (1 - \text{number of cells in co-culture that divide} / \text{number of responder cells that divide})$]. For T-bet assays, T-bet expression in responder and suppressor cells was evaluated using intracellular flow cytometry following fixation, permeabilization and incubation with anti-[T-bet (4B10)]–PE conjugate.

Cytokine measurement

The spontaneous production of IFN- γ was measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. IFN- γ concentrations were determined from the regression line for a standard curve generated by using highly purified recombinant cytokine at various concentrations performed contemporaneously with each assay. The intra-assay and inter-assay coefficients of variation were 6% and 7%, respectively. The standard curve also served as an internal control over the sensitivity and range of each assay. All samples were assayed in duplicate.

Statistical analysis

Differences in variables among groups were tested by two-way analysis of variance (ANOVA). Results are expressed as mean \pm standard error of the mean (SEM) and were considered to be statistically significant at a *P*-value of < 0.05 . Group comparisons (in the suppression assay) were

performed using the Student's *t*-test, and a *P*-value of < 0.05 was taken as significant.

Results

Patients

We studied 40 untreated RRMS patients in different phases of the disease [20 in remission (remitting patients) and 20 in relapse (relapsing patients)] and 25 gender- and age-matched controls. There were no significant differences in clinical features such as age, gender and disease duration between the two groups of patients. The EDSS score was similar in remitting patients and relapsing patients before the last relapse, while the EDSS score was higher in relapsing patients during relapse than in remitting patients (*P* = 0.019) (Table 1). Five patients were studied both in the relapse and in the remission phases of the disease.

Foxp3 expression in circulating CD4⁺ CD25⁺ T cells from MS patients in different phases of disease and in controls

Foxp3 was mainly expressed in CD4⁺ CD25⁺ T cells in both relapsing and remitting patients and in controls. Few CD4⁺ CD25⁻ T cells expressed Foxp3 at low levels in relapsing and remitting patients and in controls (0.8%, 0.6% and 0.8%, respectively). There was no difference in the frequency of CD4⁺ CD25⁺ Foxp3⁺ cells between all patients and controls (data not shown). A

Table 1. Demographic and clinical features of relapsing-remitting multiple sclerosis (RRMS) patients and healthy subjects included in the study

	Relapsing patients	Remitting patients	Controls
Number	20	20	25
Age (years)	28.5 ± 11.5	32.9 ± 8.7	27.8 ± 6.2
Gender (male/female)	8/12	8/12	11/14
Disease duration (months)	34.7 ± 28.4	42.7 ± 34.3	NA
Annualized relapse rate	1.22 ± 0.76	1.03 ± 0.65	NA
EDSS score (pre-relapse)	3.2 ± 1.6* (1.8 ± 1.4)	1.6 ± 1.1*	NA
Gd-enhancing lesions (number)	1.72 ± 1.28	0	NA

EDSS, Expanded Disability Status Scale; Gd, gadolinium; NA, not applicable.

All data are mean ± standard deviation (SD).

Group comparisons were performed using the Student's *t*-test.

*A *P*-value of < 0.05 was taken as significant.

lower percentage of circulating CD4⁺ CD25⁺ Foxp3⁺ T cells was observed in relapsing patients than in remitting patients (1.69% versus 2.78% of CD25⁺ Foxp3⁺ T cells gated on CD4⁺ T cells, *P* = 0.047), while the number of CD4⁺ CD25⁺ Foxp3⁺ T cells was lower in relapsing patients than in healthy subjects, without reaching significance (1.69% versus 2.3%, *P* = 0.147) (Fig. 1a). No significant differences were observed in the percentage of CD4⁺ CD25⁺ Foxp3⁺ T cells between remitting patients and healthy subjects (2.78% versus 2.3%, *P* = 0.882) (Fig. 1a).

Foxp3 was expressed in a significantly higher percentage of CD4⁺ CD25⁺ T cells from remitting MS patients than from relapsing MS patients (23.01 ± 5.14% versus 13.42 ± 5.73% of Foxp3⁺ T cells gated on CD4⁺ CD25⁺ T cells, *P* = 0.0223), whereas no significant difference in the percentage of Foxp3-expressing cells was observed in CD4⁺ CD25⁺ T cells from controls compared with CD4⁺ CD25⁺ T cells from relapsing MS patients (18.31 ± 6.43% versus 13.42 ± 5.73%, *P* = 0.1811) and with CD4⁺ CD25⁺ T cells from remitting MS patients (18.31 ± 6.43% versus 23.01 ± 5.14%, *P* = 0.2528).

Foxp3 MFI was lower in circulating CD4⁺ CD25⁺ T cells from relapsing patients than in CD4⁺ CD25⁺ T cells from remitting patients (*P* = 0.039). There was no significant difference in the Foxp3 MFI of CD4⁺ CD25⁺ T cells between remitting or relapsing patients and controls (*P* = 0.768 and *P* = 0.236 respectively; Fig. 1b).

Representative flow cytometric analyses and immunocytochemistry of Foxp3 in CD4⁺ CD25⁺ T cells from the same RRMS patients during remission and relapse phases are shown in Fig. 2.

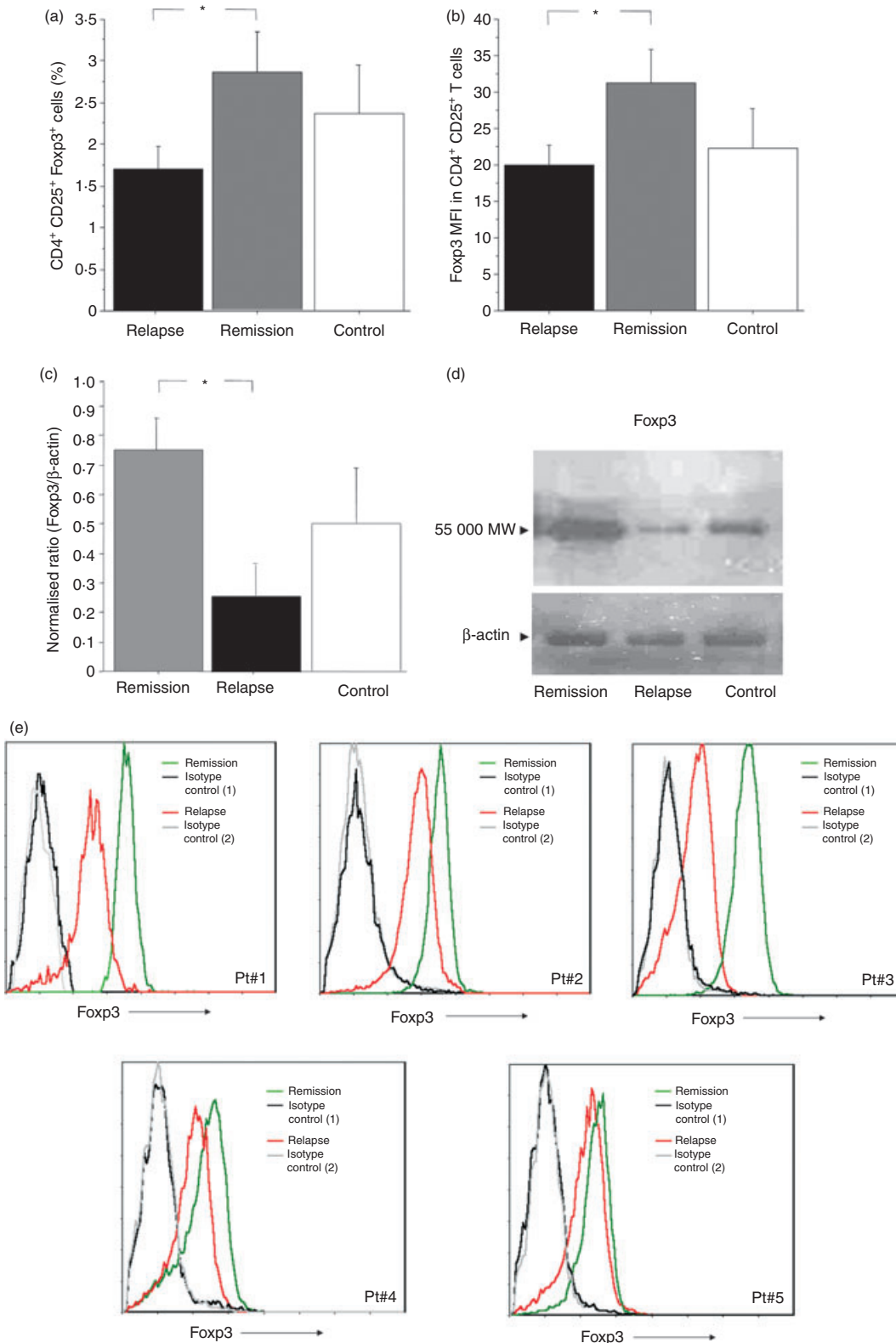
In order to confirm the increased expression of Foxp3 in bead-sorted CD4⁺ CD25⁺ cells from RRMS patients during remission compared with patients during relapse, we longitudinally studied five patients in both relapse and remission phases of the disease and in five controls by western blot analysis. Foxp3 protein levels in CD4⁺ CD25⁺ T cells were higher during remission than during relapse (*P* = 0.046; Fig. 1c). Patients in relapse showed lower Foxp3 levels than controls, but this was not significant (*P* = 0.198; Fig. 1c). There was no significant difference in Foxp3 expression between patients in remission and controls (*P* = 0.652; Fig. 1c). A representative western blot of the same patient during remission and relapse and of one control is described in Fig. 1d. Representative histograms showing Foxp3 expression in CD4⁺ CD25⁺ T cells from five RRMS patients during remission and relapse are shown in Fig. 1e.

T-bet expression in circulating CD4⁺ T cells from MS patients in different phases of disease and in controls

T-bet was mainly expressed in CD4⁺ CD25⁻ T cells in both relapsing and remitting patients and in controls. The T-bet

MFI was higher in CD4⁺ T cells from relapsing patients than in CD4⁺ T cells from remitting patients and controls (Fig. 2a; $P = 0.034$ and $P = 0.018$ respectively). The percentage of CD4⁺ T-bet⁺ T cells was significantly higher in

relapsing RRMS patients than in remitting patients and controls (Fig. 2b; $P = 0.009$ and $P = 0.008$ respectively). No significant differences were observed in the T-bet MFI in CD4⁺ T cells or in the percentage of CD4⁺ T-bet⁺ cells



between patients in remission and healthy subjects ($P = 0.589$; Fig. 2). Representative two-parameter plots showing CD4⁺ T-bet⁺ cells are described in Fig. 2c).

CD4⁺ CD25⁺ T-cell capacity to suppress CD4⁺ CD25⁻ T-cell proliferation

In the suppression assay, circulating CD4⁺ CD25⁺ T cells from healthy subjects in a suppressor/responder ratio of 1:1 induced an 80% reduction of CD4⁺ CD25⁻ T-cell proliferation, whereas CD4⁺ CD25⁺ T cells from patients in remission showed lower suppressive capacity than controls, despite the similar Foxp3 MFI, because they induced, at the same ratio, a 63% reduction of CD4⁺ CD25⁻ T-cell proliferation. CD4⁺ CD25⁺ T cells from patients in relapse, showing significantly lower Foxp3 MFI in comparison to patients in remission, clearly had less suppressive capacity than those from remitting patients and controls and, at a suppressor/responder ratio of 1:1, induced a 53% reduction of CD4⁺ CD25⁻ T-cell proliferation. A higher suppressive capacity was observed in CD4⁺ CD25⁺ T cells from healthy subjects compared with CD4⁺ CD25⁺ T cells from relapsing RRMS patients until a suppressor/responder ratio of 1:5 was reached ($P = 0.008$ at a ratio of 1:1, $P = 0.011$ at a ratio of 1:2 and $P = 0.038$ at a ratio of 1:5) and with CD4⁺ CD25⁺ T cells from remitting RRMS patients up to a ratio of 1:2 ($P = 0.026$ at a ratio of 1:1 and $P = 0.031$ at a ratio of 1:2; Fig. 3a). CFSE histograms from one representative patient in relapse and in remission phases and from one control are shown in Fig. 3d).

CD4⁺ CD25⁺ T-cell capacity to reduce T-bet expression in CD4⁺ T cells and the percentage of CD4⁺ T-bet⁺ cells

Circulating CD4⁺ CD25⁺ T cells from healthy subjects and from patients in remission clearly reduced T-bet MFI in CD4⁺ CD25⁻ T cells to a suppressor/responder ratio of 1:10, whereas CD4⁺ CD25⁺ T cells from patients in relapse were able to reduce T-bet expression only at a high ratio but failed to decrease T-bet MFI in autologous CD4⁺ CD25⁺ T cells at a low ratio (Fig. 3b). In particular,

a higher expression of T-bet was found in responder cells from relapsing RRMS patients at suppressor/responder ratios of 1:5 and 1:10 than in those from remitting RRMS patients and controls ($P = 0.028$ and $P = 0.015$ at a suppressor/responder ratio of 1:5, $P = 0.031$ and $P = 0.011$ at a suppressor/responder ratio of 1:10, respectively; Fig. 3b).

A higher percentage of CD4⁺ T-bet⁺ cells was observed in responder cells from relapsing RRMS patients than in those from remitting RRMS patients at a suppressor/responder ratio of 1:10 ($P = 0.018$), whereas controls showed a lower percentage of CD4⁺ T-bet⁺ cells compared with relapsing patients, both at 1:5 ($P = 0.021$) and at 1:10 ($P = 0.019$; Fig. 3c) suppressor/responder ratios. To verify if T-bet reduction in responder T cells in the suppressive assay was associated with a decrease of Th1 functional activity, we measured IFN- γ levels in the supernatants of T-reg cell/T-responder cell co-cultures from healthy subjects. We observed a progressive reduction of IFN- γ levels from a suppressor/responder ratio of 1:10 up to 1:1, with a parallel decrease of T-bet expression in CD4⁺ T cells (data not shown).

Discussion

T-reg cells seems to play a major role in limiting the development of autoimmune diseases, but where and when they exert their suppressive function on effector cells is still controversial.^{24,25} Some studies of EAE suggest that T-reg cells mainly act in the lymph nodes by limiting the priming of naïve T cells^{25,26} while other studies have shown that T-reg cells can proliferate and inhibit effector cells mainly at sites of inflammation.^{19,27–29} Inflammatory cytokines secreted by effector cells seem to limit the suppressive function of T-reg cells in inflammatory lesions,^{26,29–31} whereas some studies suggest that 'effector/memory-like' T-reg cells have enhanced suppressive capacity and ability to migrate to inflammatory sites.³²

Several studies have reported numeric or functional deficiencies of T-reg cells in various autoimmune diseases, including MS.^{13,22,33–36}

The mechanisms responsible for the generation of T-reg cell defects in MS are still unclear. A reduced thymic-dependent *de novo* generation of T-reg cells that

Figure 1. Frequency of circulating CD4⁺ CD25⁺ Foxp3⁺ cells and mean expression of Foxp3 [mean fluorescence intensity (MFI)] in relapsing-remitting multiple sclerosis (RRMS) patients in remission and in relapse and in age- and gender-matched healthy controls. (a) The percentage of total regulatory T (T-reg) cells (CD25⁺ Foxp3⁺ cells gated on CD4⁺ T cells) from the peripheral blood of RRMS patients and controls was significantly higher in remitting than in relapsing RRMS patients. (b) The Foxp3 MFI was higher in CD4⁺ CD25⁺ T cells from remitting than from relapsing RRMS patients. (c) Foxp3 protein levels assessed by western blot analysis of CD4⁺ CD25⁺ T cells were significantly higher in remitting RRMS patients than in relapsing RRMS patients and controls. Data are reported as mean \pm standard error of the mean (SEM). * $P < 0.05$. (d) Representative western blot of the same patient during remission and relapse (3 months after remission) and of one control. The same membranes were reprobated with anti-actin IgG immunoglobulin as a control for loading. MW, molecular weight. (e) Representative histograms showing Foxp3 expression in CD4⁺ CD25⁺ T cells from five RRMS patients during remission (green line) and relapse (red line). The black and grey lines indicate the fluorescence intensity of isotype controls in remission and relapse phases, respectively. The x-axis of each histogram represents specific fluorescence of Foxp3 on a four-decade logarithmic scale, and the y-axis represents the total number of events.

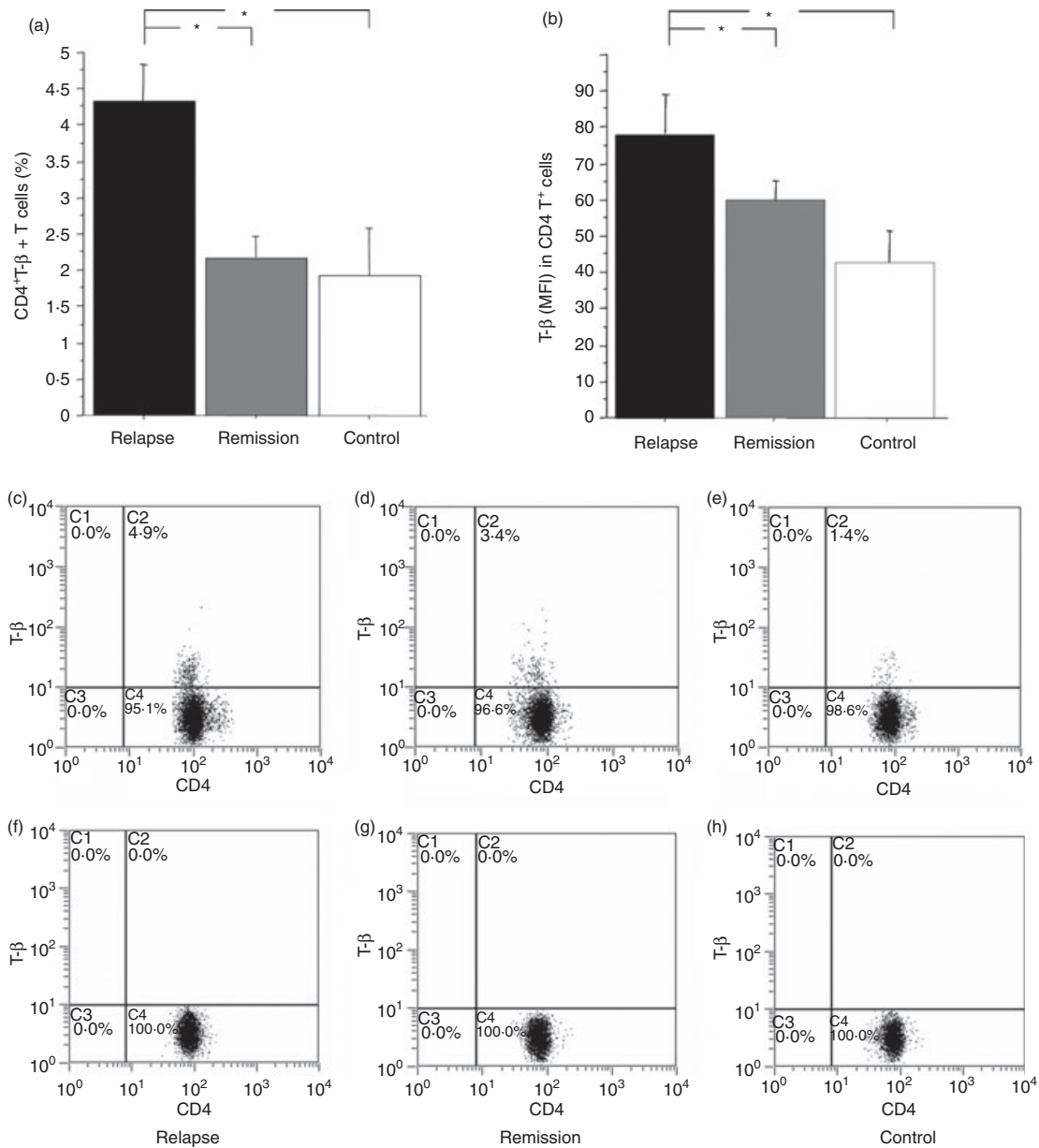


Figure 2. Mean expression of T-bet in CD4⁺ T cells and percentage of CD4⁺ T-bet⁺ T cells in relapsing-remitting multiple sclerosis (RRMS) patients in different phases of disease and in healthy subjects. (a) The percentage of CD4⁺ T-bet⁺ T cells was significantly higher in relapsing RRMS patients than in remitting RRMS patients and controls ($P = 0.009$ and $P = 0.008$ respectively). (b) Mean T-bet expression, assessed by flow cytometric analysis as fluorescence intensity, was significantly higher in CD4⁺ cells from relapsing RRMS patients than in the CD4⁺ cells from remitting patients and controls ($P = 0.034$ and $P = 0.018$ respectively). Data are reported as mean \pm standard error of the mean (SEM). * $P < 0.05$. (c–h) Representative two-parameter plots showing only cells gated on CD4⁺ lymphocyte populations (c–e) and respective phycoerythrin (PE) isotype controls for intracellular antibody (f–h). The y-axis of each plot represents specific fluorescence of T-bet–PE conjugate; the x-axis represents specific fluorescence of CD4–phycoerythrin–cyanine 5 (PC5) on four-decade logarithmic scales. These representative two-parameter plots are obtained from the same RRMS patient in relapse (c) and in remission (d) and from healthy controls. Quadrants were set by using appropriate isotype controls for each intracellular and extracellular antibody. Values in each plot are the percentage of CD4⁺ T-bet⁺ cells.

are both less sensitive to apoptosis and more effective in suppression seems to be critical for the T-reg cell dysfunction that occurs in MS patients.³⁶

In agreement with other investigators,^{21,22,35} in this study we found no difference in the frequency of circulat-

ing CD4⁺ CD25⁺ Foxp3⁺ T cells between RRMS patients and controls.

To date there are no data about the frequency of circulating CD4⁺ CD25⁺ Foxp3⁺ cells and the levels of expression of Foxp3 in untreated RRMS patients in different

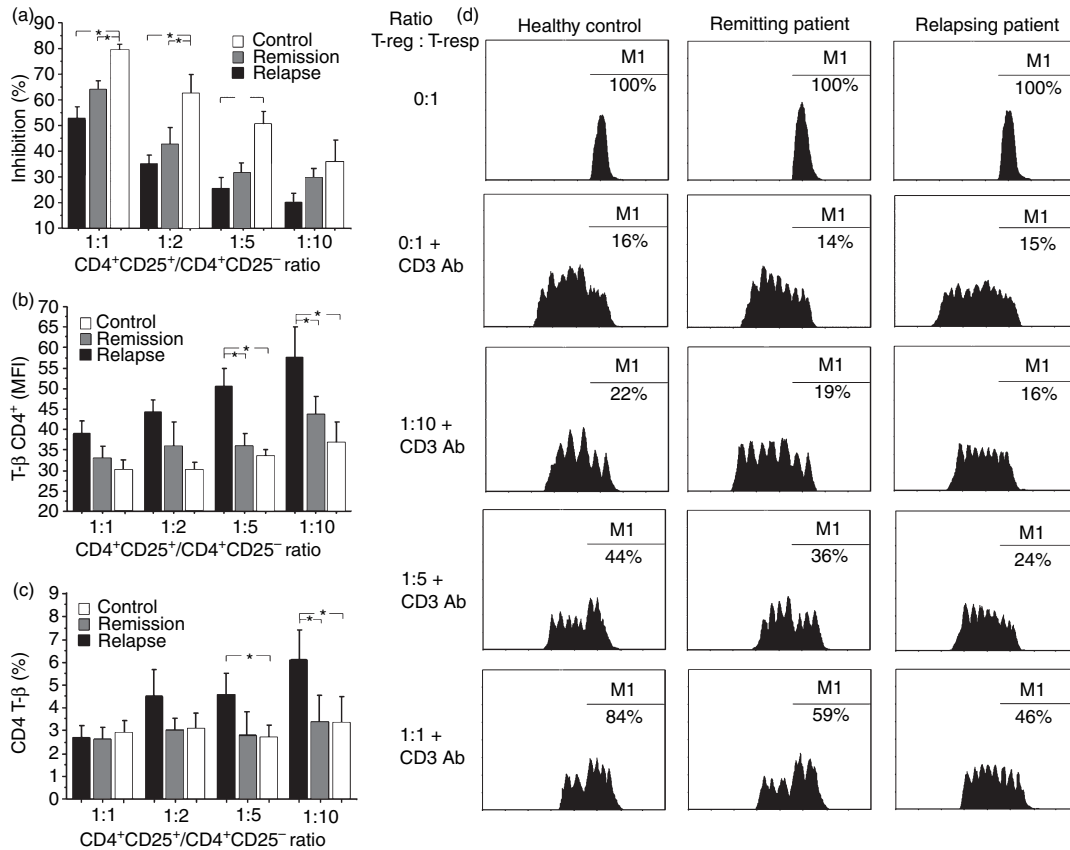


Figure 3. *In vitro* study aimed at evaluating the suppressive activity of bead-separated CD4⁺ CD25⁺ T cells isolated from relapsing-remitting multiple sclerosis (RRMS) patients and controls, and the effects on T-bet expression and on the percentage of CD4⁺ T-bet⁺ cells. (a) A higher suppressive capacity was observed in CD4⁺ CD25⁺ T cells from healthy subjects than from relapsing RRMS patients up to a suppressor/responder ratio of 1:5 ($P = 0.008$ at a suppressor/responder ratio of 1:1, $P = 0.011$ at a suppressor/responder ratio of 1:2 and $P = 0.038$ at a suppressor/responder ratio of 1:5) and from remitting RRMS patients up to a suppressor/responder ratio of 1:2 ($P = 0.026$ at a suppressor/responder ratio of 1:1 and $P = 0.031$ at a suppressor/responder ratio of 1:2). Suppression of proliferation of CD4⁺ CD25⁻ T cells is shown at CD4⁺ CD25⁺/CD4⁺ CD25⁻ ratios 1:1, 1:2, 1:5 and 1:10; the mean inhibition for the different study groups is also presented. Data from all controls, and from relapsing and remitting RRMS patients, are shown as the mean of triplicate wells. (b) Effects of regulatory T (T-reg) cells on decreasing the expression of T-bet in responder T cells. A higher expression of T-bet was found in responder cells from relapsing RRMS patients (at suppressor/responder ratios of 1:5 and 1:10) than from remitting RRMS patients and from controls ($P = 0.028$ and $P = 0.015$ at a suppressor/responder ratio of 1:5, and $P = 0.031$ and $P = 0.011$ at a suppressor/responder ratio of 1:10, respectively). (c) Effects of T-reg cells on reducing the percentage of CD4⁺ T-bet⁺ T cells. A higher percentage of T-bet⁺ cells was observed in responder cells from relapsing RRMS patients than from remitting RRMS patients ($P = 0.018$ at a suppressor/responder ratio of 1:10) and from controls ($P = 0.021$ at a suppressor/responder ratio of 1:5 and $P = 0.019$ at a suppressor/responder ratio of 1:10). Data are reported as mean \pm standard error of the mean (SEM). * $P < 0.05$. (d) Carboxy fluorescein diacetate succinimidyl ester (CFSE) histograms from one representative patient in relapse and in remission phase and from one control. The CFSE signal was measured as mean fluorescence intensity. Cells under the gated region M1 (%) represent no-divided CFSE-labeled responder cells. Ab, antibody; T-resp, responder T cell.

phases of disease (relapse and remission). We observed that the percentage of CD4⁺ CD25⁺ Foxp3⁺ cells was lower in relapsing patients than in remitting patients. In addition, Foxp3 MFI and protein expression was significantly lower in CD4⁺ CD25⁺ T cells from relapsing patients than in CD4⁺ CD25⁺ T cells from remitting patients. Likewise, in EAE the expression level of Foxp3 on a per cell basis was lower in CNS-derived T-reg cells at the peak of disease as compared with recovery.²⁶ We did not find significant differences in

CD4⁺ CD25⁺ Foxp3⁺ T-cell frequency and Foxp3 MFI in CD4⁺ CD25⁺ cells between remitting or relapsing patients and healthy subjects. Venken *et al.* showed decreased Foxp3 expression at the single-cell level in circulating in CD4⁺ CD25^{high} cells from untreated RRMS patients in the stable phase of the disease compared with controls. Some other authors suggest that not only CD4⁺ CD25^{high} T cells, but also a portion of Foxp3⁺ cells present within the CD4⁺ CD25^{low} T-cell subset showing suppressive activity on effector T cells in a transforming growth

factor- β (TGF- β)-dependent manner, should be considered regulatory T cells.³⁷ However, in humans there are a considerable number of activated, non-regulatory CD4⁺ T cells that express CD25⁺ and it must be noted that the CD4⁺ CD25⁺ T cells used in our suppressive assay might also include non-regulatory activated T cells that might be more frequent in RRMS patients than in controls. Recent data have suggested that not only is the presence of Foxp3 important for the function of T-reg cells, but the level of its expression is also crucial.³⁹ Foxp3 is transiently up-regulated in human CD4⁺ CD25⁻ T cells upon stimulation, but at lower levels than in T-reg cells,^{40,41} and a reduced expression of Foxp3 seems to correlate with decreased T-reg-cell suppressive function.^{42,43} In our population of untreated RRMS patients the loss of functional suppression by CD4⁺ CD25⁺ T cells was not strictly correlated with the Foxp3 protein levels expressed by CD4⁺ CD25⁺ T cells. RRMS patients and controls showed a similar single-cell level of Foxp3 in CD4⁺ CD25⁺ T cells, but CD4⁺ CD25⁺ T cells from RRMS patients showed less suppressive capacity than those from controls. These data are in agreement with a previous study³⁴ showing that the impaired suppressive function of CD4⁺ CD25⁺ T cells from RRMS patients may be caused by a defect in the regulatory subset. In addition, we found similar levels of Foxp3 in circulating CD4⁺ CD25⁺ cells from RRMS patients and from controls, and consistently with other studies^{40,44} our data suggest that factors other than Foxp3 may be involved in the suppressive function of CD4⁺ CD25⁺ Foxp3⁺ T cells. A recent report showed that IL-35, a novel inhibitory cytokine specifically produced by T-reg cells and a downstream target of Foxp3, is required for maximal T-reg cell suppressive activity.⁴⁵

We found an inverse association between Foxp3 MFI in CD4⁺ CD25⁺ T cells and T-bet MFI in CD4⁺ T cells from RRMS patients in different phases of the disease. Relapsing patients showed lower Foxp3 expression in CD4⁺ CD25⁺ T cells and higher T-bet expression in CD4⁺ T cells than remitting patients. In contrast, the increase of Foxp3 expression in CD4⁺ CD25⁺ T cells in remitting patients was associated with a reduced T-bet expression in CD4⁺ T cells. In a suppressive assay, T-bet expression of autologous CD4⁺ CD25⁻ cells progressively decreased in the presence of an increasing number of CD4⁺ CD25⁺ T cells from remitting patients; in contrast, CD4⁺ CD25⁺ T cells from relapsing patients failed to reduce the high level of T-bet expression in autologous CD4⁺ CD25⁻ cells at a low suppressor/responder ratio. Our data suggest that, in RRMS patients, the inhibitory function of circulating CD4⁺ CD25⁺ Foxp3⁺ cells is mainly impaired during relapse, in the presence of a higher inflammatory burden. Several mechanisms of suppression, which are not mutually exclusive, have been proposed: inhibition by cell–cell contact between T-reg cells and target T cells or APC, consumption of limiting

growth factors such as IL-2 and consequent apoptosis of target T cells,⁴⁶ and secretion of inhibitory cytokines.⁴⁵ We hypothesize that in RRMS patients during relapse, in contrast to what has been shown in EAE studies,⁴⁷ not only CNS-derived CD4⁺ CD25⁻ T cells but also circulating CD4⁺ CD25⁻ T cells expressing high levels of T-bet, may produce soluble factors and/or excessive amounts of inflammatory cytokines that impair T-reg-cell-mediated suppression. Conversely, during remission, circulating CD4⁺ CD25⁻ cells expressing low levels of T-bet still seem to be susceptible to T-reg cell regulation.

In conclusion, our data confirm a defective function of T-reg cells in MS even if peripheral CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells play an important role in the natural extinction of autoimmune inflammation in MS. The increased number of circulating CD4⁺ CD25⁺ cells expressing higher levels of Foxp3 seems to contribute to the maintenance of tolerance during the remission phase of RRMS, while, in the relapse phase of the disease, the small number of CD4⁺ CD25⁺ cells expressing low levels of Foxp3 are unable to inhibit the activated effector T cells that express a high level of T-bet. Further studies are necessary to clarify in greater detail the role of T-reg cell dysfunction on the complex mechanisms determining ongoing inflammation in MS.

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