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$CD4^+CD25^+FoxP3^+$ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis

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Abstract

Multiple sclerosis (MS) is an autoimmune disorder directed against self antigens of the central nervous system. $CD4^+CD25^+FoxP3^+$ regulatory T cell (T_{reg}) mediated suppression is an essential mechanism of self-tolerance. We studied whether changes in the suppressive function of a mixture of $CD25^{high}$ and $CD25^{intemediate}$ expressing T_{reg} cells in myelin basic protein (MBP)-induced proliferation occurred in untreated MS patients. Suppression of MBP-induced proliferation was observed in 13 out of 29 (45%) MS patients; this was significantly ($p<0.05$) less compared with 17 out of 19 (89%) healthy individuals. Relative T_{reg} counts was significantly increased in MS patients (mean ± S.D.; 20 ± 8%) compared with healthy individuals (15 ± 5%). These findings suggest that impaired T_{reg} function may be involved in pathogenesis of MS.

Keywords: Multiple sclerosis; Myelin basic protein; Proliferation; Regulatory T cells; FOXP3

1. Introduction

Multiple sclerosis (MS) is a disease of the central nervous system characterized by inflammation, demyelination, gliosis and axonal damage (Noseworthy et al., 2000; Raine and Cross, 1989; Trapp et al., 1998). In many ways, MS is remarkably heterogeneous. According to the histopathological features, MS may be subgrouped in four different pathogenetic subtypes. Moreover, MS patients differ in their clinical presentation and response to treatment (Lassmann et al., 2001).

Immunological research has focused on the characterization of autoreactive T cells against protein components of the myelin sheath such as myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG). Especially the encephalitogenicity and immunodominance of MBP peptides has been studied.

These studies demonstrated that MBP-reactive T cells are present in the T cell repertoire of both, MS patients and healthy individuals (Lunemann et al., 2004). This brings into question, why MS patients but not healthy individuals develop inflammatory tissue damage. Two explanations for this discrepancy have been assumed: a higher frequency of MBP reactive T cells in MS patients compared with healthy individuals and a different pattern of peptide recognition by T cells. But the evaluation of the frequency of MBP-reactive T cells (Hong et al., 2004) as well as the analyses of antigen presentation and peptide specificity have been inconclusive (Davies et al., 2005). Therefore, the control of autoreactive T cells may be different in healthy individuals and MS patients.

One of the key issues in autoimmunity is to understand, how the immune system discriminates between "self" and "non-self" to avoid autoreactivity but to allow effective immune responses against microbial antigens (Sakaguchi, 2005). The immune system has evolved different mechanisms to establish and sustain unresponsiveness towards self antigens such as elimination (deletion) or functional inactivation

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(anergy) of self-reactive lymphocytes. Furthermore, T cell-mediated active suppression by regulatory T cells (T_{reg}) is another essential mechanism to sustain self-tolerance. Depletion of $CD4^+CD25^+$ T_{reg} results in the onset of systemic autoimmune diseases in mice (Sakaguchi et al., 1985). In addition, active suppression by T_{reg} cells plays a role in the control of human self-antigen-reactive cells as it has been shown *in vitro* and *in vivo* (Maloy and Powrie, 2001). Recently, it was shown that *in vivo* depletion of $CD25^+$ T_{reg} cells lowers the threshold to trigger autoreactive T cell responses. Under these conditions, murine experimental autoimmune encephalomyelitis increased in severity, both in terms of mean clinical scores and mortality (Stephens et al., 2005). Therefore, impaired function of T_{reg} cells may contribute to the predisposition of a multifactorial autoimmune disease. T_{reg} cells are characterized by the expression of $CD4^+$ and $CD25^+$ and more specifically they express the message for the transcription factor forkhead box P3 (FOXP3) as a key control gene for development and function (Sakaguchi, 2005). Therefore, $CD25^+$ and $FoxP3^+$ can serve as specific molecular markers to detect and manipulate naturally occurring T_{reg} cells.

There is now accumulating evidence that FOXP3 expressing T_{reg} cells are engaged in the suppression of a variety of physiological and pathological immune responses. Impaired generation of T_{reg} cells or their effector activity may contribute to autoimmune diseases such as MS.

Compared with healthy individuals, the number of circulating T_{reg} cells was decreased (Khoury et al., 2000) or unchanged (Putheti et al., 2004; Viglietta et al., 2004; Haas et al., 2005) in MS patients. However, mitogen stimulated lymphocytes of MS patients showed an impaired suppressive function of T_{reg} cells (Viglietta et al., 2004). Moreover, reduced antigen-specific suppression has been described in myelin oligodendrocyte glycoprotein (MOG) stimulated lymphocytes (Haas et al., 2005). In these studies only $CD4^+$ T cells highly expressing CD25 were considered as T_{reg} cells and assayed for their suppressive function *in vitro*. However, it was shown that a mixture of $CD4^+$ lymphocytes, which expressed $CD25^{high}$ or $CD25^{intermediate}$ was suppressive as well (Wing et al., 2003). The aim of our study was to clarify whether a mixture of $CD25^{high}$ and $CD25^{intermediate}$ $CD4^+$ T lymphocytes expresses FOXP3 and suppresses mitogen (pokeweed mitogen; PWM) or antigen (myelin basic protein; MBP)-induced proliferation in MS patients or in healthy individuals.

2. Materials and methods

2.1. Patients and healthy individuals

The study was approved by the local ethics committee. All patients and healthy blood donors gave written informed consent prior to the study.

Thirty-five patients (13 males, 22 females) diagnosed as clinical definite MS according to the McDonald criteria (McDonald et al., 2001) were included in our study. During the time of the study, we included consecutive patients visiting our MS center who gave informed consent and who fulfilled our predefined inclusion criteria: i) patients had to suffer from clinical definite relapsing remitting or secondary progressive MS according to the McDonald criteria, ii) they had to be untreated for at least 6 months, iii) they had to be relapse free for at least 8 weeks and iv) they did not receive steroid treatment for at least 8 weeks. Patients had a relapsing remitting ($n=25$) or secondary progressive ($n=10$) course of the disease. The mean and standard deviation (mean+S.D.) was calculated for patients age (40±10 years, range 20–69 years), disease duration (7.4±6.9 years, range 0.5–25 years) and expanded disability status score (EDSS; 3.7±2, range 1.0–7.5). Thirty-four healthy individuals (17 males, 17 females: 40± 12 years, range 21–62 years) served as controls. There was no significant difference for age and sex between MS patients and healthy individuals.

2.2. Isolation of T_{reg} and $CD4^+CD25^-$ cells

Thirty milliliters of heparinized venous blood samples were used for isolation of T_{reg} and $CD4^+CD25^-$ cells. In brief, 40 µl "RosetteSep™ $CD4^+$ cell enrichment cocktail" (CellSystems Biotechnologie, St. Katharinen, Germany) was added per milliliter of blood and incubated for 15 min. The antibody-based enrichment cocktail crosslinks unwanted cells ($CD8^+$, $CD16^+$, $CD19^+$, $CD36^+$, $CD56^+$, and $CD66b^+$) to multiple red blood cells to pellet them along with free red blood cells by density centrifugation (negative selection for $CD4^+$). After incubation, blood samples were diluted with an equal volume of phosphate buffered saline (PBS; Gibco, Karlsruhe, Germany) enriched with 2% foetal calf serum (FCS; Biochrom, Berlin, Germany). Diluted blood samples were layered onto 15 ml Ficoll-Paque™ plus (Amersham Biosciences, Uppsala, Sweden) density medium and centrifuged. Cells removed from the density medium/plasma interface contained enriched $CD4^+$ cells. They were washed 2 times with PBS plus 2% FCS, resuspended at 1×10^7 cells per 100 µl PBS plus 2% FCS and incubated with 100 µl of "EasySep™ CD25 positive Selection Cocktail" per milliliter of sample for 15 min. $CD25^+$ cells were isolated by adding 50 µl of "EasySep™ SA Magnetic Nanoparticles" (CellSystems Biotechnologie) per milliliter of sample. After incubation for 10 min, 2.1 ml PBS plus 2% FCS was added. The tube was placed in the EasySep magnet (CellSystems Biotechnologie) for 5 min. Thereafter, the supernatant containing the $CD4^+CD25^-$ cells was poured into another tube by inverting magnet and tube in one continuous motion. Magnet bound T_{reg} cells were washed twice with PBS plus 2% FCS and all supernatants were combined to receive the $CD4^+$ $CD25^-$ cells. These $CD4^+CD25^-$ cells were centrifuged and resuspended in 1 ml RPMI 1640 medium containing 25 mM Hepes (Gibco), supplemented with 2 mM L-glutamine (Sigma-Aldrich Chemie, Steinheim, Germany), 200 U/ml

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Penicillin and 200 μg/ml Streptomycin (both Gibco). The tube containing T_{reg} was removed from the magnet and the cells were resuspended in 1 ml RPMI 1640 medium. T_{reg} and $CD4^+CD25^-$ cells were characterized by flow cytometry as described under Section 2.5.

2.3. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from 7 ml of heparinized whole blood by Ficoll-Paque™ plus density gradients as described, except that FCS-free PBS buffer was used.

2.4. Cell proliferation assays

To assess the functional activity of T_{reg} cells, 1×10^5 PBMCs plus 1×10^4 $CD4^+CD25^-$ cells were cultured in absence (CD25NEG) or presence of 1×10^4 T_{reg} cells (CD25MIX) in 230 μl RPMI 1640 medium supplemented with 10% heat-inactivated pooled human serum (Institut fuer Transfusionsmedizin, Universitaetsklinikum Essen) in U-bottom wells (96-well plate; Becton Dickinson Labware, Heidelberg, Germany).

Cells (CD25NEG or CD25MIX) were cultured in 230 μl RPMI 1640 medium without any stimulation (autologous cultures) or stimulated with either 5 μg/well of MBP as antigen (Acris Antibodies, Hiddenhausen, Germany) or 1 μg/well of PWM as mitogen (Sigma-Aldrich Chemie) in U-bottom wells. Cultures were set up as triplicates and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 5 days of culture, 1 μCi of ^{3}H-thymidine (Amersham Buchler, Braunschweig, Germany) per well was added and incubated for further 16 h. Using a multiple automatic sample harvester (Mach III Harvester96; Tomtec, Hamden, UK), cells were harvested on glass fiber paper and counted in scintillation fluid (Betaplate-Scint; Wallac, Turku, Finland). ^{3}H-thymidine incorporation was measured as counts per minute (cpm). The stimulation index (SI) was calculated by dividing the mean (cpm) of triplicate cultures (MBP or PWM-induced proliferation) by the mean (cpm) of triplicate autologous cultures. The percentage of suppression of T_{reg} cells (CD25MIX) was calculated by setting the SI values of CD25NEG cultures to 100% proliferation.

2.5. Cell counting in whole blood and flow cytometry

In whole blood, relative counts of $CD3^+$ lymphocytes and their subpopulations ($CD4^+$ or $CD8^+$ cells) were assessed by the four colour flow cytometry using the MultitestTest™ antibody cocktail: $CD3^+$ fluorescein isothiocyanate (FITC), $CD8^+$phycoerythrin (PE), $CD45^+$ peridinin chlorophyll protein (PerCP), $CD4^+$ allophycocyanin (APC) and $CD14^+$APC. Relative numbers of CD25 expressing $CD3^+$ cells were measured either in whole blood cells using BD Oncomark™ $CD4^+$FITC, $CD25^+$PE, $CD3^+$PerCP-Cy5.5 and $CD8^+$APC. HLA-DR expression of $CD3^+$ cells were measured in whole blood using Tritest $CD4^+$FITC, $CD8^+$PE, $CD3^+$PerCP and HLA-DR^+APC.

After addition of antibody cocktail, whole blood or isolated cells (50 μl) were incubated for 15 min. Thereafter, 500 μl of BD FACS™ lysing solution (Becton Dickinson Biosciences) was added, mixed and incubated for 15 min. Samples were measured in a FACSCalibur and analyzed utilizing CellQuest software (Becton Dickinson Biosciences).

To analyze $CD3^+$ cells and their subpopulations, lymphocytes were gated. For analysis of CD25 and HLA-DR expressing $CD3^+$, $CD3^+$ cells were gated. Three thousands events were acquired on gated cells. HLA-DR positivity was defined as described in Ditschkowski et al. (1999). The relative counts of $CD3^+$ cells are given as percentages of lymphocytes. For T cells expressing $CD4^+$, $CD8^+$, $CD25^+$or HLA-DR^+, percentages of $CD3^+$ cells in whole blood were calculated. All monoclonal antibodies were obtained from Becton Dickinson Biosciences.

2.6. Evaluation of FOXP3 expression

Total RNA was isolated from T_{reg} or $CD4^+CD25^-$ cells using the RNeasy Kit (Qiagen, Hilden, Germany) including the optional DNase digest (RNase-free DNase set). Utilizing the Gold RNA PCR core kit (Applied Biosystems, Foster City, CA, USA), 150–600 ng of RNA were reverse transcribed in a total volume of 20 μl in a Gene-Amp PCR system 9700 (Applied Biosystems). Conditions were as follows: 25 °C for 10 min, 42 °C for 15 min, 95 °C for 5 min, and 5 °C for 5 min.

The amount of FOXP3 and β-actin (ACTB; house keeping gene) message was quantified with an ABI 7000 Real-Time PCR system using Taqman Gene Expression Assays (Assay IDs: Hs00203958_m1 for FOXP3, Hs99999903_m1 for ACTB; Applied Biosystems). Twenty-five microliters containing 2.5 μl cDNA were run in duplicates. ΔC_t values for FOXP3 were calculated taking the house keeping ACTB as standard gene. The difference in FOXP3 expression between T_{reg} and $CD4^+CD25^-$ cells was calculated as fold difference $=2^{\Delta\Delta C}{}_t$ where $\Delta\Delta C_t=(\Delta C_t\ CD4^+CD25^-)-(\Delta C_t\ T_{reg})$.

2.7. Statistics

Data of patients and healthy individuals (age, sex, subpopulations of T lymphocytes and stimulation index) are given as mean ± standard deviation (S.D.). The comparison of age, sex, T lymphocytes and their subpopulations e.g. T_{reg} as well as SI values of MS patients with the appropriate values of healthy individuals was performed using Wilcoxon two sample tests (Mann Whitney *U*-test). Analysis of differences of SI values between CD25NEG and CD25MIX within the group of MS patients or healthy individuals was calculated by {[(cpm CD25NEG/cpm autologous CD25NEG)/(cpm CD25MIX/cpm autologous CD25MIX)] − 1} and evaluated using the signed rank Wilcoxon test. The percentage of suppression of T_{reg} cells (CD25MIX) was calculated by setting

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Table 1
Relative counts of T lymphocytes and their subpopulations in whole blood of healthy individuals ($n=33$) and patients suffering from multiple sclerosis ($n=34$)

T lymphocytes	Healthy individuals	MS patients	p value
$CD3^+$	71±8[a]	72±8	ns[b]
$CD3^+CD4^+$	46±7	47±10	ns
$CD3^+CD8^+$	23±6	23±8	ns
$CD3^+CD25^+$	17±6	22±10	0.016
$CD3^+CD4^+CD25^+$(Treg)	15±5	20±8	0.029
$CD3^+CD8^+CD25^+$	1.4±1.3	2.3±2	0.050
$CD3^+HLA^-DR^+$	2.6±1.5	2.4±1.4	ns
$CD3^+CD4^+HLA^-DR^+$	1.5±0.8	1.3±0.7	ns
$CD3^+CD8^+HLA^-DR^+$	1±0.9	1±0.8	ns

[a] Relative counts are given as mean±standard deviation.
[b] ns: Not significant.

the SI values of CD25NEG cultures to 100% proliferation. The percentage of suppression of T_{reg} cells was compared between MS patients and healthy individuals using Fisher's exact test. The level of significance was designated as $p<0.05$ (two-tailed tests). A trend of increase assigned as p value is 0.05–0.1. The Statistical Analysis System software (SA, Cary, NC, USA) was used to calculate statistical significance.

3. Results

3.1. Threshold for CD25 expression

Based on routine measurements of $CD4^+CD25^+$ T lymphocytes in whole blood samples, we set the threshold of CD25 expression to define T_{reg} cells to >369 channels (fluorochrome PE).

3.2. Counts of T lymphocytes and their subpopulations

Comparing the relative counts of T lymphocytes and their $CD4^+$ or $CD8^+$ subpopulations, no significant differences could be found between MS patients and healthy individuals in whole blood (Table 1). Relative counts of both CD25 positive T lymphocytes ($CD3^+CD25^+$) and T_{reg} cells ($CD3^+CD4^+CD25^+$) were significantly increased in whole blood of MS patients compared with healthy individuals.

Table 2
Stimulation indices of CD25NEG and CD25MIX cells of healthy individuals and patients suffering from multiple sclerosis

	Healthy individuals		MS patients	
	CD25NEG	CD25MIX	CD25NEG	CD25MIX
PWM[a]	235±158[b]	125±60[c]	313±245	223±154[c, d]
MBP[e]	2.9±1.6	1.7±1.1[c]	3.8±4.6	2.9±2.5

[a] PWM: Pokeweed mitogen.
[b] Stimulation indices are given as mean±standard deviation.
[c] $p<0.05$ compared with the appropriate CD25NEG values.
[d] $p<0.05$ compared with CD25MIX of healthy individuals.
[e] MBP: Myelin basic protein.

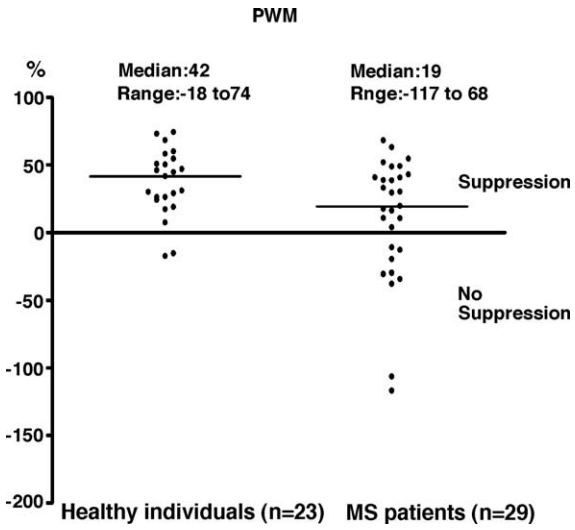

Fig. 1. Suppressive activity of regulatory T cells using co-cultures of lymphocytes derived from healthy individuals or multiple sclerosis patients after stimulation with pokeweed mitogen. PWM: pokeweed mitogen.

The relative counts of $CD3^+CD8^+CD25^+$ lymphocytes tend to increase numbers in MS patients compared with those of healthy individuals.

No significant difference in HLA-DR expression was found in lymphocyte subpopulations ($CD3^+$, CD4+, $CD8^+$) between MS patients and healthy individuals. In T_{reg} cells, HLA-DR expression was not restricted to $CD25^{high}$ expressing cells but also present in $CD25^{intermediate}$ cells (data not shown).

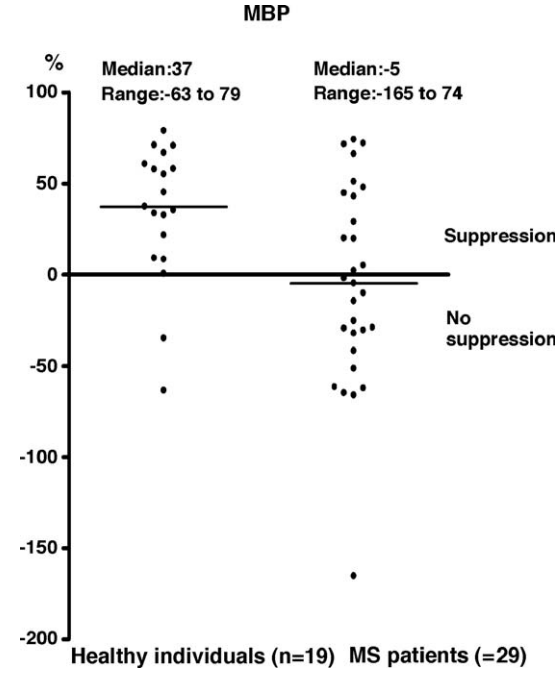

Fig. 2. Suppressive activity of regulatory T cells using co-cultures of lymphocytes derived from healthy individuals or multiple sclerosis patients after stimulation with myelin basic protein. MBP: myelin basic protein.

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3.3. Function of T_{reg} cells

Statistical analysis revealed that mitogen (PWM) and antigen (MBP)-induced proliferation in cells from MS patients as well as healthy individuals were significantly different from the appropriate autologous counts (data not shown). MBP and PWM-induced proliferation of CD25NEG cells of MS patients and healthy individuals were not significantly different (Table 2). CD25MIX cells of MS patients showed significantly higher proliferation rates for PWM stimulated cultures of lymphocytes than healthy individuals.

Co-culturing of T_{reg} cells from healthy individuals with CD25NEG cells (=CD25MIX) significantly reduced PWM and MBP-induced proliferation compared with CD25NEG cells alone (Table 2). PWM-induced proliferation of CD25MIX of MS patients was significantly reduced compared with the appropriate values of CD25NEG cells. Data obtained from MS patients displayed a broad range in proliferation and suggested that the functional activity of T_{reg} cells may differ between individual MS patients. Therefore, the suppressive activity of T_{reg} cells was calculated individually and compared.

In PWM-induced co-cultures, T_{reg} cells from healthy individuals (21 of 23 cases, 91%) showed a trend ($p=0.086$) to higher suppressive activities compared with MS patients (20 of 29 cases, 69%) (Fig. 1). However, T_{reg} cells of 13 out of 29 MS patients (45%) suppressed MBP-induced proliferation (Fig. 2), this is significantly ($p=0.022$) less than T_{reg} cells from healthy individual (17 out of 19, 89%).

3.4. FOXP3 mRNA expression

In both MS patients and healthy individuals T_{reg} cells expressed about 13-fold more FOXP3 gene transcript than $CD4^+CD25^-$ cells (Fig. 3). The amount of FOXP3 gene transcript was not significantly different between MS patients and healthy individuals.

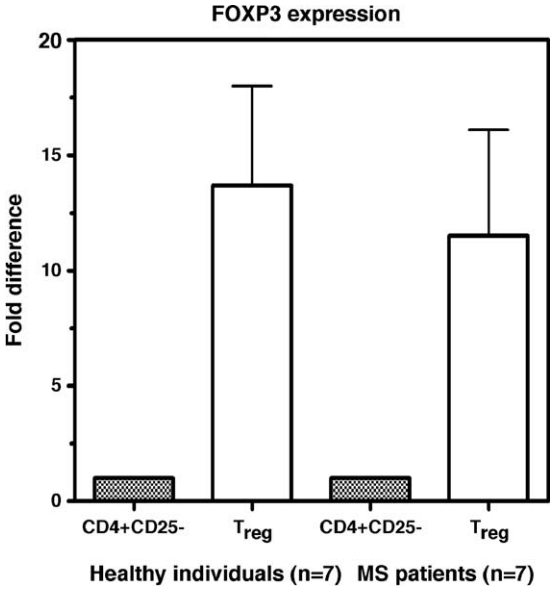

Fig. 3. Expression of FOXP3 in T_{reg} ($CD4^+CD25^+$) and $CD4^+CD25^-$ lymphocytes of healthy individuals and multiple sclerosis patients.

4. Discussion

The main finding in our study is a reduction or loss of suppressive activity of T_{reg} cells from MS patients. More specifically, PWM (mitogen)-induced proliferation showed less suppressive activity, whereas MBP (antigen)-induced suppression of T_{reg} cells is lost in 55% of MS patients.

This effect can not be due to a lack of T_{reg} cells because compared with healthy individuals MS patients had a significantly higher relative count of T_{reg} cells in their peripheral blood.

These data are in contrast to previous findings. Three studies (Haas et al., 2005; Putheti et al., 2004; Viglietta et al., 2004) did not report significant changes in frequency of $CD25^{high}$ cells between MS patients and healthy individuals. When the analyses were extended to $CD25^{intermediate}$ cells (Haas et al., 2005) or $CD4^+CD25^+$ (including all $CD25^+$) cells (Putheti et al., 2004), comparable numbers in MS patients and healthy individuals were also found. On the other hand, it was shown that MS patients had significantly reduced relative counts of $CD4^+CD25^+$ T cells (Khoury et al., 2000).

One possible explanation for this discrepancy could be the definition of T_{reg} cells according to the level of CD25 expression. We defined T_{reg} cells by a threshold for CD25 expression of >369 PE fluorescence channels. So we did not only include cells expressing $CD25^{high}$ but also those $CD4^+$lymphocytes with $CD25^{intermediate}$.

Another explanation may be that different subgroups of MS patients were included in the studies. The MS patients analyzed in our study had a mean age of 40 years, an average disease duration of 7.4 years and a mean EDSS of 3.7. The clinical features are representative for MS patients with a relapsing remitting or secondary progressive MS (Limmroth et al., 2006).

Aside from the increased count of T_{reg} cells in MS patients compared with healthy individuals, we also found a tendency to increased values for the $CD8^+CD25^+$ cell population. This may be of interest because $CD8^+CD25^+$ thymocytes with regulatory function and a phenotype similar to T_{reg} cells including FOXP3 expression has been described (Cosmi et al., 2003). This activity has been found in peripheral blood T cells, too (Jarvis et al., 2005).

Another aspect in our study was the measurement of T_{reg} activity in antigen (MBP) as well as mitogen (PWM) driven responses. A significant decrease in the suppressive activity of T_{reg} lymphocytes expressing $CD25^{high}$ has been shown either in mitogen or in anti-CD3 and anti-CD28 polyclonal stimulated cultures of lymphocytes from MS patients (Viglietta et al., 2004). This finding was confirmed in polyclonally activated as well as in MOG-antigen stimulated cultures using an isolation procedure similar to us (Haas et al., 2005). Although we used T_{reg} cells expressing either

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$CD25^{high}$ or $CD25^{intemediate}$, we observed an overall decrease in the functional activity of T_{reg} cells in PWM stimulated cultures from MS patients compared with healthy individuals. Moreover, in MBP-antigen stimulated co-cultures, T_{reg} cells obtained from about 55% of MS patients displayed no suppressive activity.

Mitogen or polyclonal (CD3, CD28) stimulation may be less efficient to detect impaired activity of T_{reg} cells in MS patients. As shown in our study, suppressive activity was lost to a higher degree in MBP-antigen induced cultures than in PWM stimulated cultures. We can not judge from the publication by Haas et al. (2005), whether in MOG-driven responses functional activity of T_{reg} cells is lacking in all patients. A failure in the regulation of MBP-specific responses may have more impact on the pathogenic process than regulation of MOG-specific responses.

In our study, T_{reg} cells defined as a mixture of $CD25^{high}$ and $CD25^{intemediate}$ showed similar suppression as $CD25^{high}$ cells in other studies. Suppressive activity of the mixture of $CD25^{high}$ and $CD25^{intemediate}$ is supported by the findings of Wing et al. (2003), who analyzed $CD4^{+}CD25^{+}$ expressing cells. They reported that 40% of the cells showing bright immunofluorescence (i.e. including intermediate CD25 expressing cells) are probably suppressive for MOG-induced proliferation in healthy individuals. They used an immunomagnetic separation procedure to obtain T_{reg} cells by positive selection, while we isolated $CD4^{+}$ lymphocytes by a negative selection procedure. Due to the low number of T_{reg} cells in the peripheral blood and the selection procedure it is not possible to compare the functional activity of T_{reg} cells with high or intermediate CD25 expression directly.

T_{reg} cells may be defined by additional markers such as HLA-DR or FOXP3. Despite Baecher-Allan et al. (2001) reported that expression of HLA-DR is restricted to $CD25^{high}$ lymphocytes we found HLA-DR expression on $CD25^{intermediate}$ cells as well. In our study determination of FOXP3 mRNA message in T_{reg} cells revealed a 13-fold higher expression compared to the appropriate $CD25^{-}$ negative cell population of MS patients as well as healthy individuals. Our results are in concordance with the findings by Haas et al. (2005) who reported nearly the same ratio of FOXP3 mRNA expression between T_{reg} $CD25^{high}$ and $CD4^{+}CD25^{-}$ cells. We and Haas et al. (2005) did not find any difference in FOXP3 expression between MS patients and healthy individuals. However, there was one report on decreased FOXP3 levels of $CD4^{+}CD25^{+}$ lymphocytes in MS patients (Huan et al., 2005). The possible explanation is that the MS patients they analyzed probably belong to a different subgroup. These patients had average disease durations of 15.3 years compared with 7.4±6.9 years in our study. In T_{reg} consisting of a mixture of $CD25^{high}$ and $CD25^{intemediate}$ cells, we observed suppressive activities up to 80% in co-cultures from healthy individuals. These data suggest but finally do not prove, that the functional activity of $CD25^{high}$ and a mixture of $CD25^{high}$ and $CD25^{intemediate}$ cells may be comparable. A further study using FoxP3 measurement by flow cytometry can clarify if these cells may be different for FoxP3 intracellular protein levels.

A complete loss of the functional activity of T_{reg} cells in MBP-stimulated cultures from MS patients may explain the manifestation and maintenance of the disease. So far we have no explanation for the failure of T_{reg} cells in MS patients.

Several factors could account for deficient function of T_{reg} cells in MS patients. First, a higher frequency of MBP reactive T cells in MS patients compared with healthy individuals may explain loss of suppression in MBP-induced proliferation. But so far according to Hong et al. (2004) estimation of frequency of MBP-reactive T cells had been inconclusive. A second explanation may be that MS patients have higher numbers of activated cells, thus "diluting" the numbers of T_{reg} cells and accounting for the apparent deficient function in *in vitro* assays. Therefore, we measured HLA-DR expression of T lymphocytes as an important parameter for activation. Because we did not find differences in the relative counts of HLA-DR expressing T cells between MS patients and healthy individuals it seems unlikely that our *in vitro* results may be explained by a "dilution effect".

Huan et al. (2005) reported a higher proliferation rate of T_{reg} cells from MS patients compared with healthy individuals. Moreover, these authors provide evidence that proliferating T_{reg} cells are less suppressive than non-proliferating T_{reg} cells. We also found that CD25MIX cells have higher PWM-induced proliferation compared with healthy individuals. These findings may explain a surplus of proliferation in those MS patients who present with a complete loss of the functional activity of T_{reg} cells. These data support our observation that MS patients compared with healthy individuals harbour proliferating albeit non-functional T_{reg} cells.

Another aspect is that aside the natural $CD3^{+}CD4^{+}$ $CD25^{+}FoxP3^{+}$ T_{reg} cells several other $FoxP3^{+}$ and non-$FoxP3^{+}$ subsets of induced suppressor T cells (Tr1, Th3) are also players of the immune tolerance network (Wing et al., 2006). Since we used PBMCs in our *in vitro* system we could not absolutely exclude that suppression of T_{reg} might be influenced by such other cells.

We provide experimental evidence for a higher number of T_{reg} cells in a subpopulation of MS patients compared with healthy individuals and impaired suppressive activity of T_{reg} cells from MS patients in an *in vitro* assay system.

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