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Reduced suppressive effect of CD4$^+$CD25^{high} regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis

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Immunoregulatory T cells of CD4$^+$CD25$^+$ phenotype suppress T cell function and protect rodents from organ-specific autoimmune disease. The human counterpart of this subset of T cells expresses high levels of CD25 and its role in human autoimmune disorders is currently under intense investigation. In multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS), the activation of circulating self-reactive T cells with specificity for myelin components is considered to be an important disease initiating event. Here, we investigated whether MS is associated with an altered ability of CD4$^+$CD25^{high} regulatory T cells (T_{reg}) to confer suppression of myelin-specific immune responses. Whereas T_{reg} frequencies were equally distributed in blood and cerebrospinal fluid of MS patients and did not differ compared to healthy controls, the suppressive potency of patient-derived CD4$^+$CD25^{high} T lymphocytes was impaired. Their inhibitory effect on antigen-specific T cell proliferation induced by human recombinant myelin oligodendrocyte protein as well as on immune responses elicited by polyclonal and allogeneic stimuli was significantly reduced compared to healthy individuals. The effect was persistent and not due to responder cell resistance or altered survival of T_{reg}, suggesting that a defective immunoregulation of peripheral T cells mediated by CD4$^+$CD25^{high} T lymphocytes promotes CNS autoimmunity in MS.

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Abbreviations: **CNS:** central nervous system · **CSF:** cerebrospinal fluid · **GAPDH:** glyceraldehyde-3-phosphate-dehydrogenase · **MFI:** mean fluorescence intensity · **MOG:** myelin oligodendrocyte glycoprotein · **MS:** multiple sclerosis · **T_{eff}:** effector T cell · **TREC:** T cell receptor excision circles · **T_{reg}:** CD4$^+$CD25^{high} regulatory T cells

Introduction

Multiple sclerosis (MS) is an inflammatory and predominantly demyelinating disease of the central nervous system (CNS). The precise pathogenic mechanisms are undetermined. In early stages, neural damage is considered to arise from T cell-mediated autoimmunity targeted against myelin components [1, 2]. In experimental models of autoimmune demyelination a critical determinant for disease induction is the activation of circulating self-reactive T cells with specificity for myelin

peptides [1]. Immunological tolerance and protection from autoimmunity are conferred by central and peripheral mechanisms such as clonal deletion of self-reactive T cells in the thymus and the induction of anergy upon encounter with autoantigens in the periphery [3, 4]. Nevertheless, autoaggressive T lymphocytes may escape these control mechanisms and T cells with specificity against a variety of self antigens are universally detectable within the circulating T cell pool of healthy individuals. More recently, the potential role of specialized suppressor T cells in the maintenance of peripheral immune tolerance has been reappraised. There is experimental evidence that a distinct subset of T cells residing in the $CD4^+$ subpopulation is prominently involved in preventing organ-specific autoimmune disease. These naturally occurring immunoregulatory T cells originate in the thymus, constitutively express CD25 and comprise 5–10% of murine and approximately 2% of human peripheral $CD4^+$ T cells [5, 6]. Within the human $CD4^+$ subset only cells coexpressing high levels of CD25 appear to have potent regulatory function [6, 7]. $CD4^+CD25^{high}$ regulatory T cells (T_{reg}) are anergic to antigenic stimulation and actively downregulate activation and expansion of conventional T lymphocytes in a cell-contact-dependent and cytokine-independent manner [5, 8–13]. Although the molecular interactions and signaling pathways that are critical for their generation and function are nut fully elucidated, T_{reg} require forkhead transcription factor scurfin encoded by the Foxp3 gene, which appears to control their development and regulatory properties [14–16]. Implication of T_{reg} in human autoimmune disease is currently under intense investigation. Either reduced numbers or suppressive capacities of T_{reg} were recently reported in patients affected by type-1 diabetes [17, 18], juvenile idiopathic arthritis [19], systemic lupus erythematosus [20], hepatitis C-associated mixed cryoclobulinemia vasculitis [21], autoimmune polyglandular syndrome type II [22], and psoriasis [23].

Viglietta *et al*. [24] described an impaired inhibitory effect of polyclonally stimulated T_{reg} from the peripheral blood of a small cohort of patients suffering from MS, a chronic inflammatory and predominantly demyelinating disease of the CNS. In murine experimental autoimmune encephalomyelitis, the mouse model for MS, myelin oligodendrocyte glycoprotein (MOG) has the highest capacity of inducing demyelination in mice [25]. Importantly, T_{reg} can delay the onset and progression of autoimmune demyelination [26]. In this context it is interesting that also in humans, MOG is one of the most potent inducers of *in vitro* $CD4^+$ T cell responses among various foreign and self antigens [7, 13, 27]. Importantly these responses could also be blocked by T_{reg} from healthy individuals [27]. Hence, T_{reg} may be critically important to prevent autoimmunity within the CNS. Since myelin-specific T cells are detectable at similar frequencies in the circulation of both MS patients and healthy persons [1], any impairment of T_{reg} function may facilitate the activation of potentially harmful T cells and may promote their entrance into the CNS.

In the present study we isolated T_{reg} from the circulation of 73 patients with early, clinically active and untreated relapsing remitting MS and 73 age-matched healthy controls to determine their prevalence and to assess their downregulatory effects on responder T cell proliferation using recombinant human MOG (rhMOG) as stimulus. We also measured T_{reg} mediated suppression towards T cell responses elicited by antigen-nonspecific polyclonal or by allogeneic stimuli.

Results

The frequency of T_{reg} is not altered in MS patients

We screened peripheral blood samples from 73 MS patients and 73 healthy individuals for the presence of T_{reg} by flow cytometry. As only $CD4^+$ T cells that coexpress high levels of CD25 exhibit regulatory function [6, 7], the number of T_{reg} was determined as the frequency of CD4-FITC positive cells with the highest CD25-PE fluorescence intensity (Fig. 1A). We did not find significant differences in percentages of $CD25^{high}$ cells between MS patients (mean 2.3%, range 0.4–4.9) and normal individuals (mean 2.5%, range 0.5–5.1)

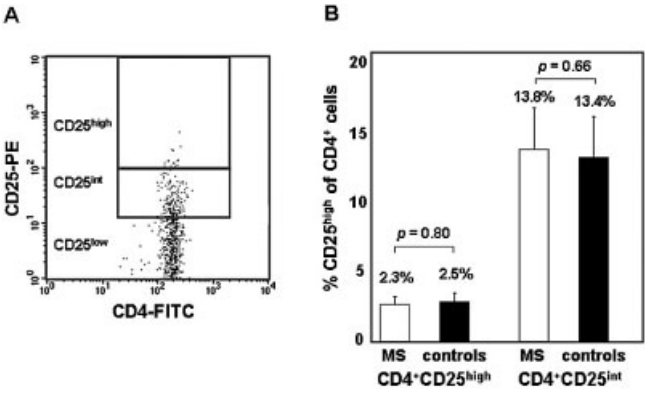

Figure 1. The frequency of T_{reg} is not altered in MS patients. (A) Negatively selected $CD4^+$ lymphocytes from peripheral blood obtained from MS patients and healthy controls were double-stained with FITC-labeled anti-CD4 and PE-labeled anti-CD25. After gating on $CD4^+$ cells, the frequencies of $CD4^+CD25^{high}$ and $CD4^+CD25^{int}$ T cells was determined as percentage of $CD4^+$ cells with highest and intermediate CD25 staining, respectively. (B) Numbers of $CD4^+CD25^{high}$ cells and $CD4^+CD25^{int}$ cells in peripheral blood samples of 73 MS patients and 73 healthy controls were at comparable levels. The mean percentages are depicted by the bars.

(Fig. 1B). Recently activated helper T cells, defined as cells expressing intermediate levels of CD25 ($CD4^+CD25^{int}$) [28], were also detectable in comparable numbers in the peripheral blood of MS patients (mean 13.8%, range 4.4–24.5) and control donors (mean 13.4%, range 3.3–28.2). In a previous study $CD4^+CD25^{int}$ cells did not exhibit regulatory cell characteristics such as hyporesponsiveness and suppression [6].

The frequency of T_{reg} in blood and cerebrospinal fluid is equivalent

To assess whether T_{reg} are sequestered in the CNS during an acute MS relapse, we analyzed the percentages of T_{reg} in parallel in peripheral blood and cerebrospinal fluid (CSF) specimens of 15 MS patients using flow cytometry as described above. Within the CSF, $CD25^{high}$ cells constituted 3.0% (range 0.1–8.6) of $CD4^+$ T cells. This was comparable to T_{reg} frequencies of 2.4% (range 0.5–6.2) among blood-derived $CD4^+$ T cells. There was no correlation between T_{reg} numbers, $CD4^+$ numbers, and total cell counts in the 15 CSF specimens analyzed (data not shown).

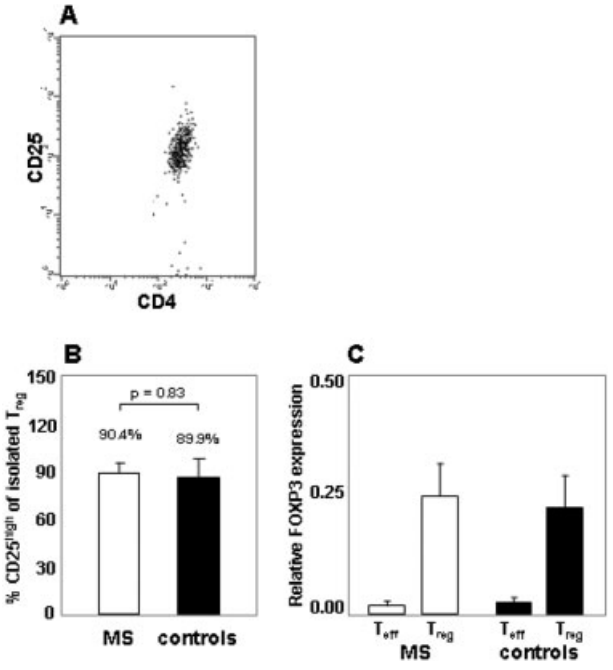

Figure 2. Immunomagnetic separation reveals highly pure fractions of T_{reg} and T_{eff}. (A) Purity of sorted T_{reg}, as determined by FACS staining with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25. (B) Mean percentage of $CD4^+CD25^{high}$ cells is equally high among T_{reg} preparations obtained from 73 MS patients and 73 healthy donors. (C) Quantitative RT-PCR reveals a markedly higher Foxp3 mRNA expression in purified T_{reg} than in T_{eff} in 13 MS patients (white bars) and 13 controls (black bars). Expression levels do not differ between T_{reg} derived from MS patients and healthy controls (black bars). Bars represent mean data plus SEM of Foxp3 mRNA expression divided by GAPDH expression.

Immunomagnetic separation yields highly pure T_{reg} and effector T cell subsets

We tested the purity of freshly isolated T_{reg} by determining the $CD4^+CD25^{high}$ cell count as described above (Fig. 2A). All 146 samples were highly enriched for $CD4^+CD25^{high}$ cells [purity: MS patients $90.4 \pm 7.8\%$ ($n=73$); controls $89.9 \pm 9.3\%$ ($n=73$)] (Fig. 2B). Bead separated T_{reg} showed the same purity as compared to T_{reg} isolated by cell sorting (see Supplementary material, figure 1).

The mean fluorescence intensity (MFI) of CD25 expression in purified $CD4^+CD25^{high}$ subsets was equal in both experimental groups indicating a similar amount of $CD25^{high}$ expression (data not shown). Effector T cell (T_{eff}) subsets obtained from both MS patients and healthy individuals were equally depleted of $CD25^{high}$ cells (MS $0.2 \pm 0.2\%$; controls $0.3 \pm 0.2\%$) and contained similar numbers of $CD25^{int}$ T cells (MS $13.4 \pm 5.0\%$; controls $14.0 \pm 4.3\%$).

Since T_{reg} require expression of transcription factor scurfin to exert their regulatory properties [14–16], we determined Foxp3 gene transcripts in purified T_{reg} and T_{eff} subpopulations derived from 13 MS patients and 13 normal control individuals. As expected, $CD4^+CD25^{high}$ cells from MS patients as well as from healthy controls showed a markedly higher expression of Foxp3 mRNA [MS $0.25 \pm 0.08\%$ (Foxp3/GAPDH), controls $0.22 \pm 0.06\%$] than $CD4^+CD25^-$ cells (MS $0.02 \pm 0.01\%$, controls $0.02 \pm 0.01\%$). There were no significant differences in Foxp3 expression levels between T cells from both study cohorts (Fig. 2C).

Similar frequencies of T cell receptor excision circle-expressing T_{reg} in MS patients and control donors

As T_{reg} have a memory phenotype [8], we additionally studied intracellular levels of T cell receptor excision circles (TREC) in purified T_{reg} derived from 10 MS patients and 10 normal controls. TREC are a marker for recent thymic emigrants and are influenced by both the thymic-dependent T cell generation and the degree of peripheral T cell proliferation [29]. TREC were detectable at very low and comparable levels in T_{reg} derived from both cohorts. Mean frequencies of TREC-expressing T_{reg} were $3.3 \times 10^3/10^6$ cells (range 0.1–16.0) in MS patients versus $1.9 \times 10^3/10^6$ cells (range 0.1–5.5) in control individuals (see Supplementary figure 2). This contrasts to TREC levels of 200×10^3in 10^6 cells $CD4^+$ T cells of healthy donors [30]. Thus, T_{reg} are likely to have undergone several cell divisions in the periphery as

expected for memory T cells. In concordance with this finding >93% of $CD4^+CD25^{high}$ T cells derived from both cohorts expressed CD45RO.

T_{reg} derived from MS patients exhibit reduced suppressive properties

To assay the inhibitory effects of T_{reg} on responder T cell proliferation we used primary *in vitro* proliferation assays. T_{reg} were co-cultured with autologous T_{eff} under either rhMOG-specific (n=35, MS and controls) or, alternatively under polyclonal (n=21, MS and controls), or allogeneic (n=17, MS and controls), stimulation conditions. There was no statistical difference in levels of T_{eff} cell proliferation between MS patients and healthy controls (Fig. 3A–C). In particular, we did not find differences in proliferative responses of myelin-specific and presumably autoreactive T_{eff} cells obtained from MS patients and healthy control individuals. The degree of antigen-specific T_{eff} proliferation was in concordance with proliferative T cell responses towards rhMOG observed in other studies [31–34]. rhMOG preparations used in this study were tested to be free of endotoxin by HPLC, and *in vitro* stimulation with β2-microglobulin obtained from the same *E. coli* expression system did not induce detectable T cell proliferation as previously demonstrated [27]. Furthermore, T_{eff} did not react when either APC or rhMOG were omitted in co-culture experiments (data not shown). These observations strongly support that rhMOG-induced T cell responses were specific and not caused by contaminants.

Moreover, T_{reg} from both cohorts invariably exhibited anergy when stimulated alone. To account for differences in the activation of responder T cells within the T_{eff} subset we analyzed the cytokine gene expression profiles in freshly isolated $CD4^+CD25^{low/int}$ T cells following depletion of T_{reg} derived from 10 MS patients and 10 healthy individuals. IFN-γ, IL-2, and TNF-α transcripts were barely detectable and comparable in T_{eff} isolated from both study groups, indicating the presence of very low numbers of activated T cells within the T_{eff} subpopulations. Relative cytokine mRNA expressions from MS-derived T_{eff} in relation to mean mRNA levels of controls were 0.8, range 0.2–2.8, p=0.64 (IFN-γ), 1.6, range 0.6–4.3, p=0.25 (IL-2), and 1.6, range 0.3–13.0, p=0.49 (TNF-α).

Under all stimulation conditions, increasing concentrations of patient- and control-derived T_{reg} decreased T_{eff} proliferation in a dose-dependent manner (representative examples are shown in Supplementary figure 3). T_{reg} derived by magnetic isolation showed similar suppressive capacities as compared to T_{reg} purified by cell sorting (see Supplementary figure 1).

However, in marked contrast to inhibition rates obtained with donor T_{reg}, suppression of responder T cell proliferation was significantly less pronounced with T_{reg} isolated from peripheral blood of MS patients independent of the stimulation signal applied in co-culture experiments (Fig. 3A–C). In concordance with other reports suppression by T_{reg} was specific as the addition of 10^5 T_{eff} did not inhibit the proliferation (data not shown).

In the presence of rhMOG stimulation co-culture of T_{eff} with T_{reg} at a 4:1 ratio, T_{reg} obtained from the MS group reduced mean T_{eff} proliferation to a limited extent (from 4.6×10^3 to 3.7×10^3 cpm). In contrast, control donor-derived T_{reg} suppressed T_{eff} proliferation more efficient (from 5.8×10^3 to 3.0×10^3 cpm). This is equivalent to T_{reg}-mediated inhibition of 19.6% (range 0.0–71.0) in MS patients versus 48.2% (range 12.8–98.0) in controls (Fig. 3A).

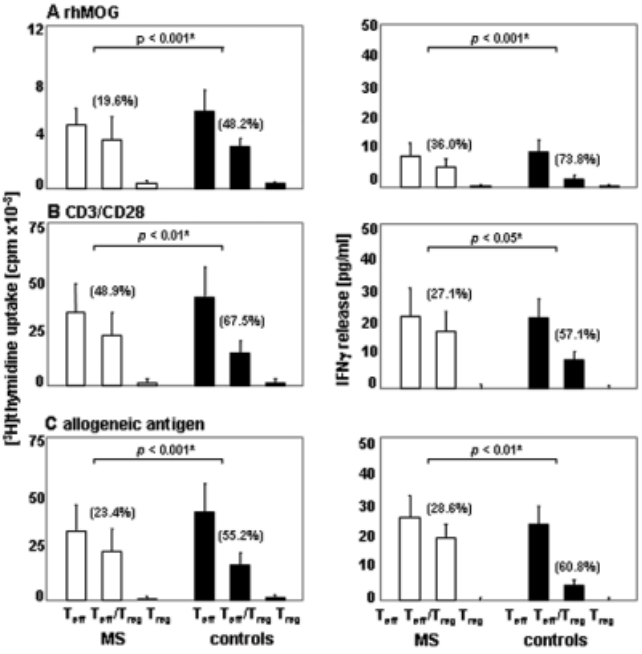

Figure 3. Reduced inhibitory effect of T_{reg} isolated from MS patients. Mean proliferative responses (left side) and IFN-γ production (right side) of T_{eff} and T_{reg} derived from peripheral blood samples of MS patients (white bars) and control donors (black bars). T_{eff} (10^5 cells) were stimulated alone or in co-culture with autologous T_{reg} with 10 μg/mL rhMOG (n=35, MS patients and controls, T_{eff}:T_{reg} ratio 4:1), 1 μg/mL soluble anti-CD3/anti-CD28 (n=21, MS patients and controls, T_{eff}:T_{reg} ratio 4:1), or 10^5 allogeneic irradiated PBMC (n=17, MS patients and controls, T_{eff}:T_{reg} ratio 1:1). Supernatants were harvested on day 4 and analyzed by ELISA. The bars depict the mean data of proliferation and IFN-γ release plus SEM for (A) rhMOG, (B) anti-CD3/anti-CD28, and (C) allogeneic stimulation, respectively. The mean T_{reg}-mediated suppression of T_{eff} proliferation and IFN-γ secretion was calculated and added in parenthesis. While T_{eff} proliferative responses and IFN-γ secretion are equivalent between MS patients and controls, inhibition by T_{reg} originating from MS patients is significantly reduced as indicated by asterisks.

Table 1. Inhibitory activities of T_{reg} from MS patients and control individuals[a)]

		rhMOG		CD3/CD28		allogeneic antigen	
Suppression		MS	controls	MS	controls	MS	controls
Low	0-33%	26	6	10	0	11	1
Moderate	33-67%	7	19	7	10	6	9
High	67-100%	2	10	4	11	0	7

[a)] Percent inhibition achieved by purified T_{reg} in *in vitro* proliferation assays as described in Material and methods. Suppression is indicated as low (0–33%), moderate (33–67%) and high (67–100%).

Upon polyclonal stimulation of T_{eff}/T_{reg} subsets at a 4:1 ratio, mean T_{eff} proliferation was downregulated from 33.3×10^3 to 22.1×10^3 cpm in the MS group and from 43.1×10^3 to 18.1×10^3 cpm in the control group. This corresponds to a T_{reg}-induced suppression of 48.9% (range 0.0–83.8, MS) and 67.5% (range 38.1–89.5, controls), respectively (Fig. 3B). These findings confirm recent observations by Viglietta *et al.* [24], who tested T_{reg} in a cohort of 15 MS patients using polyclonal stimulation with plate-bound anti-CD3 and found a consistently reduced suppressive effect mediated by $CD4^+CD25^{high}$ T cells.

In response to allogeneic T cell stimulation 1:1 co-culture of T_{eff} with patient-derived T_{reg} induced a mean reduction of responder T cell proliferation from 31.9×10^3 to 22.3×10^3 cpm in the MS group and from 42.5×10^3 to 18.5×10^3 cpm in the control group. This is equivalent to a T_{reg}-induced suppression of 23.4% (range 0.0–60.6,) in MS patients versus 55.2% (range 10.1–90.6) in controls, respectively (Fig. 3C).

When analyzed individually, 60 donor T_{reg} samples assessed in a total of 73 experimental pairs revealed a superior suppressive ability compared to MS patient-derived T_{reg} tested in parallel (see Supplementary figure 4). The degree of suppression achieved by patient- and donor-derived T_{reg} is indicated in Table 1. Weak T_{reg} activity (<33% inhibition) was found in 47 of 73 MS patients and in 7 of 73 healthy controls.

The determination of IFN-γ release in cell culture supernatants after 4 days of co-culture yielded similar results (Fig. 3A–C). The suppression of T_{eff} proliferation by increasing concentrations of T_{reg} coincided with their ability to inhibit IFN-γ secretion in a dose-dependent manner (data not shown). T_{reg} originating from MS patients inhibited IFN-γ secretion only modestly by 36.0% (range 0.0–87.8, rhMOG), 27.1% (range 3.6–40.3, anti-CD3/anti-CD28), and 28.6% (range 0.5–50.4, allogeneic PBMC). In contrast, co-culture with healthy donor derived T_{reg} was associated with a more efficient reduction of IFN-γ production by T_{eff} [73.8% (range 12.9–100), 57.1% (range 41.1–80), and 60.8% (range 21.4–88.4)].

The reduced suppressive properties of T_{reg} in MS patients are related to a functional impairment rather than a resistance of T_{eff} towards suppression

To dissect whether the reduced inhibitory activity of T_{reg} in MS patients is due to a functional impairment of T_{reg} or by an altered susceptibility of responder T cells towards suppression, we performed cross-over *in vitro* proliferation assays. We reciprocally mixed T_{reg} from healthy individuals with anti-CD3/anti-CD28-stimulated T_{eff} from MS patients or from control donors,

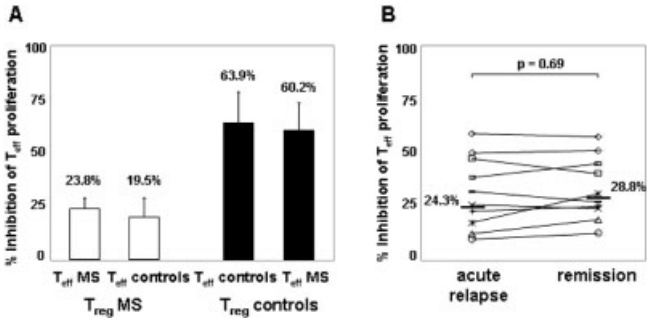

Figure 4. Reduced suppression in MS is due to dysfunction of T_{reg} and independent from disease activity. (A) In three independent cross-over experiments, T_{reg} obtained from three different healthy donors were co-cultured with anti-CD3/anti-CD28-stimulated T_{eff} obtained from three different individuals with MS, and *vice versa* (T_{eff}:T_{reg} ratio 4:1). Patient-derived T_{reg} (white bars) exhibited weak suppression on both autologous T_{eff} (23.8%, range 17.5–29.7, right white bar) and responder cells from control donors (19.5% range 8.3–31.1, left white bar). In reciprocal assays T_{reg} derived from healthy individuals (black bars) exhibited high suppression towards T_{eff} obtained from the same donor (63.9% range 48.2–82.6, right black bar) as well as on T_{eff} isolated from MS patients (60.2% range 50.6–78.0, left black bar). Bars represent mean data plus SEM of T_{reg} suppressive activity in these three different experiments. (B) Percentages of inhibition of rhMOG-induced proliferation of T_{eff} by T_{reg} (T_{eff}:T_{reg} ratio 4:1) obtained from 10 MS patients during acute relapse (left side) and in remission 12–18 weeks thereafter (right side). Differences in mean T_{reg}-mediated suppression between both time points (depicted by the bars) were statistically not significant.

and *vice versa*, in three independent experiments. As illustrated in Fig. 4A, T_{reg} isolated from MS patients exhibited a weak anti-proliferative effect on target responder cells from either MS patients (23.8%, range 17.5–29.7) or healthy donors (19.5%, range 8.3–31.1). In contrast, T_{reg} from healthy persons markedly reduced proliferation of T_{eff} isolated from both MS patients (60.2%, range 50.6–78.0) and control donors (63.9%, range 48.2–82.6). These findings suggest that the impaired downregulatory properties detectable in $CD4^+CD25^{high}$ T cells from MS patients are most likely due to a T_{reg} dysfunction instead of responder cell resistance.

The suppressive effect of T_{reg} in MS patients is reduced to a similar extent during clinical remission

To determine, whether the impaired downregulatory abilities of T_{reg} correlate with active disease, we repeatedly isolated and tested T_{reg} and T_{eff} subsets from 10 MS patients under rhMOG stimulation co-culture conditions within 12–18 weeks following an acute relapse. In all patients relapse-associated symptoms had resolved and none of the subjects had initiated immunomodulatory therapy at second assessment. Serial magnetic resonance imaging data were not available. T_{reg} isolated in clinical remission displayed similar inhibition rates of T_{eff} proliferative responses compared to effects observed with T_{reg} selected during the acute relapse in all individuals assessed. Mean T_{reg}-mediated suppression of rhMOG-stimulated T_{eff} was 24.3%, range 8.2–59.3 in remission versus 28.8%, range 10.1–58.0 during relapsing disease phases. Importantly, in each patient the T_{reg} suppressive activity as well as the proliferation of T_{eff} stayed remarkably constant (Fig. 4B). This argues for reliable purification of T_{eff} and T_{reg} and against fluctuations of MOG-specific T_{eff} and T_{reg} in blood. Thus, it appears unlikely that T_{reg} impairment is influenced by disease activity and argues against a significant depletion of T_{reg} from peripheral blood during relapse.

T_{reg} from MS patients do not display enhanced sensitivity towards IL-2 deprivation cell death

T_{reg} promptly die *in vitro* without further stimulation or addition of exogenous IL-2. To exclude the possibility that T_{reg} derived from MS patients die more rapidly *in vitro* than normal donor T_{reg}, we determined the rate of apoptosis after 48 h of culture in medium with or without IL-2 (Fig. 5). As previously reported, T_{reg} died more quickly than T_{eff} [12]. Furthermore, IL-2 rescued significantly more T_{reg} than T_{eff} from cell death, which reflects the well known dependency of highly purified

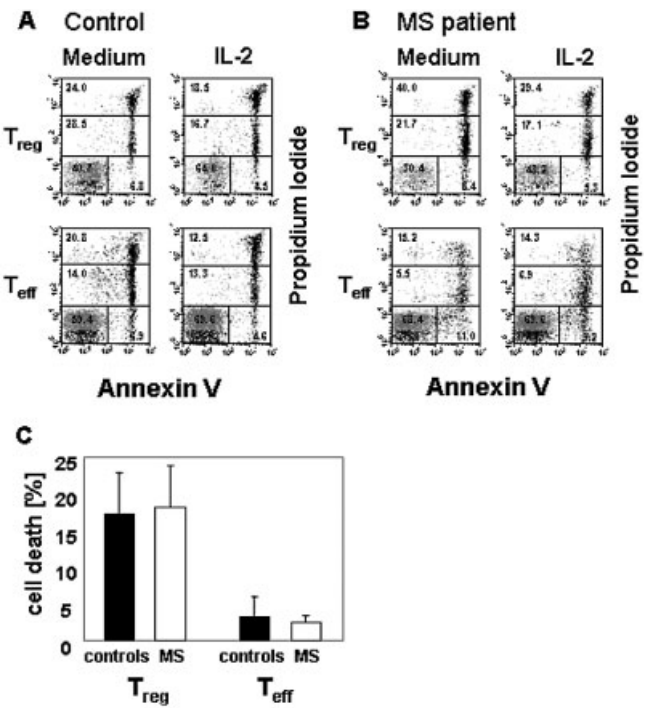

Figure 5. Apoptosis sensitivity of T_{reg} is not enhanced in MS patients. Rate of apoptosis of T_{reg} and T_{eff} from normal donors (A) and MS patients (B) after 48 h of culture in medium alone versus medium supplemented with IL-2. Four populations can be distinguished as described in Material and methods; data shown represents at least three independent experiments for each patient. (C) Effect of IL-2 deprivation on T_{reg} and T_{eff} (n=8, MS patients and normal donors); IL-2 deprivation = (cell death rate without IL-2) – (spontaneous cell death rate).

$CD4^+CD25^{high}$ cells on exogenous IL-2 *in vitro* [35, 36]. Most importantly, we did not detect an increase of apoptotic or necrotic cell death in T_{reg} from MS patients (spontaneous apoptosis 45.2 ± 7.5% (MS, n=8) *vs.* 45.7 ± 5.0% (controls, n=8) (Fig. 5A, B), and IL-2 led to a comparable increase in cell viability (Fig. 5C). Stimulation with soluble anti-CD3 and anti-CD28 under the conditions used in our proliferation assays also increased cell survival (data not shown). This is in line with other reports showing that defective suppressor function of T_{reg} in human autoimmune disease was not linked to altered survival rates of patient-derived T_{reg} under cell culture conditions [23]. T_{eff} taken from MS patients (18.4 ± 8.9%) and healthy donors (20.0 ± 9.9%) also showed similar spontaneous apoptosis (data not shown).

Discussion

Among several T cell subsets exerting a primarily suppressive action $CD4^+CD25^{high}$ T cells have recently been extensively characterized [5]. The murine counter-

part of this T cell subset has an established role in the prevention of organ-specific autoimmune disease, including experimental allergic encephalomyelitis [5, 26]. A similar subpopulation of $CD4^+CD25^{high}$ T cells is present in human peripheral blood and lymphoid organs [6–13]. From a few recent studies, it appears that T_{reg} numbers are reduced or their function is impaired in patients with various autoimmune disorders [17–24]. The impact of $CD4^+CD25^{high}$ T cells in immunoregulation is critically supported by observations of Bennett *et al.* [37] who demonstrated that mutations of Foxp3, a transcription factor which is essential for $CD4^+CD25^{high}$ T cell function, cause the immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX).

In this study, we analyzed the function of $CD4^+CD25^{high}$ T cells in patients with relapsing remitting MS in comparison to age-matched healthy individuals. The MS population consisted of treatment-naïve patients who had early stage MS and clinically active disease. This allowed the role of $CD4^+CD25^{high}$ T cells to be specifically examined in the context of acute inflammatory CNS damage. As the inflammatory process leading to myelin destruction in MS is likely to be initiated by autoimmune T cell reactivity against myelin components, we specifically tested the ability of $CD4^+CD25^{high}$ T cells to regulate antigen-specific immune responses against rhMOG. Our data are the first to demonstrate a significantly altered average ability of $CD4^+CD25^{high}$ T cells to suppress rhMOG-specific T_{eff} immune responses in MS patients compared with healthy individuals.

The reduced downregulatory effect in patient-derived $CD4^+CD25^{high}$ T cells was detectable in the presence of unaltered $CD4^+CD25^-$ T_{eff} proliferation and concomitant IFN-γ production, as previously described in several studies [8–13].

A less complete suppression of $CD4^+CD25^-$ T_{eff} expansion mediated by patient-derived $CD4^+CD25^{high}$ T cells was also detectable in co-cultures responding to stimulation with anti-CD3/CD28 monoclonal antibodies or to allogeneic stimuli thereby confirming earlier studies by Viglietta *et al.* [24]. In the vast majority of 73 experimental pairs (MS patient *vs.* control donor) tested in independent *in vitro* proliferation assays, $CD4^+CD25^{high}$ T cells purified from healthy persons exhibited a superior suppressive effector function compared to T_{reg} derived from the MS patient. The impaired downregulatory activity of T_{reg} in MS patients coincided with an equal number of $CD25^{high}$ and $CD25^{int}$ T cells within the $CD4^+$ T cell pool of MS patients and control donors. The degree of suppression observed in MS patient was not influenced by the severity of relapses, and there was also no correlation between T_{reg}-associated suppressive potencies and disease duration, relapse rate, and neurological disability as determined by the expanded disability status scale [38].

In the absence of a clear phenotypic marker other than CD25, which defines regulatory T cells within the human $CD4^+CD25^+$ T cell pool, a prerequisite to study target T_{reg} is the selection of $CD4^+$ T cells expressing high levels of CD25. Regulatory function is confined to $CD4^+CD25^{high}$ T lymphocytes as opposed to $CD4^+CD25^{low}$ and $CD4^+CD25^{int}$ T cell fractions, which do not exhibit regulatory properties [6, 7] and, within the $CD4^+CD25^{int}$ T cell subset, contain recently activated and proliferating T cells which transiently upregulate CD25 [28]. To proceed with highly purified immunoregulatory and responder T cell subsets we used low affinity-immunomagnetic beads to enrich only the brightest $CD25^+$ cells from purified $CD4^+$ T cells. In MS patients and control donors, the isolation procedure consistently selected a small $CD4^+$ cell subpopulation that coexpressed CD25 at high levels and, as a typical feature of immunoregulatory T cells, invariably exhibited anergy towards both rhMOG-specific and antigen-nonspecific polyclonal or allogeneic stimuli. Furthermore, $CD4^+CD25^{high}$ T cells purified from both study cohorts expressed equal levels of Foxp3 and had identical levels of intracellular TREC. Separate experiments comparing the suppressive activity of bead-purified *vs.* FACS-sorted $CD4^+CD25^{high}$ T cells from the same blood specimen revealed similar results (data not shown), confirming that our immunomagnetic purification yielded pure $CD4^+CD25^{high}$ T cells. These observations indicate that the functional properties of immunoregulatory T lymphocytes of patient origin were not due to a higher prevalence of activated T_{eff} cells within the isolated $CD25^{high}$ subpopulation. $CD4^+CD25^{high}$ T cells also did not differ as to their propensity to undergo apoptotic cell death, suggesting that the functional capacities of subsets isolated from MS patients were not affected by an altered survival compared to their counterparts from control individuals.

Suppression of responder T cell proliferation may not only be influenced by T_{reg}-mediated effects but also by the susceptibility of T_{eff} towards inhibitory signals. To exclude responder T cell resistance in MS patients, we mixed T_{eff} from MS patients with T_{reg} from healthy subjects, and *vice versa*, in cross-over *in vitro* proliferation assays. Whereas donor-derived T_{reg} induced an appropriate and comparable downregulation of responder T cell subsets from both study cohorts, T_{reg} isolated from MS patients exhibited weak suppressive effects when tested against both autologous and donor T_{eff}. Similar observations were recently reported by Viglietta *et al.* [24]. This clearly suggests that a functional impairment of T_{reg} rather than a responder T cell resistance accounts for the reduced inhibition. More-

over, we did not observe alterations in frequency or activation state of responder T cells originating from MS patients. Peripheral blood samples of both study groups had similar frequencies of $CD4^+CD25^{int}$ T cells as assessed by flow cytometry, and there were no differences in the proportion of $CD4^+CD25^{low/int}$ T cells among immunomagnetically separated T_{eff} subsets. Likewise, responder T cells from MS patients and control donors did not differ in the expression of T cell activation markers CD69 and CD38 (data not shown) and in both groups $CD4^+CD25^{low}$/$CD4^+CD25^{int}$ T cells expressed equivalent amounts of IFN-γ, IL-2, and TNF-α mRNA transcripts. Cytokine mRNA expressions were extremely low, suggesting that T_{eff} subsets harbored a very low number of activated T cells prior to *in vitro* stimulation. In addition, both antigen-specific and antigen-nonspecific stimuli applied in the co-culture experiments induced comparable T_{eff} proliferation levels. From these observations it appears unlikely that variations in the composition of responder T cells and, as a result, differing responses to both stimulatory and immunoregulatory signals may account for the lower inhibitory effects mediated by patient derived T_{reg}.

It is also critical to exclude that immunoregulatory T cells migrate to the CNS to locally regulate the inflammatory T cell response and, as a consequence are depleted from the peripheral blood of MS patients. $CD4^+CD25^{high}$ cells were detected in the CSF specimens from MS patients, and the proportion of $CD25^{high}$ T cells contained within total $CD4^+$ cells did not differ between CSF and peripheral blood with average frequencies of 2–3%. Furthermore, serial assessment of T_{reg} performed in 10 MS patients during active disease and again in clinical remission revealed both similar frequencies and a consistently impaired suppressive potency. These findings argue against an accumulation of T_{reg} to the CNS during relapse and suggest that the functional alteration detectable in $CD4^+CD25^{high}$ T cells from MS patients is not influenced by disease activity. However, studies with rheumatoid arthritis patients revealed thus far diverging results, showing either stable or reduced numbers of $CD25^{high}$ regulatory T cells in the peripheral blood [39, 40], therefore further studies regarding T_{reg} distribution in active disease are warranted.

In conclusion, our findings indicate that in patients with MS a persistent functional deficiency rather than a reduced survival of T_{reg} or a resistance of responder T cells accounts for the impaired suppression of autoantigen-specific and general T cell responses. The defective control of rhMOG-responsive T lymphocytes together with the beneficial effect of $CD4^+CD25^{high}$ T cells in MOG-related EAE as previously demonstrated by Kohm *et al.* [26] strongly imply that a failure to maintain peripheral immune tolerance may have a pathogenic role in MS and may contribute to the emergence of CNS autoimmunity against myelin components. Therefore, studies addressing the responsiveness of this specialized T cell subset to pharmacological modulation should be performed.

Material and methods

Patients

The study included 73 patients with definite relapsing remitting MS according to the Poser criteria [41], and a mean age of 32.6 years (range 15–58 years) as well as 73 age-matched healthy control individuals (mean 31.5 years, range 20–55 years). All MS patients had clinically active disease and had not yet received treatment with corticosteroids or immunomodulatory agents. Patients had a mean disease duration of 15 months, a mean EDSS score of 2.5, and had previously experienced an average of two relapses. Ten patients were serially tested within 12–18 weeks following treatment with a 3-day course of methylprednisolone 1000 mg daily for relapse-associated symptoms. None of these patients had initiated treatment with immunomodulatory agents at second assessment. The protocol was approved by the University of Heidelberg ethics committee and all individuals gave written informed consent.

Magnetic cell separation

PBMC were obtained from 50 mL EDTA-blood by density gradient centrifugation using Ficoll/Hypaque (Biochrom, Berlin, Germany). $CD4^+$ T cells were isolated from PBMC using a negative $CD4^+$ T cell isolation kit (Dynal, Hamburg, Germany). T_{reg} and $CD4^+CD25^{low}$/$CD4^+CD25^-$ T_{eff} were isolated from the pure, untouched $CD4^+$ T cells using CD25 magnetic beads (Dynal). Magnetic beads were removed from T_{reg} using Detachabead CD4 solution (Dynal).

CSF specimens were collected from a subset of 15 MS patients. Cells were collected by centrifugation at 800 × *g* for 10 min and resuspended in 200 μL PBS for flow cytometric analysis.

Cell sorting and flow cytometry

Alternatively, $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were separated by the use of a FACSVantage^{TM} SE cell sorter (Becton Dickinson, NJ). Briefly, freshly isolated peripheral T cells or CSF cells were directly stained for 30–45 min at 4°C with fluorescein (FITC)- or phycoerythrin (PE)-labeled antibodies specific for human CD4 (M-T477, Becton Dickinson), CD25 (M-A251, PharMingen, San Diego, CA), CD45RA (HI-100, PharMingen), and CD45RO (UCHL-1, PharMingen) as recently described [42]. Standard FACS analysis for detection of various markers and quantification of apoptosis was performed using a FACScan and the CellQuest software (Becton Dickinson).

Cell proliferation assay

T_{eff} (10^5 cells) were incubated in 96-well plates (Nunc, Wiesbaden, Germany) in 200 μL RPMI 1640, supplemented with 5% AB serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate, alone or in co-culture with 10^5 or 2.5×10^4 $CD4^+CD25^{high}$ T_{reg} (T_{eff}/T_{reg} ratios 1:1 or 4:1). For stimulation of myelin-specific T cells, 10 μg/mL recombinant MOG (rhMOG, kind gift from Bob Harris, Neuroimmunology Unit, Karolinska Sjukhuset, Stockholm, Sweden) was added to the culture medium and 2×10^5 irradiated (30 Gray) T cell-depleted PBMC served as APC. Alternatively, stimulation was performed with either 10^5 irradiated, T cell-depleted allogeneic PBMC or with soluble anti-CD3 (1 μg/mL) and anti-CD28 antibodies (1 μg/mL). After 4 days at 37°C in 5% CO_2, 1 μCi [^{3}H]thymidine per well was added for additional 16 h. Proliferation was measured using a scintillation counter. Inhibition rate (%) of T_{reg} in co-culture experiments was defined as [1 – (cpm of [^{3}H]thymidine uptake within T_{reg}–T_{eff} co-culture/cpm of T_{eff} alone)] × 100. To measure IFN-γ release, 50 μL cell culture supernatant was removed from each well after 4 days of incubation. IFN-γ concentrations were determined using an IFN-γ-ELISA kit (Biosource, Nivelles, Belgium). Cell culture medium, rhMOG-peptide, anti-CD3 and anti-CD28 antibodies were endotoxin free (<10 pg/mL) as assessed by the Limulus assay (Sigma, Taufkirchen, Germany).

Quantitation of TREC in $CD4^+CD25^{high}$ regulatory T cells

To study the degree of peripheral proliferation, we determined the number of TREC in 2×10^5 T_{reg} as described previously [30]. Results were expressed as TREC per 10^6 cells.

Quantitative real time PCR

Total RNA was isolated from freshly separated T_{reg} (2×10^5) or T_{eff} (10^6) using an Absolutely RNA Microprep kit (Stratagene, Heidelberg, Germany). cDNA conversion was performed with oligo(dT) primers using a Superscript II cDNA synthesis kit (Invitrogen, Karlsruhe, Germany).

To measure cytokine mRNA expression in T_{eff}, real time PCR was performed with primers and TaqMan® probes specific for human IFN-γ, IL-2, and TNF-α. Primers and FAM™ dye-labeled TaqMan® MGM probes were purchased as Assay-on-Demand™ Gene Expression Product from Applied Biosystems (Weiterstadt, Germany). Each PCR reaction (30 μL) consisted of 500 ng cDNA, 1× TaqMan Universal PCR Mastermix, 200 nM primers and 100 nM TaqMan probe. As a positive control for cytokine gene expression, we tested T_{eff} stimulated *in vitro* for 3 days with soluble anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL). A reaction mixture without cDNA served as a negative control in each run.

Foxp3 mRNA quantitation in T_{reg} was performed with a SYBR Green PCR Core Reagent Kit (Applied Biosystems). Reaction mixtures contained 100 ng cDNA, 1× SYBR Green PCR buffer, 3 mM $MgCl_2$, 1 mM dNTP, 900 nM of each primer (forward: 5′-AGCTGGAGTTCCGCAAGAAAC; reverse 5′-TGTTCGTCCATCCTCCTTTCC), 0.75 U AmpliTaq Gold™, and 1 U AmpErase in a total volume of 30 μL.

All real time PCR reactions were performed in triplicate by the use of a standard two-step cycle program utilizing an ABI Prism 5700 sequence detector system (Applied Biosystems). Expression of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used to correct for different levels of inhibition during reverse transcription of RNA and during PCR. Relative expression levels of cytokine and FoxP^3 mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method as outlined in the manufacturer's technical bulletin and expressed as mean target mRNA expression in patient derived T cells relative to the mean target mRNA expression in T cells obtained from control donors.

Quantification of apoptosis

Freshly isolated T_{reg} or T_{eff} (3×10^4 cells) were kept in culture medium alone or supplemented with either 100 U/mL IL-2 or soluble anti-CD3 (1 μg/mL) and anti-CD28 antibodies (1 μg/mL) for 48 h in round-bottom microtiter plates. For detection of cell death cells were washed with annexin V-binding buffer and stained with Alexa Fluor® 488 annexin V (Molecular probes) for 15 min in the dark. Cells were washed and resuspended in 3 μg/mL propidium iodide (Sigma) followed by flow cytometry analysis. Annexin V staining allows viable cells (annexin V negative and propidium iodide negative) to be distinguished from early apoptotic cell populations (annexin V positive and propidium iodide negative), whereas propidium iodide labels later stages of cell death (annexin V positive and propidium iodide positive) with necrotic cells taking up the highest amount of propidium iodide.

Statistical analysis

To analyze for differences in T_{reg}-mediated suppression, in T_{reg} and T_{eff} mRNA expression as well as in T_{reg} and T_{eff} prevalence, we used Wilcoxon-signed rank sum test and Pearson's correlation coefficients. Analysis of covariance was used to assess differences in T_{reg} TREC numbers between MS patients and healthy controls, adjusted for age. A p value of < 0.05 was considered significant.

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