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Natural Naive CD4$^+$CD25$^+$CD127^{low} Regulatory T Cell (Treg) Development and Function Are Disturbed in Multiple Sclerosis Patients: Recovery of Memory Treg Homeostasis during Disease Progression[1]

Koen Venken,* Niels Hellings,* Tom Broekmans,*† Karen Hensen,‡ Jean-Luc Rummens,‡ and Piet Stinissen[2]*

Patients with relapsing-remitting multiple sclerosis (RR-MS) show a suboptimal CD4$^+$CD25$^+$ regulatory T cell (Treg) function, whereas no Treg alterations are observed in secondary progressive MS (SP-MS) patients. To clarify the difference in Treg activity between early and chronic disease stages in MS, we analyzed the functional capacity and homeostatic parameters of naive CD4$^+$CD25$^+$CD127^{low}CD45RA$^+$ Tregs (nTregs) and their memory counterparts CD4$^+$CD25$^+$CD127^{low} CD45RO$^+$ Tregs (mTregs) in untreated MS patients and healthy controls. Interestingly, whereas the suppressive capacity of FACS-sorted nTregs was impaired in both early and chronic MS patients, only the latter group showed a restored mTreg function. Consistent with this observation, chronic MS patients had increased numbers of mTregs as compared with age-matched early MS patients, whereas nTreg frequencies did not differ significantly. TCR excision circle numbers were reduced in nTregs of early MS patients, suggestive of a diminished nTreg thymic output. Moreover, a decreased number of CD31$^+$ mTregs were observed in early vs chronic MS patients, indicating that inflammatory processes drive the homeostatic turnover of mTregs during the early disease stage. Additionally, early MS patients showed a more restricted nTreg and mTreg TCR BV gene profile as compared with healthy controls and chronic MS patients. Finally, analysis of IFN-β and glatiramer acetate-treated MS patients showed that these immunomodulatory drugs modify nTreg homeostasis. Taken together, this study provides strong evidence for a disturbed thymic nTreg development and function in MS patients. Moreover, memory Treg but not naive Treg homeostasis recovers during disease progression. ***The Journal of Immunology,*** **2008, 180: 6411–6420.**

Naturally occurring CD4$^+$CD25^{high} regulatory T cells (Tregs)[3] are considered to be the most pivotal players in the maintenance of immune tolerance. Numerous studies have indicated their role in the prevention of detrimental autoimmunity in animal models (1). Consistent with this observation, a number of alterations of Tregs, whether quantitatively or qualitatively, have been observed in patients with different autoimmune diseases (2–4).

The isolation and investigation of Tregs have been hampered due to the lack of Treg-specific markers. For instance, CD25 (the IL-2R α-chain), glucocorticoid-induced TNF receptor family-related gene (GITR), and CTLA-4, molecules constitutively expressed by Tregs, are also induced by recently activated conventional T cells (5). FOXP3, an important transcription factor in the development and function of these suppressor cells (6, 7), is a more specific marker (when measured directly ex vivo) but has the limitation to be expressed solely intracellularly, excluding an isolation of FOXP3$^+$-viable T cells for functional applications. However, recent reports indicated that Tregs express low levels of the IL-7 receptor α-chain (CD127) at their cell surface (8, 9). The expression of CD127 is inversely correlated with FOXP3 expression and with the suppressive function of CD25^{high} Tregs. CD127 expression thus permits the discrimination and isolation of viable Tregs out of blood samples (10).

Most peripheral blood Tregs appear as a mature population with a memory phenotype (CD45RO$^+$). However, a subset of naive CD4$^+$CD25$^+$CD45RA$^+$ regulatory T cells has been detected in the peripheral blood and lymphoid organs and is thought to represent precursor cells of Ag-experienced CD45RO$^+$ Tregs, analogous to the development of conventional memory T cells (11–13). CD45RA$^+$ Tregs express high levels of FOXP3 and posses an equivalently strong suppressive capacity as compared with their memory counterparts. The frequency of naive Tregs declines with age, mainly as a consequence of thymic involution (13). In contrast, memory Treg levels are maintained throughout life (11) or are even increased in elderly (14, 15). Vukmanovic-Stejic et al.

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[3] Abbreviations used in this paper: Treg, CD4$^+$CD25$^+$ regulatory T cell; FOXP3, forkhead box P3; MS, multiple sclerosis; RR-MS, relapsing-remitting MS; SP-MS, secondary progressive MS; HC, healthy control; GA, glatiramer acetate; EDSS, Expanded Disability Status Scale; nTreg, CD4$^+$CD25^{high}CD127^{low}CD45RA$^+$ naive Treg; mTreg, CD4$^+$CD25^{high}CD127^{low}CD45RO$^+$ memory Treg; Tresp, responder T cell; TREC, T cell receptor excision circle; gDNA, genomic DNA; DD, disease duration; BV, V region of the β-chain.

(16) have recently shown that memory Tregs have a very high turnover in vivo, are highly susceptible to apoptosis, and contain critically short telomeres. They further indicated that a proportion of Tregs is generated from rapidly dividing $CD4^+$ conventional T cells. It is evident that peripheral homeostatic mechanisms are very important in the control of Treg diversity and numbers and concomitantly in the maintenance of immune tolerance in healthy individuals. Disturbances within these mechanisms can have detrimental consequences and could contribute to the development of autoimmune diseases.

We and other have previously analyzed $CD4^+CD25^{high}$ Treg function in patients with multiple sclerosis (MS). A suboptimal Treg suppressive capacity was observed in patients with relapsing-remitting MS (RR-MS) (17–20). Remarkably, this Treg dysfunction seems to be restored in later disease stages, as secondary progressive MS (SP-MS) patients show a normal Treg suppressive function (19). Additionally, we recently reported that functional alterations of RR-MS-derived Tregs could be associated with a diminished expression of scurfin (*FOXP3* product) within these cells (21). At this time, the differences in Treg functionality observed between RR-MS and SP-MS patients have not been clarified.

In this study, we analyzed the suppressive capacity of both FACS-sorted naive and memory Treg subsets in RR-MS and SP-MS patients and in healthy individuals. Additionally, we further investigated whether the observed Treg functional differences in MS patients were correlated with homeostatic changes of Tregs in MS patients.

Materials and Methods

Study subjects

Peripheral blood samples were obtained from a total of 70 patients with clinically definite MS (49 RR-MS and 21 SP-MS according to Ref. 22) and 30 healthy controls (HC). Forty MS patients were defined as untreated; that is, they had not received corticosteroids or immunomodulatory drugs within (at least) 3 mo of blood samplings. Some of these MS patients (5/40) received symptomatic therapy (e.g., drugs to manage bladder dysfunction (tolterodine-L-tartrate), spasticity (baclofen), or fatigue (amantadine)) at the time of blood collection. The other 30 MS patients were under treatment with IFN-β ($n = 20$) or glatiramer acetate (GA, $n = 10$) at time of blood sampling. MS patients with a diverse range of clinical parameters were included in this study to allow an accurate investigation of Treg characteristics at different disease stages. The mean age of MS patients was 40.4 years (range of 21–58 years), the average disease duration was 9.4 years (range of 3 mo–24 years), and Expanded Disability Status Scale (EDSS) scores ranged from 1 to 6.5. MS patients were subdivided into different groups based on subtype (RR or SP), age (20–40 and 40–60 years), disease duration (less than or more than 10 years), or treatment as indicated in the text or figure legends. For each experiment, results were compared with data obtained for an age-matched control group (mean age 38 years, range of 20–57 years). This study was approved by the local Medical Ethical Committee and informed consent was obtained from all study subjects.

Cell isolation

PBMCs were isolated from whole blood by Ficoll density gradient centrifugation (Histopaque, Sigma-Aldrich). $CD4^+$ T cells were preenriched from blood by means of RosetteSep $CD4^+$ T cell enrichment mixture (StemCell Technologies) as described before (21). $CD4^+$ T cells were stained with fluorescent-labeled mAbs directed against human CD4 and CD25 (both from BD Biosciences), CD127 (eBioscience), and CD45RA (ImmunoTools) for 30 min at 4°C. $CD4^+CD25^{high}CD127^{low}CD45RA^+$ (naive Tregs, nTregs), $CD4^+CD25^{high}CD127^{low}CD45RA^-$ (memory Tregs, mTregs) and $CD4^+CD25^-CD127^{high}CD45RA^+$ (naive conventional T cells) subsets were isolated by means of FACS sorting using a FACSAria (BD Biosciences). Purity of FACS-sorted cell fractions was routinely 98–99% for each T cell fraction. After sorting, the cells were collected and directly used for suppression assays or frozen at −80°C for subsequent molecular analysis.

Flow cytometry

The following mAbs were used: anti-human CD4 (clone SK3), CD25 (2A3), CD45RO (UCHL-1) (all from BD Biosciences), CD27 (9F4), CD28 (KOLT-2), CD31 (HEC75), CD45RA (MEM-56), CD62L (SK11) (all from ImmunoTools), or CD127 (RDR5) (eBioscience). For phenotypic analysis, purified PBMCs or FACS-sorted cells were stained for 30 min, washed, and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). For intracellular staining of FOXP3, cells were fixed and permeabilized before adding anti-human FOXP3 (clone PCH101, eBioscience) as described previously (21). Statistical analysis was performed using CellQuest software.

Suppression assay

The ability of Tregs to suppress the proliferation of polyclonally activated responder T cells (Tresp) was assessed by a CFSE-based coculture assay. CFSE-labeled $CD4^+CD25^-CD127^{high}CD45RA^+$ Tresp were cultured in triplicate in 96-well round-bottom plates (Nunc) at 2×10^4 cells/well, alone or in the presence of varying amounts of autologous $CD4^+CD25^{high}CD127^{low}CD45RA^+$ nTregs or $CD4^+CD25^{high}CD127^{low}CD45RA^-$ mTregs (Tresp/Treg ratios were 1:0, 1:0.25, 1:0.5, and 1:1). In parallel, CFSE-labeled Tregs and memory Tregs were cultured alone. Autologous irradiated PBMCs (10^5 cells/well) were added as feeder cells in all cultures. As a polyclonal stimulus, we used anti-CD3 mAb (2 μg/ml; clone 2G3, BIOMED, Diepenbeek, Belgium). Cells were harvested after 5 days of culture, and suppressive capacity of Tregs was determined as the relative inhibition of the responder T cell proliferation for each Tresp/Treg ratio as described before (23). Background proliferation of Tresp (Tresp CFSE dilution in the absence of anti-CD3 Ab) never exceeded 5% ($CFSE^{low}$ cells) and was not significantly different between MS patients and controls (mean % $CFSE^{low}$ cells of 1.6 ± 0.9% vs 1.9 ± 0.2%, respectively). Additionally, proliferative responses of anti-CD3 Ab-stimulated Tresp cultured alone (no Tregs added) was not significantly different ($p > 0.05$) between MS patients (mean 70.0 ± 6.4% $CFSE^{low}$) and HC (mean 72.4 ± 4.8% $CFSE^{low}$).

Quantification of T cell receptor excision circles (TRECs)

Genomic DNA (gDNA) was extracted from FACS-sorted nTreg and mTreg cells using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. TREC numbers were quantified by real-time PCR on a LightCycler system (Roche Diagnostics) as described before (24). Results were expressed as TRECs/100 ng gDNA.

TCR BV analysis

RNA isolation (Roche Diagnostics) and cDNA synthesis (Promega) of nTreg and mTreg were performed according to the manufacturers' recommendations. The TCR BV usage of Treg subsets was evaluated by means of quantitative RT-PCR (LightCycler) using a specific forward and reverse primer for each of 23 functional TCR BV subfamily genes (25). Primers names were adapted to the current nomenclature (26). The FastStart DNA Master SYBR Green I kit (Roche Diagnostics) was used for PCR amplicon detection. The amplification protocol consisted of an initial 10 min at 95°C, followed by 45 cycles of repeated denaturation (95°C), annealing (3 s at 60°C), and elongation (10 s at 72°C). The expression of each TCR BV gene was calculated relatively as compared with the sum of all TCR BV genes.

Statistical analysis

Statistical analyses were performed using Prism software version 4.0 (GraphPad Software) and SPSS 14.0 for windows. Results are expressed as mean values ± SEM unless otherwise indicated in figure legends. For comparisons of Treg parameters between patients and controls, Student's t tests were applied. Correlations between parameters were analyzed using Pearson's correlation tests. Relationships between explanatory/independent variables (age, disease duration, and EDSS scores of patients) and response variables (Treg parameters) were further evaluated by multiple regression statistical analyses. Differences were considered significant when $p < 0.05$.

Results

Identification of natural naive and memory regulatory T cells in the peripheral blood of healthy donors and MS patients

PBMCs of MS patients and HC were stained with fluorescent-labeled Abs directed against CD4, CD25, CD45RA and CD127 and analyzed by means of flow cytometry. Within the $CD45RA^{high}$ (naive) and $CD45RA^-$ (memory) $CD4^+$ T cell populations, a

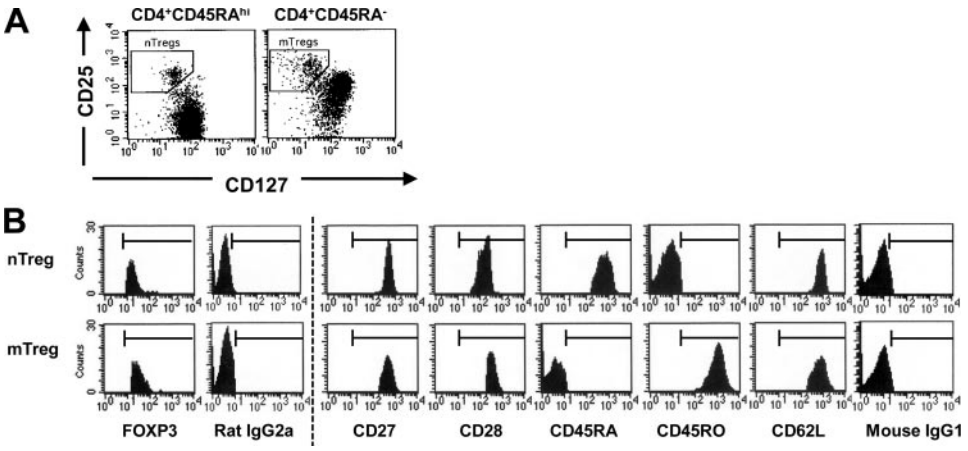

FIGURE 1. Identification of naive and memory regulatory T cells in peripheral blood of healthy individuals and MS patients. *A*, PBMCs were stained for CD4, CD25, CD45RA, and CD127 and analyzed by means of flow cytometry. Dot plots are illustrative for costaining of CD25 and CD127 in, respectively, $CD4^+CD45RA^{high}$ (naive, *left*) and $CD4^+CD45RA^-$ (memory, *right*) T cells from one MS patient. A small subpopulation of naive and memory Tregs characterized by a high CD25 and low CD127 expression is readily visualized. *B*, Naive and memory Tregs from 5 HC and 5 MS patients were further phenotypically analyzed for indicated markers (histograms represent data from one MS patient). Boundaries represent cut-offs of positive signals for each marker based on isotype control staining (rat IgG2a for intracellular FOXP3 staining and mouse IgG1 for surface markers).

small subset of cells showing a high expression of CD25 and a low expression of CD127 could be readily visualized (Fig. 1*A*). Further phenotypical analysis revealed that these $CD25^{high}CD127^{low}$ cells were $FOXP3^+$, demonstrating their regulatory phenotype (Fig. 1*B*). In agreement with recent reports (9, 11, 12), naive Tregs showed a slightly lower expression of CD25 as compared with memory Tregs (Fig. 1*A*). Additionally, $CD4^+CD25^{high}CD127^{low}CD45RA^+$ T cells of both HC and MS patients were $CD27^+$, $CD28^+$, $CD62L^{high}$, and $CD45RO^-$, indicating that terminal effector cells that regain CD45RA expression (CD45RA revertants (27)) were not included in this subset (Fig. 1*B*). There were no significant differences regarding the analyzed markers between Tregs from MS patients and HC (data not shown).

In conclusion, naive and memory regulatory $CD4^+$ T cells could be detected as, respectively, $CD4^+CD25^{high}CD127^{low}CD45RA^+$ (nTreg) and $CD4^+CD25^{high}CD127^{low}CD45RA^-$ (mTreg) cells in the peripheral blood of MS patients and healthy individuals.

Reduced suppressive function of nTregs in MS patients and restoration of mTreg function in the chronic disease phase

Previous studies of our group and others (17–19) have indicated that the suppressive capacity of the total population of $CD25^{high}$ regulatory T cells is suboptimal in RR-MS patients, whereas SP-MS patients showed a normal Treg function. Additionally, we observed a positive correlation between the suppressive capacity of Tregs and disease duration of MS patients (19). To analyze the suppressive capacity of naive and memory Tregs separately, we FACS-sorted both Treg subpopulations out of peripheral blood collected from 16 untreated MS patients (10 RR-MS and 6 SP-MS) and 12 HC and cocultured them with naive autologous responder T cells ($CD4^+CD25^-CD127^{high}CD45RA^+$) at different Tresp/Treg ratios. In light of our previous observations, we segregated MS patients based on subtype (RR-MS or SP-MS) and disease duration (<10 years or ≥10 years). For each MS subgroup, Treg suppressive values were compared with those of an age-matched control group (HC). An overview of these results is shown in Fig. 2. Both nTregs and mTregs of HC and MS patients dose-dependently inhibited the anti-CD3-induced proliferation of responder cells. However, RR-MS patients with a short disease duration (DD < 10 years; mean 3.7 ± 1.2 years) showed a significantly ($p < 0.01$) reduced nTreg as well as mTreg suppression as compared with age-matched HC for all Tresp/Treg ratios tested. SP-MS and RR-MS patients with a long disease duration (DD ≥ 10 years; mean 16.0 ± 3.5 years) also showed a suboptimal nTreg suppression as compared with age-matched HC (Fig. 2). Moreover, the nTreg suppressive capacity was not significantly different between RR-MS (DD < 10 years), RR-MS (DD ≥ 10 years), and SP-MS patients, regardless of age differences. In contrast, the mTreg function of the chronic patients (SP-MS and RR-MS DD ≥ 10 years) was similar to that of HC (Fig. 2; $p > 0.05$). Additionally, FACS-sorted nTregs as well as mTregs of MS patients and HC were anergic upon TCR stimulation (data not shown).

Taken together, RR-MS patients show an impaired nTreg and mTreg function at the early disease stage. In the chronic disease phase, mTreg function but not nTreg function is restored toward normal suppressive levels.

A reduced frequency of nTregs in early MS patients and an increase of mTreg numbers in function of disease duration of MS patients

To investigate whether the functional alterations of Tregs could be associated with homeostatic changes of Tregs in MS patients, we first analyzed the frequency of both nTreg and mTreg cells in 30 HC and 40 untreated MS patients. As age is an influencing factor, we separated our study subjects into a younger (20–40 years) and older (40–60 years) group. Based on the functional Treg analysis, we subdivided MS patients based on disease duration in early (MS < 10 years) and chronic (MS ≥ 10 years) patients as indicated in Fig. 3*A*. Early MS patients showed a significantly reduced frequency of nTregs (age 20–40 years: 1.8 ± 0.2%; $p < 0.01$) and mTregs (2.1 ± 0.3%; $p < 0.01$) as compared with an age-matched control group (nTregs: 3.2 ± 0.4%; mTreg: 3.9 ± 0.4%). Differences in nTregs (but not mTreg numbers) were no longer detectable in older subjects (age 40–60 years; nTreg frequency MS < 10 years: 1.2 ± 0.1%, HC: 1.4 ± 0.1%, $p > 0.05$; mTreg frequency MS < 10 years: 2.5 ± 0.2%, HC: 4.5 ± 0.5%, $p < 0.01$). Indeed, when nTreg frequencies were plotted against the age of subjects, a significant age-associated decline in nTreg frequency could be detected (Fig. 3*B*; HC: $r = 0.63$, $p < 0.001$; total MS: $r = 0.39$, $p = 0.01$). As a result, nTreg numbers of HC and MS patients converged at older age. Numbers of mTregs in both HC and MS patients only slightly increased with age (Fig. 3*B*; HC: $r = 0.29$,

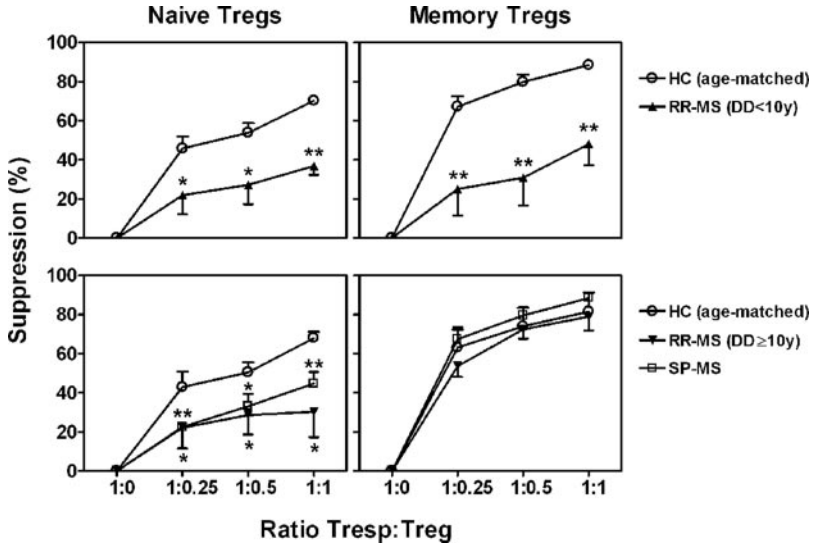

FIGURE 2. Analysis of the suppressive function of naive and memory Tregs of MS patients and control individuals. FACS-sorted naive and memory Tregs (as defined in Fig. 1) of untreated RR-MS ($n = 10$) and SP-MS ($n = 6$) patients and HC ($n = 12$) were cocultured with autologous CFSE-labeled naive conventional T cells ($CD4^+CD25^-CD127^{high}CD45RA^+$, Tresp) at different ratios. Cultures were stimulated with anti-CD3 Abs, and the CFSE signal of responder cells was analyzed on day 5 of culture. Treg-mediated suppression is expressed as the relative inhibition of the responder cell proliferation for each Tresp/Treg ratio. MS patients were subgrouped based on disease type (RR-MS and SP-MS) and disease duration (DD), and Treg suppressive values of MS patients were compared with age-matched control groups. *Upper panels*, RR-MS DD $<$ 10 years ($n =$ 6, mean age 37 $\pm$ 5 years) and HC ($n = 6$, mean age 37 $\pm$ 5 years). *Lower panels*, RR-MS DD $\geq$ 10 years ($n = 4$, mean age 51 $\pm$ 4 years), SP-MS ($n = 6$, mean age 50 $\pm$ 4 years), and HC ($n = 6$, mean age 49 $\pm$ 3 years). $*$, $p < 0.05$; $**$, $p < 0.01$ as compared with age-matched HC.

$p = 0.12$; total MS: $r = 0.37$, $p = 0.05$). Remarkably, the frequency of mTregs in chronic MS patients (MS $\geq$ 10 years: 4.0 $\pm$ 0.6%) was significantly higher ($p < 0.05$) as compared with age-matched early MS patients and did not significantly differ from older control subjects (Fig. 3*A*). With further analysis, we observed a significant positive correlation between mTreg numbers and disease duration of MS patients (Fig. 3*B*; $r = 0.48$, $p = 0.002$). The frequency of nTreg of chronic MS patients remained low (1.4 $\pm$ 0.2%) and was

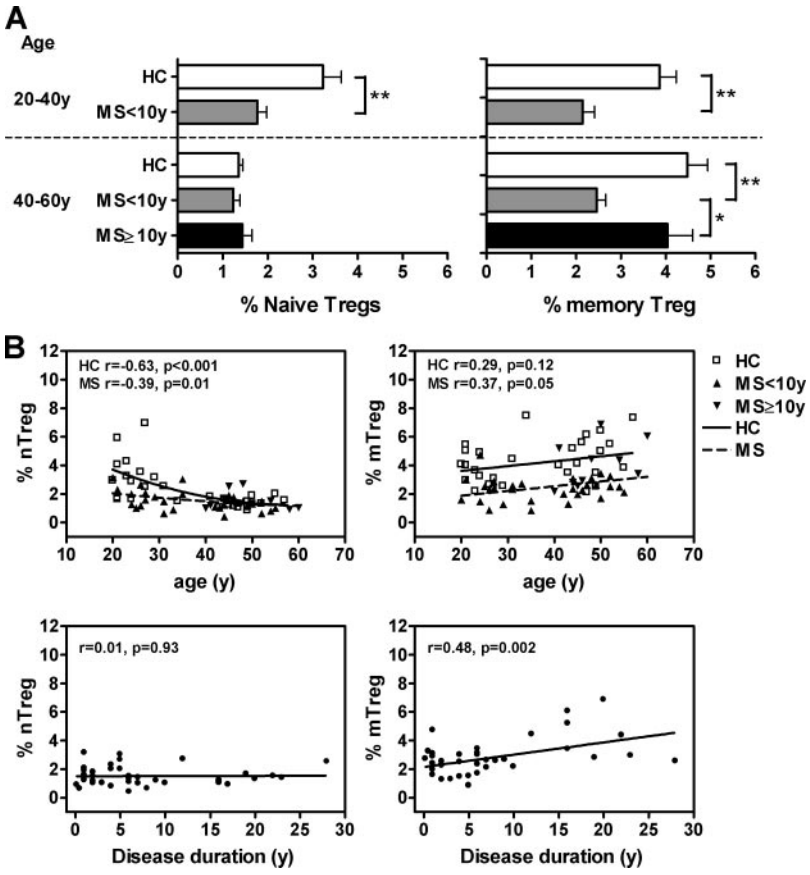

FIGURE 3. Frequency of naive and memory Tregs in the peripheral blood of MS patients and HC. *A*, PBMCs of 40 untreated MS patients and 30 HC were analyzed by flow cytometry as illustrated in Fig. 1*A*. Frequencies of nTregs and mTregs were determined as percentages of total $CD4^+$ T cells. MS patients were divided based on age (20–40 and 40–60 years) and disease duration: MS $<$ 10 years (30 RR-MS; mean DD of 3.6 $\pm$ 0.5 years) and MS $\geq$ 10 years (4 RR-MS and 6 SP-MS; mean DD of 18.8 $\pm$ 5.5 years). $*$, $p < 0.05$; $**$, $p < 0.01$ as compared with indicated study populations. *B*, Frequency of nTregs (*left*) and mTregs (*right*) was plotted against age (*upper panels*) and disease duration (*lower panels*) for MS patients and HC (age only). Indicated r and p values were measured using Pearson's correlation tests.

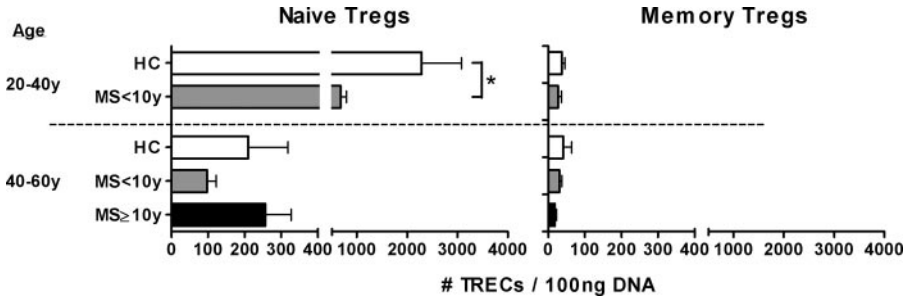

FIGURE 4. TREC numbers in naive and memory Tregs isolated from MS patients and HC. TREC numbers were determined by real-time PCR in gDNA of naive and memory Tregs FACS sorted from the peripheral blood of 22 untreated MS patients and 15 HC. Study groups were subdivided as indicated: MS $<$ 10 years (n = 12) and MS $\geq$ 10 years (n = 10). *, $p < 0.05$ as compared with age-matched HC.

not significantly reduced as compared with age-matched controls (Fig. 3*A*). Additionally, we performed multiple regression analyses to study the relationship between Treg subset frequencies and both age and disease duration in detail. The linear relationship between Treg frequencies (dependent variable) and both age and disease duration (i.e., the independent variables, considered together) was significant for both nTreg ($F = 4.611$, $p = 0.016$) and mTreg frequencies ($F = 6.434$, $p = 0.004$) of MS patients. However, coefficient analyses indicated that for nTregs of MS patients, age ($t = -3.035$, $p = 0.004$) but not disease duration ($t = 1.525$, $p = 0.136$) is the significant explanatory variable. In contrast, disease duration ($t = 2.385$, $p = 0.022$) but not age ($t = 1.277$, $p = 0.210$) significantly contributes to memory Treg frequencies in MS patients. Furthermore, memory Treg (but not nTreg) frequencies correlated with EDSS scores of MS patients (Pearson's $r = 0.39$, $p = 0.03$), but this effect was secondary to the positive correlation between disease duration and EDSS ($r = 0.677$, $p < 0.01$).

In summary, changes in nTreg and mTreg numbers in early vs chronic MS patients were consistent with differences in Treg function. Disease duration rather than age determines a recovery of mTreg numbers in MS patients.

Disturbed CD4$^+$CD25^{high}CD127^{low} regulatory T cell homeostasis in MS patients

To clarify the observed alterations in Treg numbers, we analyzed homeostatic parameters of Tregs in the same subgroups of untreated MS patients and HC. First, we measured TRECs in FACS-sorted nTregs and mTregs of HC and MS patients to investigate the thymic production of regulatory T cells. As expected, nTregs of young healthy persons (age 20–40 years) showed high TREC amounts (Fig. 4). Naive Tregs of early MS patients had a significantly reduced TREC content as compared with age-matched controls (TRECs/100 ng gDNA; MS $<$ 10 years: 674 $\pm$ 119, HC: 2286 $\pm$ 791; $p < 0.05$). In older subjects (age 40–60 years) this difference was not significant, as TREC levels decreased in both study groups (Fig. 4; MS $<$ 10 years: 97 $\pm$ 25; HC: 209 $\pm$ 109 TRECs/100 ng gDNA). Additionally, chronic MS patients also showed no significant difference in nTreg TREC levels (MS $\geq$ 10 years: 255 $\pm$ 70). Further statistical analyses indicated that TREC levels in nTregs of MS patients correlated with the age (Pearson's $r = -0.52$, $p = 0.023$) but not disease duration of patients ($r = -0.28$, $p = 0.24$). As mTregs have a high turnover rate in vivo

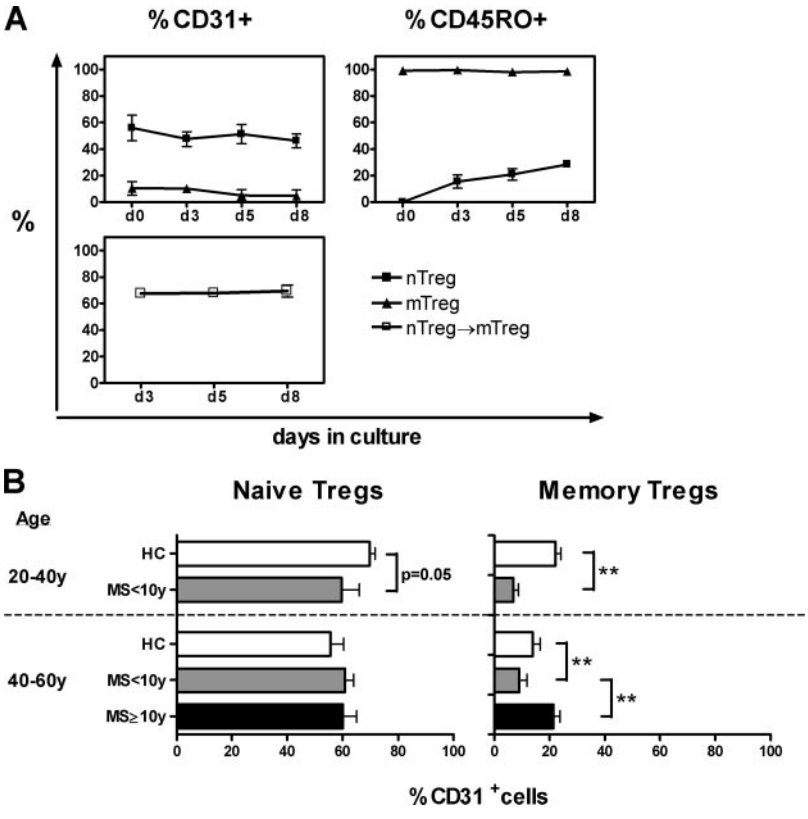

FIGURE 5. Analysis of CD31 expression by nTregs and mTregs of MS patients and HC directly ex vivo and after activation in vitro. *A*, FACS-sorted naive and memory Tregs were stimulated with anti-CD3 Ab in the presence of feeder cells. At indicated time points, cells were harvested and analyzed for CD45RO, CD31, and 7-AAD expression by means of flow cytometry. Figures show percentages of CD31$^+$ (*left*) and CD45RO$^+$ positive (*right*) cells in viable (7-AAD$^-$) nTregs and mTregs. A subpopulation of nTregs converted to mTregs after TCR stimulation (*right*), and for these cells (nTreg $\rightarrow$ mTreg) CD31 expression was shown in the lower panel. *B*, CD31 expression by nTregs and mTregs of 40 untreated MS patients and 30 HC was analyzed by flow cytometry. MS patients were subgrouped as indicated in Fig. 3*A*. *, $p < 0.05$; **, $p < 0.01$ as compared with indicated study populations.

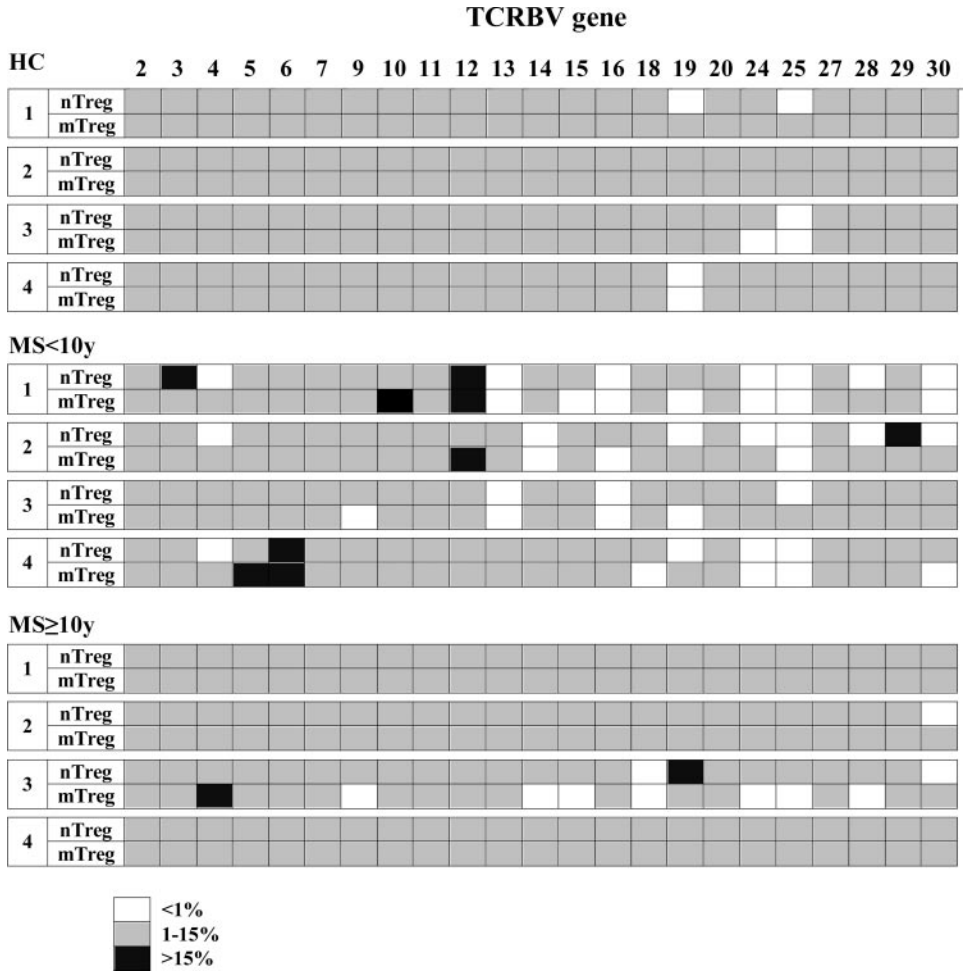

FIGURE 6. TCR repertoire of naive and memory Tregs. The expression of 23 TCR BV genes was determined by real-time PCR for FACS-sorted naive and memory Tregs from 4 HC, 4 MS $<$ 10 years patients, and 4 MS $\geq$ 10 years patients (all untreated). The relative number of genes expressed within each Treg subset is shown.

(16), only a very low number of TRECs could be observed in this subset for all study groups (Fig. 4), and therefore no significant differences were seen.

Next, we analyzed the expression of PECAM-1 (CD31) on the surface of nTregs and mTregs of HC and untreated MS patients by means of flow cytometry. This adhesion molecule is down-regulated after homeostatic proliferation (28). Therefore, a low number of $CD31^+$ cells within a T cell population is indicative for a high cell turnover in vivo. A high proportion of nTregs from HC was $CD31^+$ (Fig. 5). Interestingly, when FACS-sorted naive Tregs were activated in vitro by means of anti-CD3 Ab, we observed that a proportion of $CD31^+$ nTregs differentiated into $CD31^+$ mTregs (Fig. 5*A*). Most blood-circulating mTregs had lost CD31 expression (Fig. 5), demonstrating their highly proliferative state in vivo. We observed a small difference ($p = 0.05$) between the number of $CD31^+$ cells in nTregs of young HC (69 $\pm$ 2%) and early MS patients (60 $\pm$ 6%; Fig. 5*B*). Both young and older early MS patients showed a pronounced reduced number of $CD31^+$ cells within their mTreg populations as compared with age-matched controls (age 20–40 years: MS $<$ 10 years: 7 $\pm$ 2%, HC: 22 $\pm$ 2%; age 40–60 years: MS $<$ 10 years: 9 $\pm$ 3%, HC: 15 $\pm$ 3%). Remarkably, CD31 expression levels were normal for mTregs of chronic MS patients (21 $\pm$ 2%; $p > 0.05$ as compared with HC). There was a significant relationship between CD31 expression levels of Treg subsets and age, disease duration, and EDSS considered together (nTreg: $F = 2.992$, $p = 0.049$; mTreg: $F = 7.993$, $p = 0.001$). Coefficient analysis showed that age ($t = -2.694$, $p = 0.012$) was the explanatory variable for CD31 expression of nTregs and disease duration ($t = -3.705$, $p = 0.002$) for CD31 expression of mTregs. Individual contributions of other parameters were not significant ($p > 0.05$).

Finally, we analyzed the TCR diversity of naive and memory Tregs of 8 untreated MS patients (4 MS $<$ 10 years and 4 MS $\geq$ 10 years) and 4 healthy controls. The relative expression of TCR BV genes in each Treg population is given in Fig. 6. In general, nTregs and mTregs from HC showed a broad TCR BV usage, and the TCR BV profiles between these two Treg subsets were strikingly similar. In contrast, nTregs and mTregs from early MS patients showed a more restricted TCR BV gene usage. Most chronic MS patients showed a TCR BV profile comparable to that of Tregs from HC.

Taken together, these data suggest a disturbed nTreg development and altered mTreg homeostasis in the early MS patients.

Influence of IFN-β and GA treatment on Treg homeostasis in MS patients

Currently approved immunomodulatory therapies for MS include IFN-β and GA. A positive influence of these drugs toward Treg function and/or numbers has been described recently (21, 29, 30). However, the mechanism behind these effects is still unknown. To investigate whether these drugs have an effect on Treg homeostasis, we analyzed nTreg and mTreg frequencies (Fig. 7, *top panels*) and CD31 expression (Fig. 7, *bottom panels*) in IFN-β- ($n = 20$) and GA- ($n = 10$) treated patients and compared these results with age-matched untreated MS groups subdivided for disease duration. Naive and memory Treg frequencies were both significantly higher in GA-treated ($p < 0.01$) and IFN-β-treated ($p < 0.05$) early MS patients as compared with age-matched untreated MS patients.

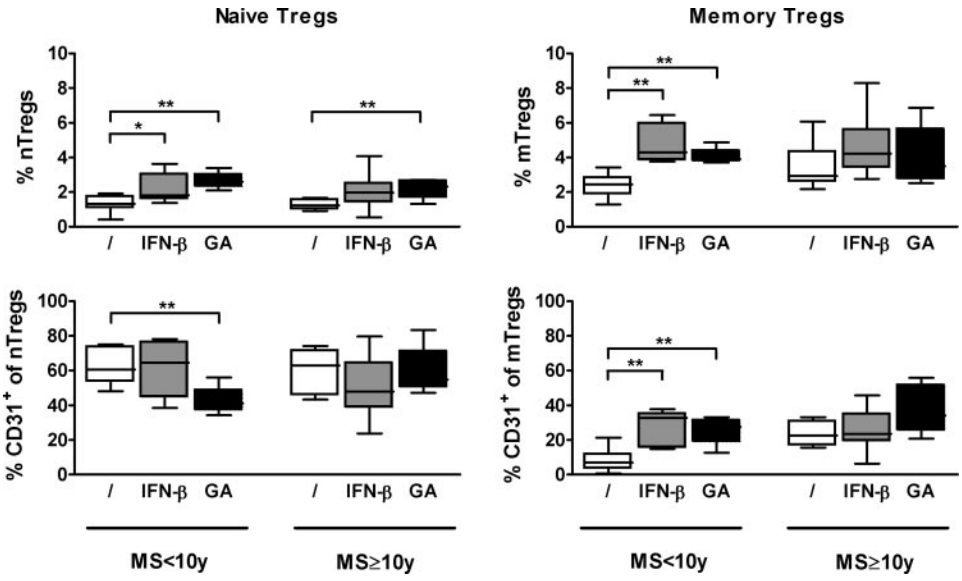

FIGURE 7. Influence of IFN-β and GA treatment on Treg homeostasis in MS patients. PBMCs of 20 IFN-β- (mean age 47 $\pm$ 9 years) and 10 GA- (mean age 45 $\pm$ 7 years) treated MS patients were analyzed by flow cytometry for CD4, CD25, CD45RA, CD127, and CD31 expression. Indicated values (shown as box and whiskers) represent the frequency (*upper panel*) and CD31 expression (*lower panel*) of nTregs (*left*) and memory Tregs (*right*) for both MS $<$ 10 years and MS $\geq$ 10 years patients. *, $p < 0.05$; **, $p < 0.01$ as compared with age- and disease duration-matched untreated MS patients.

Only nTregs were increased in GA-treated chronic MS patients. The number of CD31 expressing memory Tregs was significantly higher in GA- and IFN-β-treated early MS patients ($p < 0.01$). The number of CD31 expressing nTregs was reduced in GA-treated patients ($p < 0.01$), which could reflect an increased nTreg turnover or differentiation into mTreg.

In conclusion, these results indicate that IFN-β and GA therapy can up-regulate nTreg and mTreg numbers in MS patients probably by both an influence on Treg thymic development and peripheral cell turnover.

Discussion

Treg functional alterations in patients with RR-MS have been demonstrated by independent reports. Moreover, our previous results clearly showed that this Treg defect could not be observed in patients with SP-MS. Until this time, the reason for the discrepancy between RR-MS and SP-MS patients was not known. To answer this question, a detailed investigation of naive and memory Treg function and homeostasis in both early and chronic MS patients was performed in this study. We were able to show that Treg functional dynamics during MS progression are associated with changes in Treg homeostasis. Naive Treg function is reduced in both early RR-MS as well as in progressive MS patients. However, mTreg homeostasis is restored during the progressive disease phase and compensates for the nTreg defect.

Several studies have reported the existence of a small population of natural nTregs in the peripheral blood of healthy adult individuals, next to mTregs (11–13). To our knowledge, this is the first report of a direct ex vivo functional analysis of nTregs in patients with an autoimmune disease. We isolated nTregs and mTregs based on the expression of the recently described marker CD127 in combination with the differentiation marker CD45RA and the more commonly used Treg-associated molecules CD4 and CD25. Our data clearly show that both $CD4^+CD25^+CD127^{low}CD45RA^+$ and $CD4^+CD25^+CD127^{low}CD45RA^-$ T cell subsets are $Foxp3^+$ and are able to suppress T cell proliferation in vitro, confirming their regulatory phenotype (8, 9). Furthermore, our gating strategy excluded "CD45RA revertants", that is, T cells that are characterized by a low expression of CD28, CD27, and CD62L (27), but also T cells at an intermediate stage of maturation expressing both CD45RO and CD45RA.

It is evident that Treg homeostasis, including both thymic and peripheral processes, must be tightly regulated to maintain an optimal immune tolerance throughout life. Naive and memory Tregs are phenotypically nearly identical (i.e., $CD4^+CD25^+CD127^{low}FOXP3^+$), suggesting that nTregs constitute a direct pool of precursor cells for mTregs. Our data, in agreement with others (9), show that nTreg numbers decline progressively with age, indicating that thymic involution is influencing the size of the nTreg population in the peripheral blood. Indeed, although Tregs are present in the thymus as a $CD45R0^+$ population (13), our observation and the fact that most cord blood Tregs are $CD45RA^+$ (12, 31) strengthen the concept that thymic Tregs, in concordance with conventional T cells (32), convert to the CD45RA phenotype just before migration toward the periphery. Moreover, in nTregs of young healthy individuals we found high numbers of TRECs, and most nTregs expressed PECAM-1 (CD31), an adhesion molecule that is expressed on recent thymic emigrants. TRECs will be exponentially diluted out during cell divisions in the periphery (33). However, our data indicate that only after a high turnover of Tregs, CD31 expression is lost, thus explaining why TRECs within nTreg populations decrease relatively faster with age as compared with CD31 expression. This observation, together with the fact that CD31 can be measured at the single-cell level by means of flow cytometry, makes CD31 a sensitive marker for homeostatic proliferation (34). Measurements of TREC numbers provide a good estimation of thymic output of (especially naive) T cells, although such estimates should be used with caution as TREC content of a T cell population can be strongly influenced by peripheral cell proliferation. The differentiation of nTregs into Ag-experienced mTregs also influences the frequency of these cells. Where this conversion takes place and how it is controlled in vivo are currently unknown. Naive Tregs might be activated in the periphery by self-Ags and subsequently converted to mTregs, which are known to have a self-TCR repertoire. Our results showing an overlap in TCR BV profile between nTregs and mTregs of healthy individuals are in agreement with this hypothesis. Moreover, we observed that after activation of nTregs, a subpopulation develops into $CD31^+$ mTregs. As most blood-circulating mTregs of both young and older individuals had a low CD31 expression (and TREC content), these cells profoundly

proliferate in vivo, as recently proven by deuterium-labeling experiments (16). Whether this has functional consequences for Treg subsets in controlling autoimmunity will have to be analyzed in animal models of autoimmunity, but mTregs have been shown to be more prone to CD95L-mediated apoptosis, suggesting their vulnerability during long-term inflammation (12).

Our previous observation of a functional Treg difference between RR-MS and SP-MS patients was further clarified in this report. In the early phase of the disease, RR-MS patients show a compromised naive and memory Treg homeostasis and function that can potentially be associated with MS-related autoimmune manifestations. These data are consistent with recent observations by Haas et al. (35), who found a reduced percentage of $CD31^+CD45RA^+CD45RO^-$ Treg cells in a cohort of RR-MS patients with short disease duration (1–5 years, median 2 years). Moreover, our observation of a significantly reduced TREC content in nTregs of young RR-MS patients further suggests an age-inappropriate reduced thymic output of nTregs in MS patients. Indeed, different reports have indicated a premature immunosenescence in patients with autoimmunity, including MS (24, 36). Normally, thymic involution causes a decline in the generation of naive conventional T cells with age and progresses at a rate of ~3%/year. It is thought that this restructuring process is accelerated in MS patients. The more pronounced decline in frequency of nTregs in MS patients, especially at young age, is in line with this hypothesis. Alternatively, it is possible that nTreg development is specifically disturbed in a subset of MS patients. We have recently shown that the mean cellular FOXP3 expression (mean fluorescence intensity) is decreased in MS patients (21). Additionally, a recently published large-scale genetic analysis has identified several Treg-associated genes as heritable risk factors for multiple sclerosis (37). Naive Tregs of MS patients in this study showed a reduced FOXP3 mean fluorescence intensity (data not shown) as compared with HC, but this small effect was not significant, probably due to the low number of patients analyzed. As TREC numbers are also influenced by peripheral T cell turnover, it may be that thymic function is not altered in MS patients, but that inflammatory processes drive a clonal exhaustion of naive Tregs and conventional T cells, which could also explain the small but significant reduction in the proportion of $CD31^+$ cells in the nTreg population of early MS patients. Both possibilities (i.e., a reduced thymic output and increased proliferation of nTregs in response to the disease inflammation) are not mutually exclusive. Of note, HLA-DR2-positive MS patients showed a significantly lower frequency of nTregs when compared with HLA-DR2-negative patients (unpublished data).

The alterations in nTreg homeostasis could have led to the observed significant contraction in TCR diversity and impaired Treg function, which can also have consequences for the transformation of nTregs into mTregs in early MS patients. Our observation of a reduced number of $CD31^+$ cells in the mTreg population points toward an increased turnover of these cells, possibly as a compensatory mechanism to control inflammation. This is in agreement with the concept that during the early disease stage, peripheral inflammatory processes underlie disease manifestations in the CNS. It is thought that myelin-reactive T cells can escape peripheral immune surveillance due to the suboptimal Treg function and after crossing the blood-brain barrier will start an immune cascade in the brain tissue. In an attempt to down-regulate ongoing lesion inflammation, Tregs will be mobilized, up-regulate cell adhesion molecules (CD103 and CD49d), and will be recruited into the CNS of RR-MS patients as recently shown by our group (21). Results with the animal model for MS, experimental autoimmune encephalomyelitis, are concordant with this hypothesis (38, 39).

In the chronic disease stage, when most RR-MS patients have developed a more progressive disease (SP-MS) (40), we observed a recovery of mTreg but not nTreg function. The normal mTreg function was correlated with an increase of mTregs in chronic MS patients as compared with age-matched early MS patients, indicating that disease duration rather than age is the underlying factor. Indeed, our statistical analyses regarding TREC levels and CD31 expression of Treg subsets suggest that naive Treg development and homeostasis are mainly influenced by thymic output (which is inversely correlated with age), whereas memory Treg homeostasis is influenced by disease duration (which is correlated with disease progression). Our previous observation of a recovery of Treg function in SP-MS patients can therefore be attributed to the restoration of the memory Treg population, whereas nTreg function remained suboptimal in these patients. This latter observation probably excludes an increased conversion of naive into memory Tregs. The normal frequency of $CD31^+$ mTreg cells seems to be a reflection of a restored Treg homeostasis possibly influenced by a reduced proinflammatory cytokine environment (41, 42) and the apparent stop in Treg migration toward the CNS (21).

Because increased Treg numbers have been observed in the elderly (>70 years) (14, 15), it is possible that a physiological compensatory mechanism, which normally is particularly active at old age, is accelerated in MS patients due to pronounced immunosenescence in these patients. Moreover, Rozenkranz and coworkers recently showed an intensified $FOXP3^+$ Treg suppressive activity in patients with Alzheimer and Parkinson disease, suggesting an association between Treg function and neurodegenerative pathology (15). It is likely that some of these cells are induced regulatory T cells. Indeed, as natural Tregs are apoptosis prone (16, 43, 44), induction of Tregs from conventional T cells has been reported to be involved in Treg homeostasis. However, no clear phenotypic differences between naturally and induced Tregs have been described at present. By means of cytokine-blocking experiments and supernatant analyses, we can exclude that mTregs from chronic patients represent TGF-β producing Th3 (45) or IL-10 producing Tr1 regulatory T cells (46) (data not shown). Additionally, we also observed no difference in the percentage of $HLA\text{-}DR^+$ mTregs, a functionally distinct population of Tregs recently described by Baecher-Allan et al. (47), between MS patients and HC (Ref. 19 and unpublished data). Our analysis of TCR BV gene expression of Tregs in these patients supports a more general restored mTreg homeostasis, as Tregs induced from effector/memory $CD4^+CD25^-$ T cells in an Ag-dependent manner would probably have a more restricted TCR profile (16). However, the identification of surface markers specific for induced Tregs will be needed to fully clarify this issue in future studies. Finally, although we used a 3 mo cutoff to guarantee a washout period of any previously administered medication, it could still be possible that a restoration of mTreg function and homeostasis in chronic MS patients is related to (any possible) long-term treatment. However, results obtained from two treatment-naive chronic MS patients were similar as compared with those of the total chronic MS group recruited in this study (data not shown). This latter observation provides strong evidence that our results were not profoundly biased by potential long-term medication histories and justifies conclusions made throughout this study.

A possible restoration of the Treg homeostasis, definitely in the early phase of the disease, can be of therapeutic potential. Interestingly, patients treated with IFN-β and GA in this study

showed restored nTreg numbers as compared with age- and disease duration-matched untreated patients. Our data suggest an influence of these immunomodulatory drugs on thymic Treg development and Treg homeostasis and provide an explanation for previous observations of restored Treg numbers and function in treated MS patients (21, 29, 30). Further investigations, for instance a longitudinal follow-up of TCR BV profiles and homeostatic parameters of Tregs from GA- and IFN-β-treated patients, are necessary to unravel the molecular events underlying these results. Additionally, a specific recovery of nTreg homeostasis by stimulation of thymic nTreg generation can be a promising therapeutic strategy for MS patients as well as for patients with other autoimmune diseases known to have an impaired Treg physiology (2).

In conclusion, our results show a disturbed thymic nTreg development in MS patients and an increased mTreg turnover in the early disease phase. During the chronic disease stage, a recovery of memory Treg homeostasis compensates for this Treg defect.

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Disclosures

The authors have no financial conflicts of interest.

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