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*J Immunol* 2007; 179:1322-1330; ;  
doi: 10.4049/jimmunol.179.2.1322  
<http://www.jimmunol.org/content/179/2/1322>

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Prevalence of Newly Generated Naive Regulatory T Cells ( $T_{reg}$ ) Is Critical for $T_{reg}$ Suppressive Function and Determines $T_{reg}$ Dysfunction in Multiple Sclerosis<sup>1</sup>

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The suppressive function of regulatory T cells ( $T_{reg}$ ) is impaired in multiple sclerosis (MS) patients. The mechanism underlying the  $T_{reg}$  functional defect is unknown.  $T_{reg}$  mature in the thymus and the majority of cells circulating in the periphery rapidly adopt a memory phenotype. Because our own previous findings suggest that the thymic output of T cells is impaired in MS, we hypothesized that an altered  $T_{reg}$  generation may contribute to the suppressive deficiency. We therefore determined the role of  $T_{reg}$  that enter the circulation as recent thymic emigrants (RTE) and, unlike their CD45RO<sup>+</sup> memory counterparts, express CD31 as typical surface marker. We show that the numbers of CD31<sup>+</sup>-coexpressing CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>FOXP3<sup>+</sup>  $T_{reg}$  (RTE- $T_{reg}$ ) within peripheral blood decline with age and are significantly reduced in MS patients. The reduced de novo generation of RTE- $T_{reg}$  is compensated by higher proportions of memory  $T_{reg}$ , resulting in a stable cell count of the total  $T_{reg}$  population. Depletion of CD31<sup>+</sup> cells from  $T_{reg}$  diminishes the suppressive capacity of donor but not patient  $T_{reg}$  and neutralizes the difference in inhibitory potencies between the two groups. Overall, there was a clear correlation between  $T_{reg}$ -mediated suppression and the prevalence of RTE- $T_{reg}$ , indicating that CD31-expressing naive  $T_{reg}$  contribute to the functional properties of the entire  $T_{reg}$  population. Furthermore, patient-derived  $T_{reg}$ , but not healthy  $T_{reg}$ , exhibit a contracted TCR V $\beta$  repertoire. These observations suggest that a shift in the homeostatic composition of  $T_{reg}$  subsets related to a reduced thymic-dependent de novo generation of RTE- $T_{reg}$  with a compensatory expansion of memory  $T_{reg}$  may contribute to the  $T_{reg}$  defect associated with MS. *The Journal of Immunology*, 2007, 179: 1322–1330.

Growing evidence suggests a role for natural immunoregulatory T cells of the CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> phenotype ( $T_{reg}$ )<sup>3</sup> in the prevention of autoimmunity. Numerous studies have reported numeric or functional deficiencies of  $T_{reg}$  in various human autoimmune diseases including inflammatory demyelinating disorders of the CNS (Refs. 1–10 and M. Korporal, M., J. Haas, B. Fritzsching, S. Möller, B. Fritz, C. S. Falk, P. H. Krammer, E. Suri-Payer, and B. Wildemann, Restored suppressive capacities of regulatory T cells under therapy with IFN- $\beta$  and glatiramer acetate in patients with multiple sclerosis, submitted for publication). We and others have shown that  $T_{reg}$  derived from patients

with relapsing remitting multiple sclerosis (RRMS) display a functional impairment because they inhibit myelin-specific and Ag-nonspecific T cell proliferation less potently as compared with healthy control donors (Refs. 8–10 and M. Korporal, M., J. Haas, B. Fritzsching, S. Möller, B. Fritz, C. S. Falk, P. H. Krammer, E. Suri-Payer, and B. Wildemann, submitted for publication). The mechanisms of this  $T_{reg}$  defect are unclear. In particular,  $T_{reg}$  frequencies in the peripheral blood of multiple sclerosis (MS) patients are unaltered as is their susceptibility to undergoing CD95 ligand-mediated apoptosis (Refs. 8, 10, and 11 and M. Korporal, M., J. Haas, B. Fritzsching, S. Möller, B. Fritz, C. S. Falk, P. H. Krammer, E. Suri-Payer, and B. Wildemann, submitted for publication).

$T_{reg}$  mature in the thymus and the majority of the cells rapidly adopt a memory CD45RO phenotype (12) when released into the periphery. However, there is recent evidence that small numbers of T cells with a naive CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> surface profile and immunosuppressive properties are detectable within the peripheral  $T_{reg}$  pool (13–16). Unlike their Ag-primed memory counterpart, this naive  $T_{reg}$  subset may comprise de novo generated cells that have been recently released from the thymus and have not yet experienced Ag contact. Kimmig et al. have demonstrated that surface expression of CD31 (PECAM-1) on naive CD4<sup>+</sup> T cells distinguishes recent thymic emigrants (RTEs) from peripherally expanded naive Th cells (RTE-Th) (17). CD4<sup>+</sup>CD45RA<sup>+</sup>RO<sup>-</sup>CD31<sup>+</sup> RTEs, unlike CD45RA<sup>+</sup> Th cells lacking CD31, contain high amount of TCR excision circles (TREC). TRECs are generated as a by-product of the TCR rearrangement process in the thymus and are enriched in newly generated T cells (18). Thus, phenotypic and molecular features define

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Received for publication December 19, 2006. Accepted for publication May 11, 2007.

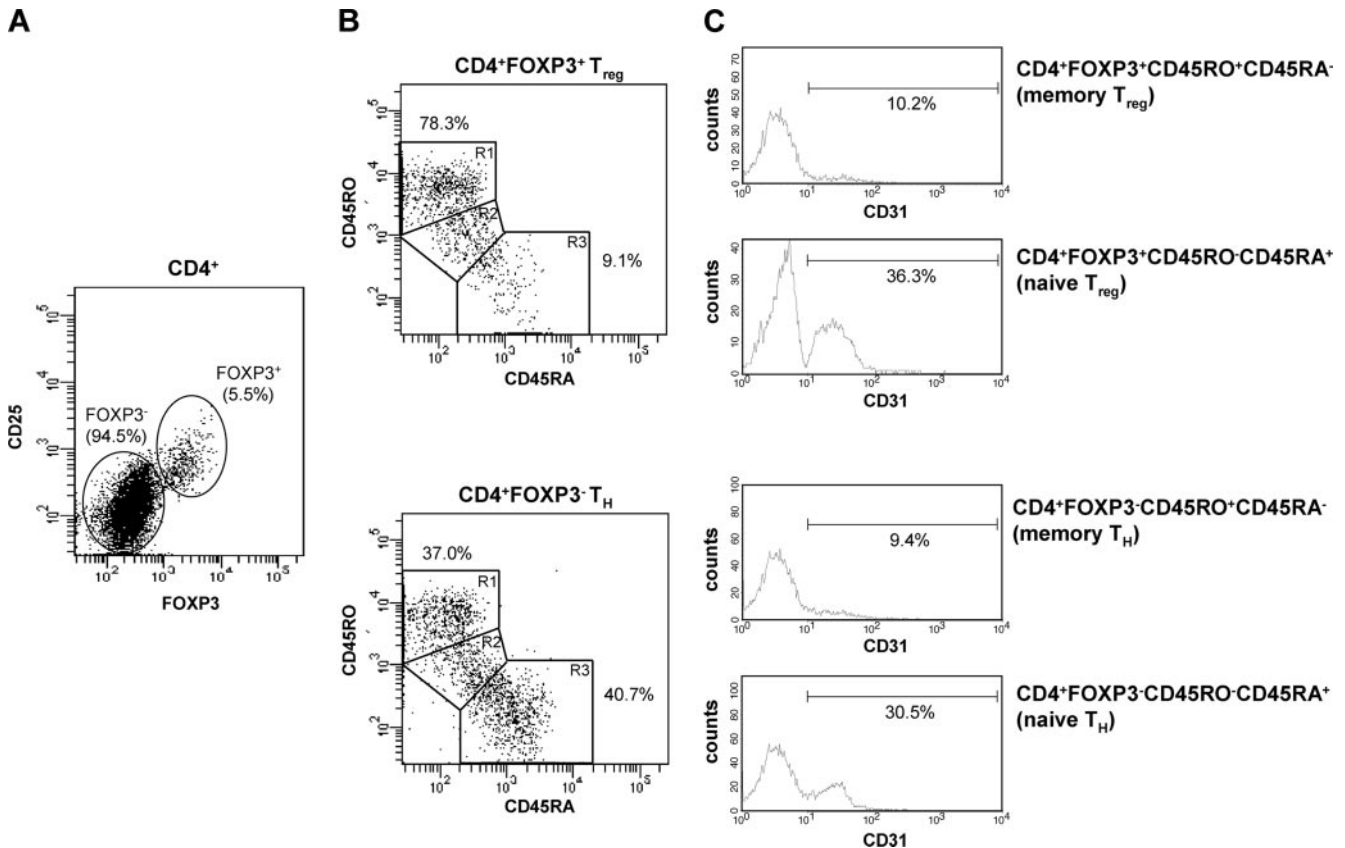
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<sup>1</sup> This work was supported by the Gemeinnützige Hertie-Stiftung (1.01.1/04/003), Deutsche Forschungsgemeinschaft (Sonderforschungsbereich (SFB) 405, 5H and SFB 571, B7), the Young Investigator Award from the Faculty of Medicine, University of Heidelberg (to B.F.), and Serono Deutschland GmbH.

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<sup>3</sup> Abbreviations used in this paper:  $T_{reg}$ , regulatory T cell; CS, complexity score; GA, glatiramer acetate; HC, healthy control; MS, multiple sclerosis; RRMS, relapsing remitting MS; RTE, recent thymic emigrant;  $T_{eff}$ , effector T cell; TREC, TCR excision circle.

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**FIGURE 1.** Coexpression of CD45RA/CD45RO and CD31 on peripheral Th and  $T_{reg}$ . PBMCs were stained with mAbs specific for CD4, CD25, CD45RA, CD45RO, CD31, and intracellular FOXP3 and analyzed by flow cytometry. A typical staining of PBMCs obtained from one healthy donor is shown. **A**, The dot plot shows the expression of CD25 and FOXP3 on PBMCs gated on  $CD4^+$  cells.  $T_{reg}$  were identified as  $FOXP3^+$  cells and Th as  $FOXP3^-$  cells. **B**, Dot plots show CD45RA vs CD45RO expression on  $FOXP3^+$   $T_{reg}$  and  $FOXP3^-$  Th. Memory and naive Th and  $T_{reg}$  were defined as  $CD45RA^-CD45RO^+$  (R1) and  $CD45RA^+CD45RO^-$  (R3), respectively. **C**, Histograms show CD31 expression on memory and naive Th and  $T_{reg}$ . Coexpression of CD31 identifies RTE-Th as  $CD4^+FOXP3^-CD45RA^+CD45RO^-CD31^+$  cells and RTE- $T_{reg}$  as  $CD4^+FOXP3^+CD25^+CD45RA^+CD45RO^-CD31^+$  cells.

$CD4^+CD45RA^+CD31^+$  T cells as RTEs and  $CD4^+CD45RA^+CD31^-$  T cells as peripherally expanded naive Th cells. As thymic function declines with age the release of RTEs also decreases. This loss of de novo generated Th cells is compensated by peripheral postthymic expansion, resulting in an increase of  $CD4^+CD45RA^+CD31^-$  T cell numbers with age (17, 19).

This age-related change in the composition of the  $CD4^+$  T cell pool may also affect the homeostatic balance and, eventually, the functional properties of  $T_{reg}$ . In concordance with this assumption, an age-dependent decline of  $T_{reg}$  suppressive potencies has been recently reported in a pilot study (20). Our own previous work suggests that the thymic export function is impaired in MS as levels of TREC-expressing peripheral T cells are significantly decreased in patients with clinically active RRMS in the absence of an accelerated T cell turnover (21). Therefore, we hypothesized that an altered  $T_{reg}$  homeostasis may contribute to the suppressive deficiency associated with MS.

To elucidate a potential imbalance in  $T_{reg}$  homeostasis in MS, we analyzed the role of naive  $T_{reg}$  that coexpress CD31 and circulate in the periphery as RTE. We measured the numbers of RTE- $T_{reg}$  within the peripheral blood of patients with RRMS and healthy persons by multicolor flow cytometry analysis and determined their contribution to the suppressive function of the entire  $T_{reg}$  population by in vitro proliferation assays. We also performed CDR3 spectratype analysis to screen  $T_{reg}$  derived from both cohorts for differences in the TCR repertoire.

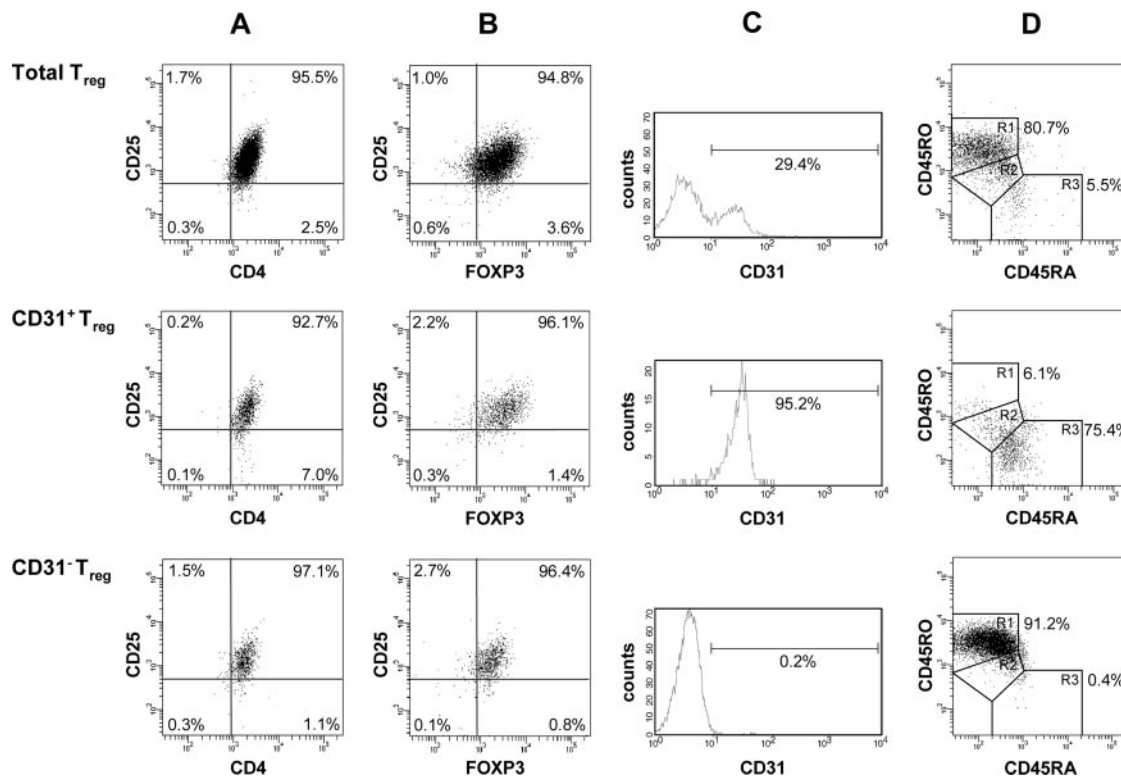
## Materials and Methods

### Subjects

Blood specimens were obtained from 40 RRMS patients (mean age 33.9 years, range 14–62 years) as well as from 49 healthy controls (HCs) (mean age 36.7 years, range 24–85 years). All patients had definite MS according to McDonald's or Poser criteria (22, 23) and had experienced an average number of 1.4 previous relapses (range 0–3). Disease duration ranged between 1 and 5 years (median 2). The mean Expanded Disability Status Scale score was 1.7 (range 1–2). All patients had a clinically active disease and had not yet received treatment with corticosteroids or immunomodulatory agents. The protocol was approved by the University of Heidelberg (Heidelberg, Germany) ethics committee and all individuals gave written informed consent.

### Flow cytometry

The mAbs used for flow cytometry were obtained from BD Pharmingen (anti-human CD4, CD45RO, CD45RA, and CD31), Miltenyi Biotec (anti-human CD25), and eBioscience (anti-human FOXP3). FOXP3 staining was performed according to the manufacturer's protocol. For six-color flow cytometric analysis, cells were first stained with a surface mAb specific for CD4, CD25, CD45RO, CD45RA, or CD31 followed by FOXP3 intracellular staining. FACS acquisition was performed immediately with a FACSCanto cytometer and analyzed with FACSDiva software (BD Biosciences). For the quantification of  $T_{reg}$  and Th subsets within peripheral blood, stained PBMCs were first gated on  $CD4^+$  cells and then analyzed for the expression of FOXP3 and CD25. Because only  $CD4^+$  T cells expressing the FOXP3<sup>+</sup> gene product scurfin exhibit suppressive function (24),  $CD4^+CD25^+FOXP3^+$  cells were defined as  $T_{reg}$  and  $CD4^+FOXP3^-$  cells as Th (Fig. 1A).  $T_{reg}$  and Th cells were further analyzed for their CD45RA/CD45RO surface expression to identify  $CD45RA^-CD45RO^+$



**FIGURE 2.** Purity and phenotype of isolated T<sub>reg</sub> and T<sub>reg</sub> subsets. *A* and *B*, Immunomagnetically isolated T<sub>reg</sub> and T<sub>reg</sub> subsets were stained with mAbs specific for CD4, CD25, CD45RA, CD45RO, CD31, and FOXP3 and analyzed by flow cytometry. A typical staining of T<sub>reg</sub> subsets immunomagnetically separated from peripheral blood of one healthy donor is shown. More than 92% of isolated T<sub>reg</sub>, CD31<sup>+</sup>T<sub>reg</sub>, and CD31-depleted T<sub>reg</sub> were CD4<sup>+</sup>CD25<sup>high</sup> (*A*) and FOXP3-positive (*B*) and therefore were highly pure. *C*, Approximately 30% of isolated T<sub>reg</sub> express CD31 on their surface. After further separation of total T<sub>reg</sub> into CD31<sup>+</sup> and CD31<sup>-</sup> cells using CD31-labeled microbeads, >95% of the CD31<sup>+</sup> fraction but only <1% of the CD31<sup>-</sup> fraction expressed CD31. *D*, Dot plots show CD45RA vs CD45RO expression, revealing that purified total T<sub>reg</sub> are predominantly CD45RA<sup>-</sup>CD45RO<sup>+</sup> memory cells (R1) with only 5–10% naive cells (R3). CD31<sup>-</sup>T<sub>reg</sub> also exhibited a memory phenotype with <1% of CD45RA<sup>+</sup>CD45RO<sup>-</sup> cells. In contrast, CD31<sup>+</sup>T<sub>reg</sub> were predominantly naive with only <10% memory cells.

memory and CD45RA<sup>+</sup>CD45RO<sup>-</sup> naive subsets (Fig. 1*B*). RTE-T<sub>reg</sub> and RTE-Th were identified by coexpression of the CD31 molecule within the naive T<sub>reg</sub> and Th subsets (Fig. 1*C*).

#### Cell separation

PBMC were isolated from 50 ml of peripheral blood by density gradient centrifugation using Ficoll/Hypaque (Biochrom). CD4<sup>+</sup> T cells were isolated from PBMCs by using a negative CD4<sup>+</sup> T cell isolation kit (Dyna Biotech). CD4<sup>+</sup>CD25<sup>high</sup> T<sub>reg</sub> and CD4<sup>+</sup>CD25<sup>low</sup>/CD4<sup>+</sup>CD25<sup>int</sup> effector T cells (T<sub>eff</sub>) were isolated from the pure untouched CD4<sup>+</sup> T cells using CD25 magnetic beads (Dyna Biotech). Magnetic beads were removed from T<sub>reg</sub> using Detachabead CD4 solution (Dyna Biotech). T<sub>reg</sub> isolated from the PBMC of nine HCs and seven patients were further separated according to their CD31 expression in CD31<sup>+</sup>T<sub>reg</sub> and CD31<sup>-</sup>T<sub>reg</sub> by the use of a FITC-labeled anti-CD31 mAb and an anti-FITC MultiSort kit (Miltenyi Biotec). Immune magnetic separation constantly yielded highly pure T<sub>reg</sub> subsets with >90% CD4<sup>+</sup>CD25<sup>high</sup> cells as previously described (Ref. 10 and M. Korporal, M., J. Haas, B. Fritzsche, S. Möller, B. Fritz, C. S. Falk, P. H. Krammer, E. Suri-Payer, and B. Wildemann, submitted for publication). The purity and phenotype of random samples were further tested by intracellular FOXP3 staining that revealed >90% FOXP3<sup>+</sup> cells in preparations of total T<sub>reg</sub> ( $n = 20$ , mean 94.7%, range 92.2–98.5% FOXP3<sup>+</sup>) as well as in CD31<sup>+</sup>T<sub>reg</sub> ( $n = 7$ , mean 94.5%, range 90.9–96.1% FOXP3<sup>+</sup>) and CD31<sup>-</sup>T<sub>reg</sub> ( $n = 11$ , mean 93.4%, range 93.0–96.4% FOXP3<sup>+</sup>). Preparations of T<sub>reg</sub> and T<sub>reg</sub> subsets isolated from patients and HCs showed similar CD25 and FOXP3 expressions (data not shown). The vast majority of isolated CD31<sup>+</sup>T<sub>reg</sub> expressed CD31 on their surface (mean 96.3%, range 92.4–99.5%), whereas only 4.6% (range 0.2–7.7%) of the CD31-depleted T<sub>reg</sub> remained CD31<sup>+</sup>. CD31<sup>-</sup>T<sub>reg</sub> exhibited a memory phenotype (mean 82.5%, range 78.3–92.0% CD45RA<sup>-</sup>CD45RO<sup>+</sup>). In contrast, CD31<sup>+</sup>T<sub>reg</sub> were predominantly naive (mean 68.5%, range 61.1–75.4% CD45RA<sup>+</sup>CD45RO<sup>-</sup>) with <10% memory cells (mean 9.7%, range 5.3–12.7% CD45RA<sup>-</sup>CD45RO<sup>+</sup>) (representative

examples showing purity and phenotype of isolated T<sub>reg</sub> subsets are shown in Fig. 2).

#### Cell proliferation assay

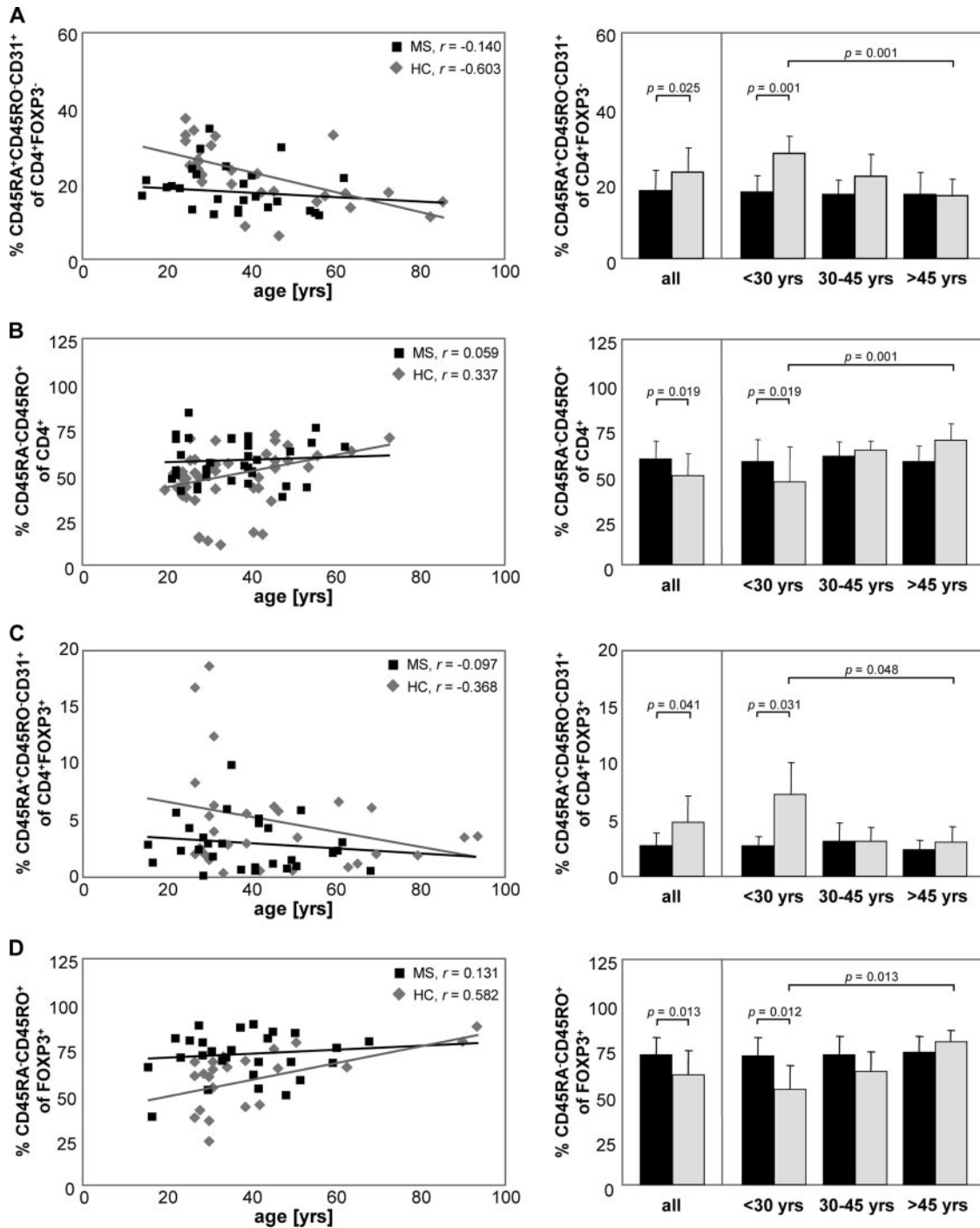
Freshly isolated T<sub>eff</sub> (10<sup>5</sup>) were incubated in 96-well plates (Nunc) in 200  $\mu$ l of culture medium alone or in 4:1 coculture with 2.5  $\times$  10<sup>4</sup> total T<sub>reg</sub> or T<sub>reg</sub> subsets. For TCR stimulation, soluble anti-CD3 (1  $\mu$ g/ml) and anti-CD28 mAbs (1  $\mu$ g/ml) were added to the culture medium. After 4 days at 37°C in 5% CO<sub>2</sub>, 1  $\mu$ Ci of [<sup>3</sup>H]thymidine per well was added for an additional 16 h. Proliferation was measured using a scintillation counter. Inhibition rate (percentage) of T<sub>reg</sub> in coculture experiments was defined as  $[1 - \text{[}^3\text{H]thymidine uptake (cpm) within a T}_{reg} \text{ plus T}_{eff} \text{ coculture} \div \text{cpm of T}_{eff} \text{ alone}] \times 100$ . Each experiment was performed in triplicate. The CD3 and CD28 mAbs used for cell culture experiments were purified from hybridoma supernatants by protein A affinity purification as described (25). Cell culture medium and mAbs were endotoxin free (<10 pg/ml) as assessed by a *Limulus* assay (Sigma-Aldrich).

#### Quantification of TRECs

Genomic DNA was extracted from 2  $\times$  10<sup>5</sup> T<sub>reg</sub> or T<sub>reg</sub> subsets by the use of a DNAzol solution (Invitrogen Life Technologies) according to the manufacturer's recommendations. The numbers of TRECs in T cell subsets were determined by real time PCR as described previously (21). Results were expressed as TRECs per 10<sup>6</sup> cells.

#### CDR3 spectratyping

We performed CDR3 spectratyping to determine the TCR V $\beta$  repertoire in patient and HC T<sub>reg</sub> and T<sub>eff</sub>. Total RNA was isolated from 2  $\times$  10<sup>5</sup> T<sub>reg</sub> or 10<sup>6</sup> T<sub>eff</sub> using Absolutely RNA Microprep kit (Stratagene) according to the manufacturer's protocol and converted to cDNA by a SuperScript first-strand synthesis kit (Invitrogen). Amplification of the entire group of 24 TCR V subfamilies was conducted with 24 V $\beta$ -specific primers and one



**FIGURE 3.** Frequencies of naive Th and T<sub>reg</sub> coexpressing CD31 are age-dependent and reduced in MS. PBMCs obtained from 28 HCs and 28 MS patients were analyzed by multicolor flow cytometry using mAbs specific for CD4, CD25, CD45RA, CD45RO, CD31, and intracellular FOXP3. Dot plots (filled square, MS; gray diamond, HCs) show relative frequencies of Th and T<sub>reg</sub> subsets in individual samples. The linear regression curves are shown. Histogram bars (black, MS; gray, HCs) represent mean percentages of Th and T<sub>reg</sub> subsets in different age groups. **A**, Percentages of FOXP3<sup>-</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD31<sup>+</sup> cells (RTE-Th) among CD4<sup>+</sup> T cells decline with age in HCs and are significantly reduced in younger MS patients (<30 and 30–45 years old). **B**, Conversely, the relative frequencies of CD45RA<sup>-</sup>CD45RO<sup>+</sup> memory cells within CD4<sup>+</sup> T cells rise with age in control donors and are clearly higher in MS patients aged under 30. **C**, Similar to RTE-Th, the proportions of CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD31<sup>+</sup> cells within total CD4<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> (RTE-T<sub>reg</sub>) decrease with age in HCs and are significant higher in younger MS patients, which is paralleled by an increase of CD45RA<sup>-</sup>CD45RO<sup>+</sup> memory T<sub>reg</sub> in elder HCs and in younger MS patients.

primer recognizing the C region (26, 27). One hundred nanograms of cDNA from each T<sub>eff</sub> and T<sub>reg</sub> sample was amplified in eight parallel PCRs each containing a set of three different V $\beta$  primers respectively and the C-primer, which was 6-FAM-labeled for CDR3 spectratyping. Size distribution of fluorescent PCR products was determined by laser-induced capillary electrophoresis using an automated DNA analyzer (A310; Applied

Biosystems) and GeneScan software (Applied Biosystems). Normal transcript size distribution consists of eight peaks for each V subfamily (28). Complexity within a V subfamily was determined by counting the number of peaks per subfamily. The complexity score (CS) was calculated as the summation of the scores of all 24 subfamilies. The maximum score would be 192.

### Statistical analysis

To determine whether differences and correlations in cell counts, inhibitory capacities, TREC levels, and TCR diversities were statistically significant, we performed the Student's *t* test using a two-tailed distribution with unpaired samples and calculated the Pearson's correlation coefficient. *p* < 0.05 was considered significant.

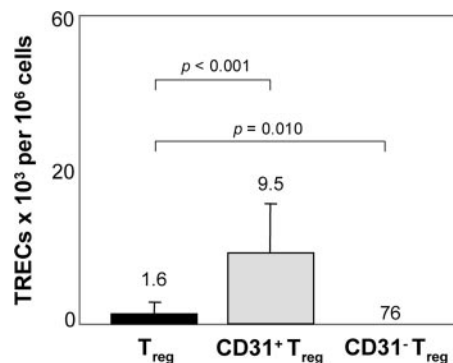
## Results

### Frequencies of naive Th cells coexpressing CD31 decrease with age and are reduced in the peripheral blood of MS patients

To determine the number of naive helper T cells that have just entered the circulation as RTEs (RTE-Th), we performed multi-color flow cytometric staining of PBMCs obtained from 28 MS patients and 28 age-matched HCs. RTE-Th frequencies were calculated as percentage of CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD31<sup>+</sup> cells within total CD4<sup>+</sup>FOXP3<sup>-</sup> T cells (Fig. 1). As expected, the proportions of RTE-Th within CD4<sup>+</sup>FOXP3<sup>-</sup> cells declined with age in HCs (Fig. 3A). In individuals <30 years of age RTE-Th constituted approximately one-third of CD4<sup>+</sup> T cells (mean 28.3%, range 20.9–37.5%). RTE-Th numbers were significantly lower in persons aged 30–45 years (mean 22.0%, range 8.9–33.0%, *p* = 0.048) and lowest in persons >45 years of age (mean 16.6%, range 6.5–33.4%, *p* = 0.001). RTE-Th frequencies in patients were significantly lower than those in HCs (MS patients: mean 18.1%, range 3.5–34.6%; HCs: mean 22.7%, range 6.5–37.5%; *p* = 0.025, Fig. 3A). However, when comparing patients and HCs aged >45 years, the difference was <1% (MS patients: mean 17.1%, range 12.1–29.4%; HCs, mean 16.6%, range 6.5–33.4%; *p* = 0.905). Similarly, the entire naive Th subset (CD45RA<sup>+</sup>CD45RO<sup>-</sup> of CD4<sup>+</sup>FOXP3<sup>-</sup> cells) decreased with age in healthy donors but was constantly low in MS patients (data not shown). Conversely, the proportions of memory cells within CD4<sup>+</sup> T cells rise with age in HCs (<30 years: mean 38.4%, range 14.6–68.2%; >45 years: mean 68.4%, range 51.0–88.3%; *p* = 0.002) and are enhanced in MS patients (MS: mean 59.4%, range 39.8–84.3%; HCs: mean 51.0%, range 14.6–88.5%; *p* = 0.050; Fig. 3B). CD31 expression on memory CD4<sup>+</sup> T cells was consistently <10% and did not differ between MS patients and HCs (data not shown). In concordance with numerous studies and our own previous observations, the distribution of total CD4<sup>+</sup> T cells was independent of age and unaltered within peripheral blood samples of MS patients (data not shown).

### Frequencies of naive T<sub>reg</sub> coexpressing CD31 are age-associated and are reduced in the peripheral blood of MS patients

Next, we screened peripheral blood samples from 28 MS patients and 28 age-matched HCs for the presence of total T<sub>reg</sub> and T<sub>reg</sub> subsets by multicolor flow cytometry. Proportions of RTE-T<sub>reg</sub> subsets were calculated as percentages of CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD31<sup>+</sup> cells within total CD4<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>+</sup> cells (Fig. 1). With regard to total T<sub>reg</sub>, we observed slightly reduced percentages of CD25<sup>+</sup>FOXP3<sup>+</sup> cells among total CD4<sup>+</sup> T cells in MS patients that were not statistically significant (MS: mean 5.0%, range 1.6–9.2%; HCs: mean 5.7%, range 2.6–12.9%; *p* = 0.107; not depicted). T<sub>reg</sub> frequencies did not change with age in both patients and HCs (data not shown). In contrast, RTE-T<sub>reg</sub> frequencies within total FOXP3<sup>+</sup> cells declined with age in HCs (all: mean 4.8%, range 0.3–19.5%; <30 years of age: mean 7.2%, range 1.5–19.5%; 30–45 years of age: mean 3.1%, range 0.3–6.1%; >45 years of age: mean 3.2%, range 0.7–6.6%; Fig. 3C). In patients the proportions of RTE-T<sub>reg</sub> were significantly reduced and not age-associated (all: mean 2.7%, range 0.0–9.8%; <30 years of age: mean 2.6%, range 0.0–5.7%; 30–45 years of age: mean 3.1%,



**FIGURE 4.** TRECs are detectable in CD31<sup>+</sup> T<sub>reg</sub> but not in CD31<sup>-</sup> T<sub>reg</sub>. Intracellular levels of TREC-DNA within T<sub>reg</sub> subsets isolated from four HCs were determined by quantitative real-time PCR. TRECs were detectable at low levels in total T<sub>reg</sub> and were virtually absent in CD31<sup>-</sup> T<sub>reg</sub>. In contrast, TREC frequencies in CD31<sup>+</sup> T<sub>reg</sub> were substantially higher as compared with the entire T<sub>reg</sub> population, reflecting a more naive status. Bars represent mean TREC numbers per 10<sup>6</sup> cells for each T<sub>reg</sub> subset.

range 0.4–9.8%; >45 years of age: mean 2.4%, range 0.4–5.9%). Differences in RTE-T<sub>reg</sub> numbers between MS and HCs were statistically significant for younger persons but were no more detectable in older subjects (<30 years of age: *p* = 0.034; 31–45 years of age: *p* = 0.799; >45 years of age: *p* = 0.348; Fig. 3C). The age-dependent loss of RTE-T<sub>reg</sub> in HCs is paralleled by a moderate increase of naive T<sub>reg</sub> lacking CD31 expression (<30 years of age: mean 5.7% of total FOXP3<sup>+</sup> cells, range 1.9–23.2%; >45 years of age: mean 8.6%, range 2.5–14.7%, *p* = 0.115; not depicted). Percentages of CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD31<sup>-</sup> within FOXP3<sup>+</sup> cells were also slightly higher in younger MS patients as compared with HCs (MS patients <30 years of age: mean 9.7%, range 0.7–19.4%; *p* = 0.107; not depicted). Proportions of CD45RA<sup>-</sup>CD45RO<sup>+</sup> memory T<sub>reg</sub> increased with age in HCs (<30 years of age: mean 60.6%, range 36.4–72.3%; >45 years of age: mean 78.6%, range 64.5–88.5%; *p* = 0.013; Fig. 3D) and were enhanced in younger MS patients. CD31 expression on memory T<sub>reg</sub> was consistently low in blood samples of both MS patients and HCs (data not shown).

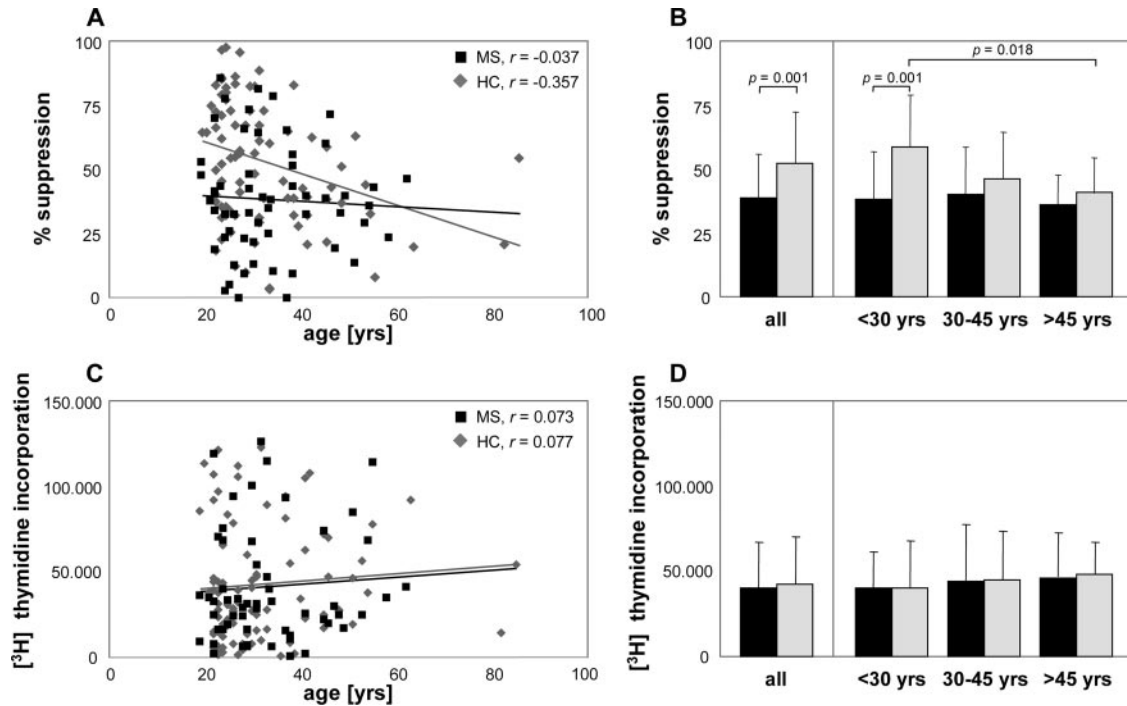
### TRECs are detectable in CD31<sup>+</sup> T<sub>reg</sub> but not in CD31<sup>-</sup> T<sub>reg</sub>

Because TRECs are highly enriched in RTEs (17) as a result of the accomplished TCR gene rearrangement process without further dilution due to proliferation (18), we studied the intracellular levels of TRECs in T<sub>reg</sub> and T<sub>eff</sub> subsets isolated from four HCs (mean age 29.0 years, range 26–41 years of age).

In concordance with our previous findings (10) total T<sub>reg</sub> had a very low TREC content (mean  $1.6 \times 10^3/10^6$  cells, range  $0.8$ – $2.5 \times 10^3/10^6$  cells), suggesting that the T<sub>reg</sub> pool in the periphery mainly consists of rapidly dividing memory T<sub>reg</sub> (Fig. 4). As expected, TRECs were virtually absent in the purified CD31<sup>-</sup> T<sub>reg</sub> subset predominantly exhibiting a memory phenotype (mean  $0.1 \times 10^3/10^6$  cells, range  $0.0$ – $0.1 \times 10^3/10^6$  cells). In contrast, mean frequencies of TRECs in CD31<sup>+</sup> T<sub>reg</sub> were substantially higher as compared with the entire T<sub>reg</sub> population ( $9.5 \times 10^3/10^6$  cells, range  $7.1$ – $11.6 \times 10^3/10^6$  cells; *p* < 0.001), reflecting the more naive status of this distinct T<sub>reg</sub> subset.

### T<sub>reg</sub> suppressive function is age dependent in healthy individuals

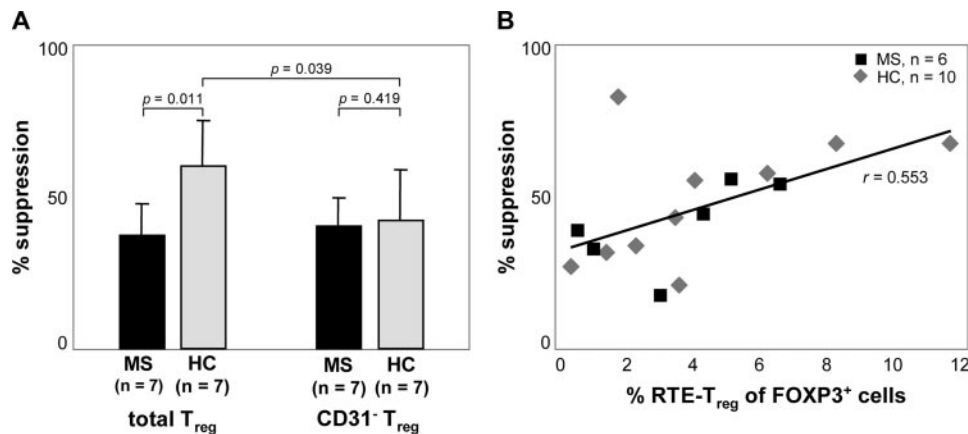
We evaluated the data of proliferation assays performed with T<sub>reg</sub> and T<sub>eff</sub> isolated from a total of 89 HC donors and 56 patients. Some were from the present study (HCs: *n* = 18; MS: *n* = 14),



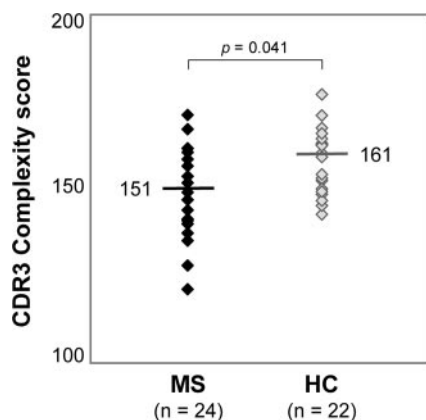
**FIGURE 5.**  $T_{reg}$  suppressive function is age-associated in healthy individuals. Inhibitory capacities of  $T_{reg}$  isolated from peripheral blood samples of 89 HCs and 56 MS patients were determined by in vitro proliferation assays with 1:4 coculture of  $2.5 \times 10^4 T_{reg}$  and  $10^5$  TCR-stimulated  $T_{eff}$ . *A*, Dot plots (filled squares, MS; gray diamonds, HCs) show the percentage of suppression in individual samples. The linear regression curves are shown.  $T_{reg}$ -mediated suppression declines with age in HCs but not in MS. *B*, Bars (black, MS; gray, HCs) denote the mean percentage of suppression per age group. Inhibitory capacities differ in the younger MS patients and the HCs but not in subjects of higher ages. *C*, Filled squares (MS) and gray diamonds (HCs) show  $T_{eff}$  proliferation (counts per minute) in individual samples. The linear regression curves demonstrate the age independence of proliferative responses in both HCs and MS patients. *D*, Bars (black, MS; gray, HC) denote that mean counts per minute also did not differ between different age groups of MS patients and HC.

others from subjects included in earlier studies (HCs:  $n = 71$ ; MS:  $n = 42$ ). Average ages of the entire cohort were 33.6 years (range 19–62 years; MS) and 32.4 years (range 19–85 years; HCs). All assays were conducted as a 1:4 coculture of  $2.5 \times 10^4 T_{reg}$  with  $10^5$  TCR-stimulated autologous  $T_{eff}$  and, similarly as in recent re-

ports (Refs. 8–10 and M. Korporal, M., J. Haas, B. Fritzsching, S. Möller, B. Fritz, C. S. Falk, P. H. Krammer, E. Suri-Payer, and B. Wildemann, submitted for publication), revealed mean  $T_{reg}$ -mediated suppressions of 38.4% in MS and 52.0% in HCs (MS: range 0.0–86.4%; HCs: range 4.1–98.4%; Fig. 5*B*). Interestingly,



**FIGURE 6.** Prevalence of RTE- $T_{reg}$  within the total  $T_{reg}$  population is critical for  $T_{reg}$  function. *A*,  $T_{reg}$  and  $T_{reg}$  subsets isolated from peripheral blood samples of seven patients and seven HCs were tested in parallel in vitro proliferation assays. *Left hand side*, Total  $T_{reg}$  derived from patients exhibited a reduced inhibitory activity (mean 37.5%, range 19.3–56.1%) when compared with HC  $T_{reg}$  (mean 60.0%, range 34.5–83.0%). In contrast, inhibition rates did not differ when using CD31-depleted  $T_{reg}$  obtained from the same patients and HCs (MS: mean 38.7%, range 25.9–59.0%; HCs: mean 42.6%, range 8.7–71.8%; *right hand side*). Thus, the difference in inhibitory potencies of MS  $T_{reg}$  and HC  $T_{reg}$ , detectable when using total  $T_{reg}$  in the coculture experiments, was neutralized by the depletion of CD31<sup>+</sup> cells. Bars denote mean percentage of suppression rates for  $T_{reg}$  (*left hand side*) and CD31-depleted  $T_{reg}$  (*right hand side*) obtained in parallel from seven MS patients (black) and seven HCs (gray). *B*, Percentage of inhibition and percentage of RTE- $T_{reg}$  obtained from six patients and 10 HCs were plotted against each other, revealing clear correlation between  $T_{reg}$  suppressive capacities and the prevalence of RTE- $T_{reg}$ .



**FIGURE 7.** TCR-diversity of  $T_{reg}$  is contracted in MS. TCR-diversity of  $T_{reg}$  isolated from 24 MS patients and 22 HCs was determined by CDR3 spectratyping. Mean complexity score of MS  $T_{reg}$  was significantly lower as compared with HC  $T_{reg}$ , suggesting a restricted clonality of  $T_{reg}$  in MS.

inhibitory capacities in younger HCs were higher as compared with persons of higher age (<30 years of age: mean 58.4%, range 10.7–98.4%; 30–45 years of age: mean 46.0%, range 3.9–89.5%,  $p = 0.026$ ; >45 years of age: mean 40.6%, range 21.2–59.6%;  $p = 0.018$ ; Fig. 5B). Such a decline of suppressive capacity was not seen in patients, who showed the same low suppressive capacity in all age groups (<30 years of age: mean 36.6%, range 0.0–86.4%; 30–45 years of age: mean 41.6%, range 0.0–81.9%; >45 years of age: mean 41.8%, range 8.5–63.7%; Fig. 5, A and B). Differences in  $T_{reg}$ -mediated suppression between MS patients and HCs were statistically significant for younger persons but were no more detectable in older subjects (<30 years of age:  $p = 0.001$ ; 30–45 years of age:  $p = 0.369$ ; >45 years of age:  $p = 0.520$ ). In contrast with the  $T_{reg}$ -mediated suppression rates, the  $T_{eff}$  proliferative responses were not age dependent in HCs and did not differ between MS patients and HCs (Fig. 5, C and D).

#### Depletion of CD31<sup>+</sup> $T_{reg}$ neutralizes the differences in $T_{reg}$ function between MS patients and healthy individuals

To determine whether the  $T_{reg}$  suppressive function correlates with the percentage of cells expressing CD31, we obtained  $T_{reg}$  and  $T_{reg}$  depleted of CD31<sup>+</sup> cells from seven patients (mean age 30.7 years) and seven HCs (mean age 28.7 years). Total  $T_{reg}$  and CD31<sup>-</sup>  $T_{reg}$  were tested in parallel in primary in vitro proliferation assays by 1:4 cocultures with  $10^5$  TCR-stimulated autologous  $T_{eff}$ . As shown above for the entire cohort, again total  $T_{reg}$  derived from patients exhibited a reduced inhibitory activity in vitro when compared with  $T_{reg}$  isolated from HCs (MS: mean 37.5%, range 19.3–56.1%; HCs, mean 60.0%, range 34.5–83.0%;  $p = 0.011$ , Fig. 6A). CD31<sup>-</sup>  $T_{reg}$  obtained from HCs exhibited a less potent inhibitory capacity toward activated  $T_{eff}$  with 42.6% (range 8.7–71.8%) suppression only (Fig. 6A). This inhibition rate was comparable to patient-derived CD31<sup>-</sup>  $T_{reg}$ , which inhibited  $T_{eff}$  proliferation to the same extent compared with total  $T_{reg}$  (38.7%, range 25.9–59.0%;  $p = 0.837$ ).

Thus, the difference in inhibitory potencies of patient and HC  $T_{reg}$  cells detectable when using total  $T_{reg}$  in the coculture experiments was neutralized by the depletion of CD31<sup>+</sup> cells.

From 16 subjects (MS:  $n = 6$ ; HCs:  $n = 10$ ) parallel data from both primary in vitro proliferation assays and multicolor flow cytometry were available. When the percentage of inhibition was plotted against the percentage of RTE- $T_{reg}$  a clear correlation between  $T_{reg}$  suppressive capacities and the prevalence of RTE- $T_{reg}$  was obtained ( $r = 0.553$ ; Fig. 6B).

#### TCR-diversity of $T_{reg}$ is contracted in MS

To analyze, whether an altered de novo generation of  $T_{reg}$  also influences the  $T_{reg}$  clonal distribution, we determined the CDR3 spectratype CS to compare the TCR diversity of  $T_{reg}$  from MS patients and HCs (Fig. 7). The mean CS of  $T_{reg}$  obtained from 22 HCs was 161 (range 138–185). CDR3 spectratyping of patient- $T_{reg}$  ( $n = 24$ ) resulted in lower CS values (mean 151, range 101–173). The difference of complexity scores between both study cohorts was statistically significant, with  $p = 0.041$ .

## Discussion

In previous work, we and others have demonstrated that  $T_{reg}$  from MS patients are functionally impaired (Refs. 8–10 and M. Korporal, M., J. Haas, B. Fritzsching, S. Möller, B. Fritz, C. S. Falk, P. H. Krammer, E. Suri-Payer, and B. Wildemann, submitted for publication). In the present study we provide evidence that the diminished suppressive  $T_{reg}$  potencies in MS are related to disequilibrium in the homeostatic composition of circulating  $T_{reg}$  and most likely result from an altered thymic release of newly formed T cells into the periphery. Because  $T_{reg}$  frequencies in peripheral blood are normal in MS patients as reported in a number of independent studies (Refs. 8–10 and M. Korporal, M., J. Haas, B. Fritzsching, S. Möller, B. Fritz, C. S. Falk, P. H. Krammer, E. Suri-Payer, and B. Wildemann, submitted for publication) and the susceptibility of patient-derived  $T_{reg}$  to undergo apoptotic cell death is also unaltered (11), we wondered whether any imbalance in T cell neogenesis contributes to the  $T_{reg}$  defect detectable in MS patients. The rationale to assess this issue in more detail was based on our earlier observation that the thymic export function and the ability to maintain T cell homeostasis is disturbed in MS (21). This may also affect  $T_{reg}$ , which is generated in the thymus as a naturally suppressive T cell subset. Kimmig et al. showed that only naive CD4<sup>+</sup>CD45RA<sup>+</sup> cells coexpressing CD31 harbor extrachromosomal deletion circles as traceable molecular markers of newly generated T lymphocytes and phenotypically define RTE (17). The prevalence of CD4<sup>+</sup>CD45RA<sup>+</sup>CD31<sup>+</sup> cells declines with age, reflecting the thymic-dependent neogenesis of this subset (17, 19). In the present work we confirmed these findings, because the proportion of CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD31<sup>+</sup> cells within the peripheral CD4<sup>+</sup>FOXP3<sup>-</sup> Th pool decreased with age whereas pure CD4<sup>+</sup> T cell numbers remained at constant levels. Moreover, the frequencies of CD4<sup>+</sup>FOXP3<sup>-</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD31<sup>+</sup> cells were significantly decreased in peripheral blood samples of MS patients, thus establishing the presence of an age-inappropriately reduced neogenesis of CD4<sup>+</sup>FOXP3<sup>-</sup> Th cells in MS. In concordance with our present and previous results, decreased levels of naive CD4<sup>+</sup> T cells in MS were also reported by Crucian et al. (29) and recently by Duszczyszyn and coworkers (30). To demonstrate that the altered Th homeostasis also affects the  $T_{reg}$  compartment, we analyzed the CD4<sup>+</sup>FOXP3<sup>+</sup> cells of patient and control-derived PBMCs. In striking contrast to the total  $T_{reg}$  quantities that were unaltered in MS, the subpopulation of naive  $T_{reg}$  coexpressing CD31 was markedly reduced in patient-derived peripheral blood specimens, with most prominent alterations in younger subjects. This  $T_{reg}$  subset encompasses up to 20% of the entire  $T_{reg}$  pool and is phenotypically defined by the expression of CD4, CD25, FOXP3, CD45RA, and CD31. CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD31<sup>+</sup>  $T_{reg}$  decline with age in healthy persons and, unlike their CD31<sup>-</sup> “memory- $T_{reg}$ ” counterparts, harbor TRECs. Therefore, they are most likely naive  $T_{reg}$  recently released from the thymus and are denoted here as “RTE- $T_{reg}$ .” The reduced de novo generation of RTE- $T_{reg}$  in MS occurred independently of clinical parameters,



including an expanded disability status scale score, disease duration, and number of relapses, and was compensated by higher proportions of CD45RA<sup>-</sup>CD45RO<sup>+</sup>FOXP3<sup>+</sup> cells resulting in a stable cell count of the total T<sub>reg</sub> population.

This shift from naive toward memory T<sub>reg</sub> is also detectable in healthy elder individuals. In MS patients the lower levels of RTE-T<sub>reg</sub> coincide with a moderately contracted T<sub>reg</sub> TCR Vβ repertoire. Together with our observation that the T<sub>reg</sub> inhibitory function seems to be reduced in age, which was also reported previously in a small number of healthy individuals (20), this raised the possibility that the prevalence of RTE-T<sub>reg</sub> critically affects total T<sub>reg</sub> function.

Accordingly, the comparative functional analysis of total T<sub>reg</sub> and T<sub>reg</sub> subsets revealed differing suppressive abilities. When assessing immunomagnetically separated total CD25<sup>high</sup> T<sub>reg</sub>, we found the expected functional defect in T<sub>reg</sub> obtained from MS patients. Interestingly, the difference in T<sub>reg</sub>-mediated suppression between patient and control-derived T<sub>reg</sub> was totally neutralized when T<sub>reg</sub> depleted of CD31<sup>+</sup> cells were used in the coculture assays. The absence of CD31<sup>+</sup> cells markedly reduced the inhibition of T cell proliferation mediated by donor T<sub>reg</sub> but had no effect on the suppressive function of patient T<sub>reg</sub>. Overall, there was a clear correlation between T<sub>reg</sub>-mediated suppression and the prevalence of RTE-T<sub>reg</sub> within the T<sub>reg</sub> pool. Taken together, this clearly indicates that CD31-expressing naive T<sub>reg</sub> contribute to the functional properties of the entire T<sub>reg</sub> population and further illustrates the obvious lack of RTE-T<sub>reg</sub> in MS patients.

Fritzsching et al. recently reported that human naive T<sub>reg</sub> are less sensitive to apoptotic cell death as compared with conventional T<sub>reg</sub> and suggested that naive T<sub>reg</sub> are in an earlier state of differentiation whereas “classic” memory T<sub>reg</sub> represent fully mature suppressor cells, which can be easily eliminated by apoptotic signals from conventional T cells (16). Moreover, in vitro expansion of human naive T<sub>reg</sub>, but not memory T<sub>reg</sub>, generates fully suppressive T<sub>reg</sub> clones as recently demonstrated (31). Therefore, although it seems counterintuitive, it is possible that also in vivo the naive or especially the CD31<sup>+</sup>CD45RA<sup>+</sup>T<sub>reg</sub> compartment exerts the most effective suppression. In contrast, preactivated T<sub>reg</sub> might be exhausted, prone to apoptosis, and less effective in suppression. This idea should be addressed in additional experiments in murine models.

Thus, we claim, that CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD31<sup>+</sup> T<sub>reg</sub> represent a group of immature, natural regulatory T cells recently released from the thymus that are characterized by normal suppressive potential and insensitivity against apoptotic elimination. A constant supply of RTE-T<sub>reg</sub> may be essential to compensate exhausted or eliminated memory T<sub>reg</sub> and to maintain full inhibitory capacity of the entire T<sub>reg</sub> compartment. Any down-regulation of RTE-T<sub>reg</sub> release in the context of physiological thymic atrophy or pathological processes may impair T<sub>reg</sub> homeostasis in the periphery and is likely to promote alterations in T<sub>reg</sub> function in magnitudes that are detectable in elderly individuals and also in MS patients. Concordant with homeostatic shifts within the T<sub>reg</sub> subset, Vukmanovic-Stejic et al. recently described a population of short-lived memory T<sub>reg</sub> induced from primed/memory CD4<sup>+</sup>CD25<sup>-</sup> T cells in the periphery that, via rapid peripheral turnover, is responsible for the maintenance of T<sub>reg</sub> numbers during aging (32).

The reconstitution of T<sub>reg</sub> homeostasis either by adoptive transfer of naive T<sub>reg</sub> or by modulation of thymic function should have the potential to restore the impaired T<sub>reg</sub> suppressive capacities in MS patients back to normal levels.

We have recently shown that prolonged treatment of MS patients with glatiramer acetate (GA) and IFN-β restores T<sub>reg</sub> func-

tion via a direct targeting of the T<sub>reg</sub> compartment (M. Korporal, M., J. Haas, B. Fritzsching, S. Möller, B. Fritz, C. S. Falk, P. H. Krammer, E. Suri-Payer, and B. Wildemann, submitted for publication). In addition to their anti-inflammatory effects (33, 34), immunomodulatory drugs, including GA, up-regulate numbers of naive T cells in MS patients (35, 36). Our own preliminary data indicate that RTE-T<sub>reg</sub> numbers increase under GA treatment (data not shown) and possibly suggest a direct effect of this agent on thymic function. Therefore, we believe that pharmacologic modulation of the thymus and the reconstruction of peripheral T<sub>reg</sub> homeostasis may trigger the augmentation of T<sub>reg</sub> inhibitory capacities and may be a promising treatment strategy in MS and other autoimmune disorders.

## Disclosures

The authors have no financial conflict of interest.

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