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Patients With Severe Multiple Sclerosis Exhibit Functionally Altered CD8⁺ Regulatory T Cells

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Abstract

Background and Objectives

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the CNS. Studies of immune dysfunction in MS have mostly focused on CD4⁺ Tregs, but the role of CD8⁺ Tregs remains largely unexplored. We previously evidenced the suppressive properties of rat and human CD8⁺CD45RC^{low/neg} Tregs from healthy individuals, expressing Forkhead box P3 (FOXP3) and acting through interferon-gamma (IFN- γ), transforming growth factor beta (TGF β), and interleukin-34 (IL-34) secretions to regulate immune responses and control diseases such as transplant rejection. To better understand CD8⁺CD45RC^{low/neg} Tregs contribution to MS pathology, we further investigated their phenotype, function, and transcriptome in patients with MS.

Methods

We enrolled adults with relapsing-remitting MS and age-matched and sex-matched healthy volunteers (HVs). CD8⁺ T cells were segregated based on low or lack of expression of CD45RC. First, the frequency in CSF and blood, phenotype, transcriptome, and function of CD8⁺CD45RC^{low} and ^{neg} were investigated according to exacerbation status and secondarily, according to clinical severity based on the MS severity score (MSSS) in patients with nonexacerbating MS. We then induced active MOG₃₅₋₅₅ EAE in C57Bl/6 mice and performed adoptive transfer of fresh and expanded CD8⁺CD45RC^{neg} Tregs to assess their ability to mitigate neuroinflammation in vivo.

Results

Thirty-one untreated patients with relapsing-remitting MS were compared with 40 age-matched and sex-matched HVs. We demonstrated no difference of CSF CD8⁺CD45RC^{low} and CD8⁺CD45RC^{neg} proportions, but blood CD8⁺CD45RC^{low} frequency was lower in patients with MS exacerbation when compared with that in HVs. CD8⁺CD45RC^{neg} Tregs but not CD8⁺CD45RC^{low} showed higher suppressive capacities in vitro in MS patients with exacerbation than in patients without acute inflammatory attack. In vitro functional assays showed a compromised suppression capacity of CD8⁺CD45RC^{low} Tregs in patients with nonexacerbating severe MS, defined by the MSSS. We then characterized murine CD8⁺CD45RC^{neg} Tregs and demonstrated the potential of CD45RC^{neg} cells to migrate to the CNS and mitigate experimental autoimmune encephalomyelitis in vivo.

Discussion

Altogether, these results suggest a defect in the number and function of CD8⁺CD45RC^{low} Tregs during MS relapse and an association of CD8⁺CD45RC^{low} Tregs dysfunction with MS severity. Thus, CD8⁺CD45RC^{low/neg} T cells might bring new insights into the pathophysiology and new therapeutic approaches of MS.

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Glossary

APCs = antigen presenting cells; **ARMSS** = age-related MS severity; **CFA** = complete Freund adjuvant; **CFSE** = carboxyfluorescein succinimidyl ester; **DAPI** = 4',6-diamidino-2-phenylindole; **EAE** = experimental autoimmune encephalomyelitis; **FACS** = fluorescence activated cell sorting; **FOXP3** = Forkhead box P3; **GA** = glatiramer acetate; **GVHD** = graft versus host disease; **HVs** = healthy volunteers; **IFN- γ** = interferon-gamma; **IL-34** = interleukin-34; **MAIT** = mucosal-associated invariant T cells; **MOG** = myelin oligodendrocyte glycoprotein; **MS** = multiple sclerosis; **MSSS** = MS severity score; **PBMC** = peripheral blood mononuclear cell; **PBS** = phosphate-buffered saline; **RRMS** = relapsing-remitting multiple sclerosis; **TCR** = T cell receptor; **TGF β** = transforming growth factor beta.

T cells play key roles not only in the defense against infectious agents and tumors but also in the pathogenesis of autoimmune diseases, including multiple sclerosis (MS). MS is a chronic inflammatory disease of the CNS affecting approximately 2.8 million people around the world and the first cause of non-traumatic acquired disability in adults.¹ Although considered as an autoimmune disease, MS complex pathophysiology has not been fully elucidated yet. However, it is widely acknowledged that immune cells infiltrate the CNS to cause demyelination and axonal injuries.^{2,3} CD4⁺ and CD8⁺ T cells have been considered as main mediators of MS neuropathology.² One hypothesis to explain the emergence of dysimmunity is the alteration of regulatory pathways, especially regulatory T cells (Tregs). Different populations of Tregs have been described as being capable of inducing tolerance; among them are CD4⁺CD25⁺Foxp3⁺ Tregs and CD8⁺CD45RC^{low/neg} Tregs.^{4,5} CD4⁺ Tregs distribution in CSF and blood, their dysfunction, and potential instability have been described in MS,⁶⁻⁸ and as such, CD4⁺ Tregs cell therapy is currently investigated to induce tolerance in transplantation and autoimmune diseases.⁹ While the role of CD8⁺ Tregs is now described in human and experimental autoimmune diseases,^{4,10,11} it is less well defined in MS. Many evidences point out a critical role for CD8⁺ T cells in MS pathophysiology. Indeed, T-cell infiltrate of active MS lesion is dominated by CD8⁺ T cells, and analysis of their repertoire skewing showed a clonal expansion of CD8⁺ T cells in the CNS not found in CD4⁺ T cells.¹² In mice, CD8⁺ T cells can have a regulatory role in experimental autoimmune encephalomyelitis (EAE), an animal model of autoimmune neuroinflammation used to investigate some aspects of MS. Some CD8⁺ subsets such as Qa-1-restricted CD8⁺ or CD8⁺CD28⁻ Tregs can prevent and/or ameliorate EAE, whereas CD8-deficient mice present a persistent EAE with more relapses.¹³⁻¹⁵ Besides, induction of myelin oligodendrocyte glycoprotein (MOG)-reactive CD4⁺ T cells initiates a counterbalancing wave of nonmyelin-specific regulatory CD8⁺ T cells.¹⁶ In humans, first evidences about the potential implication of CD8⁺ Tregs were supported by the fact that glatiramer acetate (GA)-treated patients, a therapy inducing CD8⁺ T-cell responses and GA-induced oligoclonal expansion of CD8⁺ Treg responses, showed beneficial effect on MS,^{17,18} contrary to CD4⁺ T cells.¹⁹ These findings raised the intriguing possibility that dysfunctional CD8⁺ Tregs responses account for the shift in immune tolerance associated with increased CNS-directed autoreactivity.

We have previously demonstrated the involvement of a population of CD8⁺CD45RC^{low/neg} Tregs in rat and human transplantation tolerance.²⁰⁻²² We evidenced the expression of FOXP3 by a fraction of CD45RC^{low/neg} cells and their suppressive properties involving IFN- γ , IL-10, TGF- β , and IL-34 secretion to regulate effector T-cell immune responses and control diseases,^{20,23-25} and we are now preparing a first-in-human clinical trial in transplanted patients (reshape-h2020.eu). Although, CD8⁺CD45RC^{low/neg} Tregs exhibit tolerogenic properties in transplantation, their role in autoimmune disease has not been addressed. In patients with MS, the precise involvement of CD8⁺ Tregs and the CD45RC^{low/neg} subset has not been determined yet. Thus, we hypothesized that CD8⁺CD45RC^{low/neg} Tregs function is impaired in patients with MS.

Methods

Human Blood and CSF Collection

Blood and CSF samples from untreated patients with relapsing-remitting MS, defined by a lack of immunomodulatory treatment for at least 6 months before blood sampling, from noninflammatory neurologic disease control patients, and from age-matched and sex-matched healthy volunteers were obtained from the MS center (Centre de Ressources et de Compétences Sclérose en Plaques Pays de La Loire) of the Nantes University Hospital (ABM PFS13-003 "Collection sclérose en plaques") (eTable 2, links.lww.com/NXI/A746). Diagnosis of MS was performed according to 2010 McDonald criteria. We classified patients with MS as "exacerbating" or "nonexacerbating" according to whether they were presenting with a relapse during blood sampling. Nonexacerbating patients had not experienced any MS flare-up for at least 3 months before blood sampling. We classified patients as exacerbating when presenting with a clinical relapse, hence new acute or aggravation of previous neurologic symptoms with a change of expanded disability status scale (EDSS), lasting at least 24 hours, after exclusion of any fever or other disorder causing the symptoms. Blood sampling was performed within 4 weeks after first symptoms and before any corticosteroid treatment or plasmapheresis.

Mice

C57BL6 and Balb/C (8–12 weeks old) mice were purchased from Janvier Labs. Foxp3-GFP founder reporter mice were kindly provided by Bernard Malissen. All animals were housed

in our own animal facilities. Cell isolation procedure and expansion are detailed in eMethods, links.lww.com/NXI/A746.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) and cultured cells were processed and stained as previously described²¹ and as detailed in eMethods, links.lww.com/NXI/A746. Flow cytometry data were acquired on a fluorescence activated cell sorting (FACS) Canto II flow cytometer (BD Biosciences), and FlowJo software was used to analyze data.

Suppression Assay

Human

Frozen PBMCs were thawed and then stained with antibodies against CD45RC, CD3, CD4, CD25, CD161, V α 7.2, and 4',6-diamidino-2-phenylindole (DAPI) (eTable 1, links.lww.com/NXI/A746). Four cell population were sorted using a BD FACSAria II (BD Biosciences): CD4 effector T cells (CD3⁺ CD4⁺ CD25⁻) and non-mucosal-associated invariant T cells (MAIT) CD8⁺CD45RC^{high} (CD3⁺CD4⁻CD161^{low/neg}V α 7.2^{low/neg}), CD8⁺CD45RC^{low} (CD3⁺CD4⁻CD161^{low/neg}V α 7.2^{low/neg}), and CD8⁺CD45RC^{neg} (CD3⁺CD4⁻CD161^{low/neg}V α 7.2^{low/neg}) subsets. Purity was >90% postcell sorting.

CD8⁺CD45RC subsets were stimulated overnight with coated anti-CD3 mAbs, soluble anti-CD28 mAbs (1 μ g/mL), and IL-2 (250 UI/mL; Proleukin, Novartis). CD4⁺CD25⁻ were stained with carboxyfluorescein succinimidyl ester (CFSE) (0.5 μ mol/L; ThermoFisher Scientific). 2.5 \times 10⁵ syngenic CD4⁺CD25⁻, referred to as "responders," were cocultured with CD8⁺CD45RC^{high} or CD8⁺CD45RC^{low} or CD8⁺CD45RC^{neg} in the presence of 2.5 \times 10⁵ allogenic-irradiated antigen presenting cells (APCs) (CD3-depleted PBMC pooled from 3 different HVs) at an increasing ratio of CD8⁺CD45RC cells in V-bottom 96-well plates in RPMI1640 medium with 5% heat-inactivated human AB serum for 5 days.

At day 5, cells were stained for CD3 and CD4 to assess CD4⁺CD25⁻ T cells proliferation on a Canto II (BD Biosciences). CFSE gates were drawn on FlowJo v.10 software according to a negative control well where only CD4⁺CD25⁻ T cells were cultured for each donor. Proliferation rates were normalized to the proliferation of a CD4⁺CD25⁻ T cells-APC positive control well for each donor. The average proliferation rate of CD4⁺CD25⁻ T cells was 51%.

Mouse

Fresh and expanded CD8⁺CD45RC^{neg} and CD4⁺CD25⁺ Tregs were labeled with CPD-V450 and tested for suppressive activity. In brief, Tregs were mixed with a set number of 5 \times 10⁵ CFSE labeled responder CD4⁺CD25⁻ T cells stimulated with a set number of 5 \times 10⁵ allogenic APCs at 1:1 to 1:32 Tregs:responders ratios. The proliferation of responder T cells was analyzed by flow cytometry after the exclusion of CPD-stained Tregs on CFSE dilution after 5 days of coculture in complete RPMI 1640 medium supplemented with 10% fetal calf serum.

3' Digital Gene Expression Sequencing (DGE-Seq)

PBMCs from 19 patients with relapsing-remitting MS (RRMS) and 12 HVs were thawed and then sorted using a BD FACS ARIAII (BD Biosciences). Doublets were removed based on lymphocyte morphology (forward scatter-wide, forward scatter-height, side scatter-wide, and side scatter-height). Living cells were selected based on DAPI staining. A CD3⁺/CD4⁻ gating strategy was used, and MAIT cells and natural killer T cells were excluded based on CD161, T cell receptor (TCR) V α 7.2, and CD56 expression. CD8⁺CD45RC^{high}, CD8⁺CD45RC^{low}, and CD8⁺CD45RC^{neg} were determined based on CD45RC expression. Purity was >90% postcell sorting.

Total RNA was isolated from sorted cells using RNeasy-Micro kit columns (Qiagen). Purified RNA was quantified using a Nanodrop Spectrophotometer (ThermoFischer Scientific) and was processed for 3'DGE-sequencing as previously described.²⁶ In summary, 10⁴ to 3 \times 10⁵ cells were used for library preparation. The RNAs sequencing library was processed by the GenoBIRD facility (SFR Bonamy, Nantes) for quality check (BioAnalyzer 2100; Agilent) and for paired-end 350–800 bp read sequencing on an Illumina HiSeq 2500 sequencer using a HiSeq Rapid SBS Kit (Illumina). Analyses were performed using RStudio v.1.1.477 (R v.3.3.1). The detailed procedure of DGE-seq analysis is available in Supplement.

EAE Induction and Treg Cell Administration

EAE in C57BL/6 female was induced by subcutaneous immunization in the flank with 200 μ g of MOG₃₅₋₅₅ peptide in complete Freund adjuvant containing 3.5 mg/mL of *Mycobacterium tuberculosis*. Pertussis toxin (200 ng intraperitoneally) was injected on days 0 and 2 postimmunization. Disease severity was monitored daily for EAE symptoms and scores (Cf Supplement). On the day of immunization, 2.5 to 5 \times 10⁵ fresh or 2.5 \times 10⁵ expanded CD8⁺ Tregs suspended in 100 μ L of phosphate-buffered saline (PBS) 1X were IV injected. Exposure to treatment was randomly assigned within a given cage leading to a mix of the animal groups in each individual cage. Scoring was performed independently by 2 researchers.

Standard Protocol Approvals, Registrations, and Patient Consents

All patients and HVs provided written informed consent in compliance with our University Hospital ethic committee. This study was approved by the University Hospital ethic committee of Nantes.

Mouse studies were performed according to license number APAFIS 25313 from the French ministry of Research. All animal experiments were conducted in accordance with guidelines established by our Institutional Animal Care.

Statistical Analysis

Statistical analyses and graphs were performed on GraphPad Prism v.7 software (GraphPad Software Inc) for flow cytometry results and on RStudio v.1.1.477 (R v.3.3.1) for DGE sequencing data. Data were represented as mean \pm SEM.

Data Availability

Anonymized data will be shared by request from any qualified investigator.

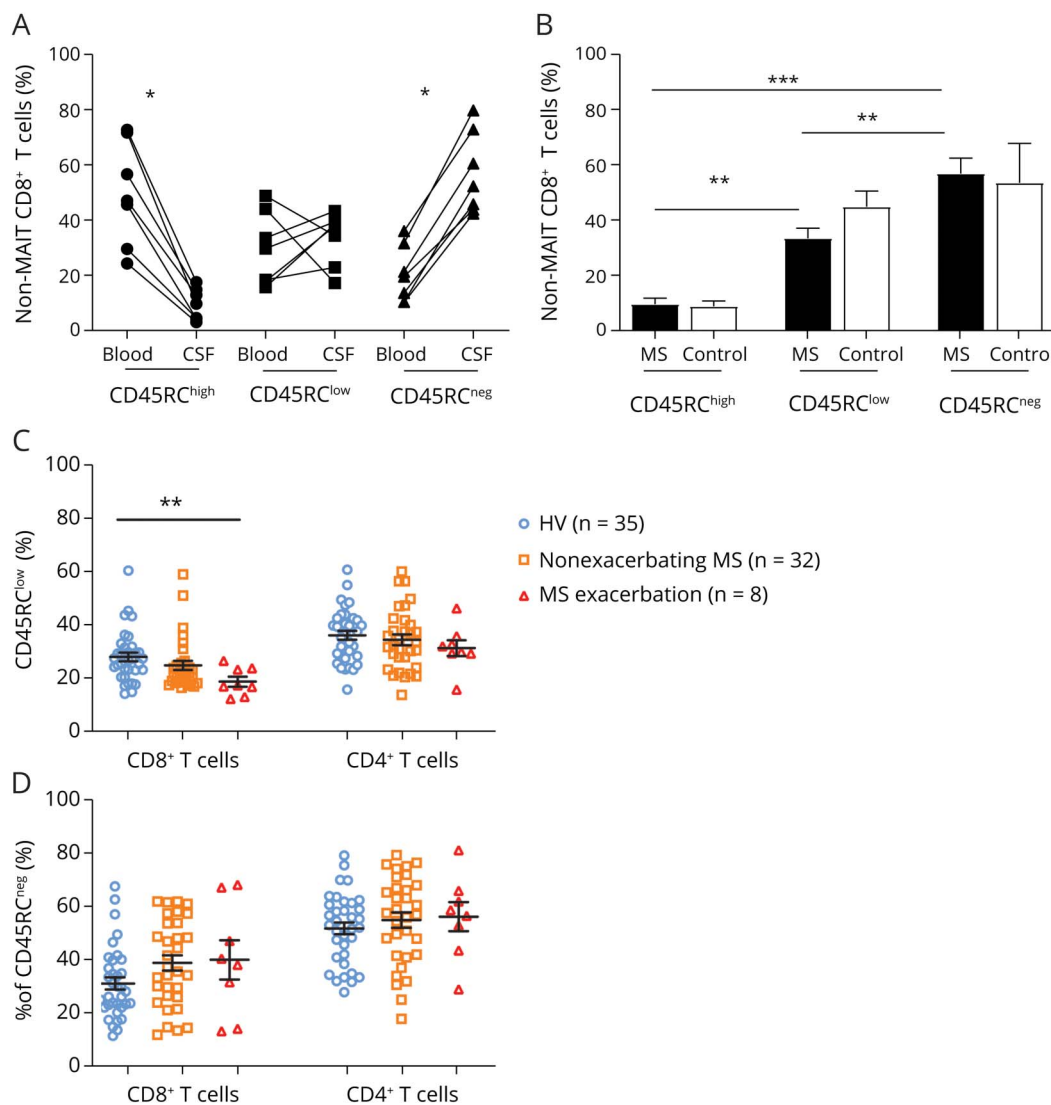
Results

The Proportion of Blood CD8⁺CD45RC^{low} Tregs, But Not CD4⁺CD45RC^{low} T Cells, Is Significantly Reduced During Exacerbation in Patients With Untreated MS

Because the CD45RC marker has never been analyzed in patients with MS while it allows the identification of suppressive CD8⁺

Tregs in human,^{5,21,27} we first assessed the frequency of this marker expressed by CD8⁺ T cells in blood and CSF of patients with MS vs control patients, i.e., patients experiencing non-inflammatory neurologic disease (Figure 1 and eTable 2, links.lww.com/NXI/A746). We excluded MAIT cells with a gating strategy of lived CD3⁺CD8⁺CD161[−]Vα7.2[−] and then divided non-MAIT CD8⁺ T cells based on CD45RC expression (eFigure 1A, links.lww.com/NXI/A746). Analysis of CD45RC subpopulations in blood vs CSF from patients with MS (Figure 1A) revealed significant differences in the pattern of CD45RC subsets with mostly the presence of CD45RC^{low} and CD45RC^{neg} cells, including memory and regulatory cells, in the CSF vs CD45RC^{high} cells that are mostly naive, Th1

Figure 1 Distribution of Blood and CSF CD8⁺ T Cells Depend on CD45RC Expression in Patients With Untreated MS and CD8⁺CD45RC^{low} Tregs Frequency Is Reduced in Blood During MS Exacerbation



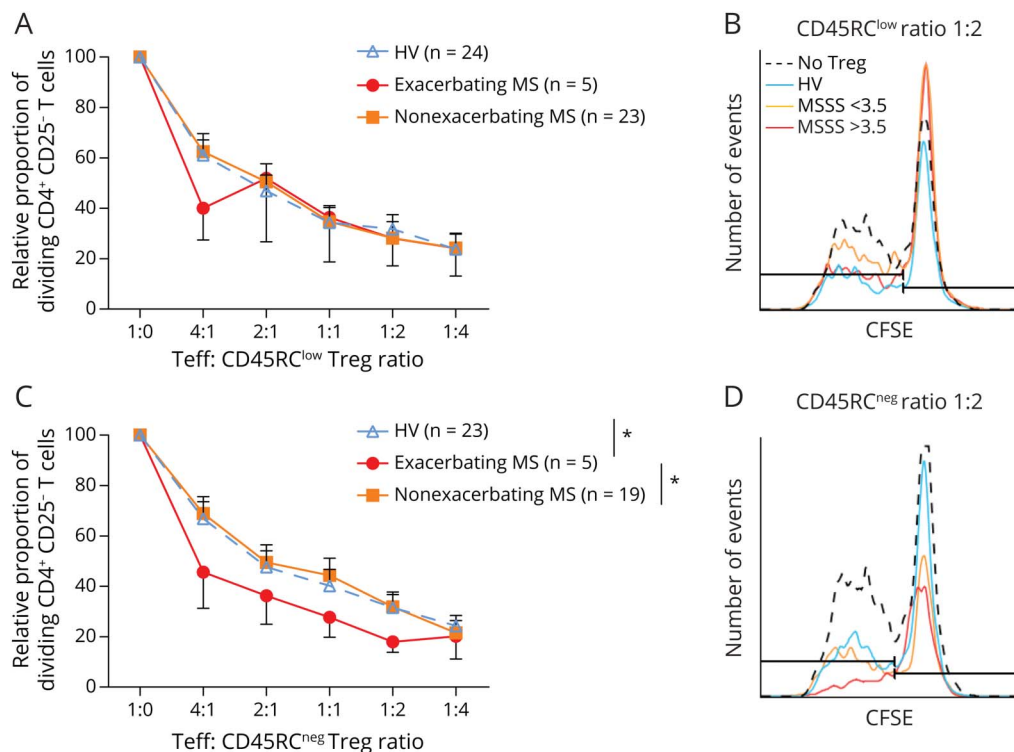
(A) Matched-paired frequency of CD8⁺ T cells excluding MAIT cells according to the CD45RC expression in blood vs CSF of patients with nonexacerbating MS (n = 7). Statistical analysis was performed using a Wilcoxon signed rank test. (B) Frequency of CD8⁺CD45RC subsets in CSF in patients with MS (n = 7) and noninflammatory neurologic controls (n = 4). Statistical analysis was performed using a Mann-Whitney test. (C, D) Frequency of blood CD45RC^{low} (C) and CD45RC^{neg} (D) in CD8⁺ and CD4⁺ T cells in HVs (n = 35), RRMS patients undergoing exacerbation (n = 8) or nonexacerbation (n = 32) was analyzed. Statistical analysis was performed using a Kruskal-Wallis test adjusted with Dunn post hoc test. *p < 0.05; **p < 0.01; ***p < 0.001. MS = multiple sclerosis; ns = nonsignificant.

precursors and Terminally Differentiated Effector Memory cells in the blood.²¹ These results seemed non-MS-specific because we observed a trend to a similar pattern of cell subset frequency in blood and CSF compartments in noninflammatory control patients (eFigure 1B, links.lww.com/NXI/A746). We found a similar frequency of blood (Figure 1A; eFigure 1B, links.lww.com/NXI/A746) and CSF (Figure 1B) CD45RC subsets between patients with MS and noninflammatory neurologic control patients. This suggests that CD45RC subset distribution in the blood and CSF is not affected by neuroinflammation but rather reflects T-cell differentiation status. We then analyzed the frequency of blood CD8⁺CD45RC^{low} (Figure 1C), CD8⁺CD45RC^{neg} (Figure 1D), and CD8⁺CD45RC^{high} (eFigure 1C, links.lww.com/NXI/A746) T cells and their CD4⁺ counterparts in patients with exacerbating and nonexacerbating MS compared with healthy volunteers. We observed that CD8⁺CD45RC^{low} Tregs were decreased in patients during exacerbation when compared with HVs, whereas CD4⁺CD45RC^{low} T cells were not modified (Figure 1C). CD8⁺ and CD4⁺ CD45RC^{neg} and CD45RC^{high} subsets were unaffected between MS patient groups and HVs (Figure 1D; eFigure 1C, links.lww.com/NXI/A746). Altogether, this suggests a reduced proportion of blood CD8⁺CD45RC^{low} Tregs during MS exacerbation.

Patients With Exacerbating MS Displayed Enhanced Ex Vivo Suppressive Function of CD8⁺CD45RC^{neg} T Cells

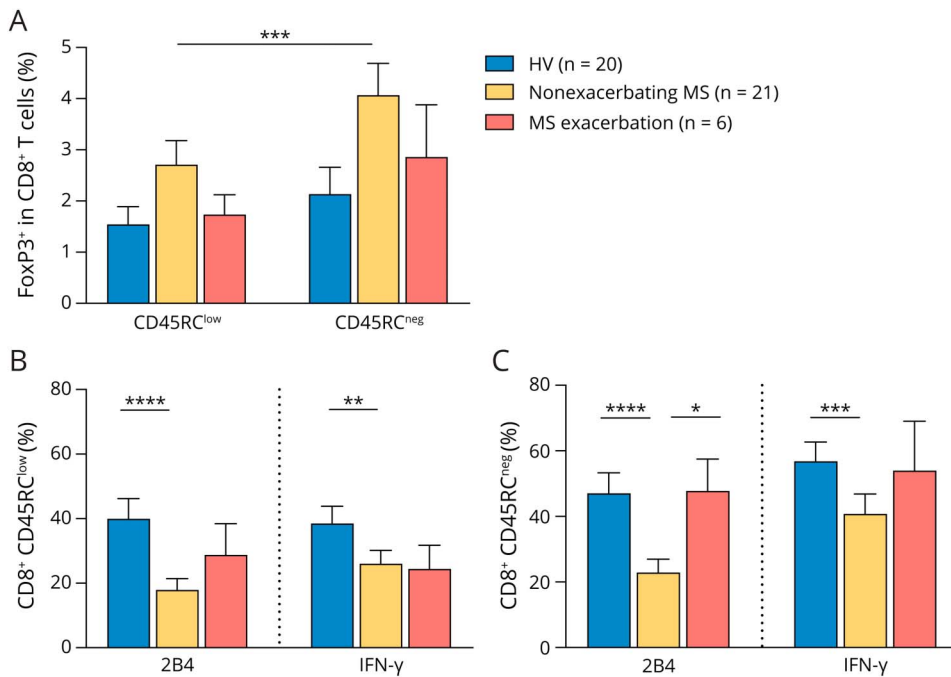
To further understand whether suppressive activity of CD8⁺ Tregs was affected in patients with exacerbating MS vs those with nonexacerbating MS compared with HVs, we performed suppressive assays where CD8⁺CD45RC^{low} or CD8⁺CD45RC^{neg} T cells were incubated with syngeneic CFSE-labeled CD4⁺ effector T-cell and allogeneic APCs for 5 days, as previously described^{21,22} (eFigure 2A, links.lww.com/NXI/A746). We observed that the proliferation of CD4⁺ effector T cell following an ascending concentration of CD8⁺CD45RC^{low} (Figure 2, A and B) or CD8⁺CD45RC^{neg} (Figure 2, C and D) from patients with nonexacerbating MS was not different when compared with HVs. However, a significantly higher suppressive ability of CD8⁺CD45RC^{neg} Tregs from patients undergoing exacerbation was observed when compared with nonexacerbating patients and HVs (Figure 2, C and D), but not for CD8⁺CD45RC^{low} Tregs (Figure 2, A and B). CD8⁺CD45RC^{high} T cells from HVs or patients with MS did not show any suppressive activity (eFigure 2B, links.lww.com/NXI/A746). This result suggests that CD8⁺CD45RC^{neg} Tregs suppressive function was not impaired in patients with MS but enhanced during ongoing neuroinflammation.

Figure 2 Enhanced Ex Vivo Suppressive Function of CD8⁺CD45RC^{neg} T Cells During MS Exacerbation



In brief, CD8⁺CD45RC^{low} and CD8⁺CD45RC^{neg} T cells and CD4⁺CD25⁻ Teff were sorted from the same individuals. Teff cells were labeled with CFSE and mixed with irradiated T-depleted PBMCs for 5 days and then analyzed for CFSE dilution. (A–D) Proliferation of CFSE-labeled Teff was assessed by flow cytometry after a 5-day culture with irradiated T-depleted PBMCs and an ascending ratio of CD8⁺CD45RC^{low} (A and B) or CD8⁺CD45RC^{neg} (C and D) T cells from HVs (blue, n = 23–24), RRMS patients undergoing exacerbation (red, n = 5) or nonexacerbation (orange, n = 19–23). Relative proportion of proliferating Teff cells normalized to proliferation in the absence of Treg (dashed black), in a range of Teff:Treg ratios. Mean ± SEM and representative histograms from the 1:2 Teff:Treg ratio of CD8⁺CD45RC^{low} (B) and CD8⁺CD45RC^{neg} (D) T cells are shown. Statistical analysis was performed using 2-way analysis of variance corrected for multiple comparison with a Bonferroni post hoc test. **p* < 0.05. MS = multiple sclerosis; Teff = effector T cells; Treg = regulatory T cells.

Figure 3 CD8⁺CD45RC^{low} and CD8⁺CD45RC^{neg} T Cells Own Different Signature in Patients With MS at Baseline and During Exacerbation



(A) Expression of Foxp3⁺ cells was assessed by flow cytometry in CD8⁺CD45RC^{low} and CD8⁺CD45RC^{neg} T cells in both HV (blue), nonexacerbating (orange) and exacerbating (red) MS patients within CD3⁺CD8⁺CD161^{low/neg}TCRVα7.2^{low/-} cells. Results are shown as mean ± SEM on a summary bar plot graph. Statistical analysis was performed using a Wilcoxon matched-pair signed rank test. (B, C) 2B4 (CD244) and IFN-γ expression were analyzed by flow cytometry, after 5 hours of PMA-inomycin stimulation for IFN-γ analysis and without stimulation for 2B4, in CD8⁺CD45RC^{low} (B) and CD8⁺CD45RC^{neg} T cells (C) of HVs (blue), nonexacerbating (orange) and exacerbating (red) MS patients. Results are shown as mean ± SEM on a summary bar plot graph. Statistical analysis was performed using 2-way analysis of variance corrected for multiple comparison with a Bonferroni post hoc test. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. HV = healthy volunteers; MS = multiple sclerosis; PMA = phorbol myristate acetate.

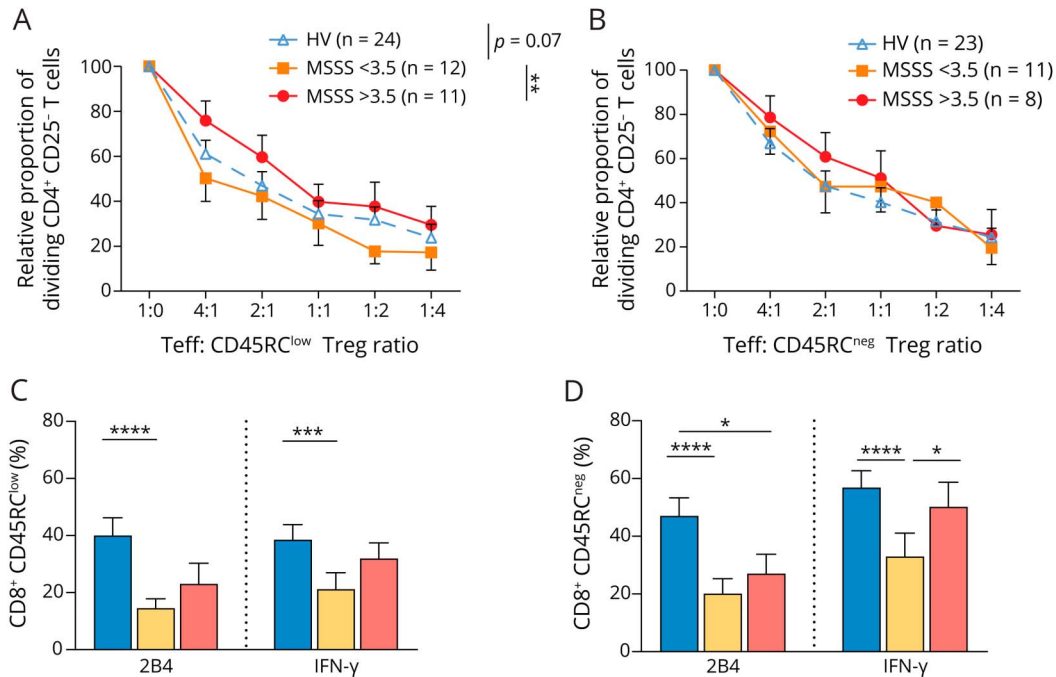
We next analyzed Foxp3 and other markers expressed by both CD8⁺CD45RC^{low} and CD8⁺CD45RC^{neg} Tregs during MS exacerbation (Figure 3; eFigure 3 and 4, links.lww.com/NXI/A746). Of interest, we observed a significant higher frequency of FOXP3⁺ by CD8⁺CD45RC^{neg} Tregs vs CD8⁺CD45RC^{low} Tregs only in patients with nonexacerbating MS. We did not observe any significant difference of FOXP3 expression in patients with exacerbating MS (Figure 3A; eFigure 3A, links.lww.com/NXI/A746). The overall expression of FOXP3 was restricted to a small fraction of circulating CD8⁺ Tregs, in both CD45RC^{low} and CD45RC^{neg}, as previously described^{21,22} (Figure 3A; eFigure 3A, links.lww.com/NXI/A746). Further analysis of markers involved in T-cell function and differentiation status did not reveal major differences between HVs, patients with nonexacerbating MS, and patients with exacerbating MS for both CD8⁺CD45RC^{low} Tregs (Figure 3B; eFigure 3B and C; eFigure 4A and B, links.lww.com/NXI/A746) and CD8⁺CD45RC^{neg} Tregs (Figure 3C; eFigure 3D and E; eFigure 4C and D, links.lww.com/NXI/A746). However, we observed for both CD8⁺ Treg subsets a significant downregulation of the inhibitory receptor 2B4 (CD244) and a downregulation of IFN-γ, a cytokine critical for the suppressive activity of CD8⁺CD45RC^{neg} Tregs, in patients with nonexacerbating MS (Figure 3, B and C; eFigure 3 B–E, links.lww.com/NXI/A746). Apart from 2B4 expression, which was higher in CD8⁺CD45RC^{neg} Tregs during MS exacerbation when compared with that in patients with nonexacerbating MS, we observed no association between the gain of function of CD8⁺CD45RC^{neg} Tregs in patients with exacerbating MS vs HVs and patients with nonexacerbating MS and their phenotype.

Severity of MS Disease Correlates With Altered Ex Vivo Suppressive Function of the CD8⁺CD45RC^{low} Treg Subset

To further understand whether CD8⁺ Treg function was altered in MS, we then further focused on patients with nonexacerbating MS and investigated the frequency and function of both CD8⁺ Treg subsets according to the MS severity score (MSSS),^{28,29} a clinical score representing disability and disease duration. We split our cohort of patients with nonexacerbating MS according to the median and mean MSSS of the cohort into 2 groups, low MSSS (<3.5, n = 12) and high MSSS (>3.5, n = 11) (eTable 2, links.lww.com/NXI/A746).

We observed that CD45RC subsets frequencies were not different between MS patient groups (low and high MSSS) and HVs within CD8⁺ or CD4⁺ T cells (eFigure 5A and B, links.lww.com/NXI/A746). Analysis of suppression of the proliferation of stimulated CD4⁺ effector T cells following an ascending concentration of CD8⁺CD45RC^{low} (Figure 4A; eFigure 5C, links.lww.com/NXI/A746) or CD8⁺CD45RC^{neg} (Figure 4B; eFigure 5D, links.lww.com/NXI/A746) T cells from low MSSS and high MSSS untreated nonexacerbating MS patients was not different when compared with that from HVs. However, CD8⁺CD45RC^{low} Tregs from patients with severe MS (MSSS>3.5) were less suppressive than those from patients with less severe MS (MSSS<3.5, *p* < 0.01) (Figure 4A; eFigure 5C, links.lww.com/NXI/A746). This difference was specific of the CD8⁺CD45RC^{low} Treg subset because there was no difference of suppressive function for the CD8⁺CD45RC^{neg} Tregs between the severe MS group, the milder form group,

Figure 4 Ex Vivo Suppressive Function of CD8⁺CD45RC^{low} T Cells Is Altered in MS Depending on the Severity of Disease But Not for CD8⁺CD45RC^{neg} T Cells



Patients with MS were assigned to groups according to MS severity based on an MSSS less than (orange) or greater than (red) 3.5 and in HVs (blue). (A, B) Proliferation of CFSE-labeled CD4⁺CD25⁻ T cells (Teff) was assayed by flow cytometry after a 5-day culture with irradiated T-depleted PBMCs and an ascending ratio of (A) CD8⁺CD45RC^{low} (n = 23) or (B) CD8⁺CD45RC^{neg} (n = 19) T cells from untreated nonexacerbating RRMS patients subdivided on disease severity using MSSS when compared with HVs (n = 24 and n = 23, respectively; blue). Statistical analysis was performed using 2-way analysis of variance corrected for multiple comparison with a Bonferroni test. Mean ± SEM. (C, D) 2B4 (CD244) and IFN-γ were analyzed by flow cytometry, after 5 hours of PMA-inomycin stimulation for IFN-γ analysis and without stimulation for 2B4, in CD8⁺CD45RC^{low} (C) and CD8⁺CD45RC^{neg} T cells (D) of HVs (blue) and MSSS <3.5 (orange) and MSSS >3.5 (red) MS patients. Results are shown as mean ± SEM on a summary bar plot graph. Statistical analysis was performed using 2-way analysis of variance corrected for multiple comparison with a Bonferroni test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. HV = healthy volunteers; MSSS = MS Severity Score; MS = multiple sclerosis; PMA = phorbol myristate acetate; Teff = effector T cells; Treg = regulatory T cells.

and HVs (Figure 4B; eFigure 5D, links.lww.com/NXI/A746). Again, CD8⁺CD45RC^{high} T cells were not capable of suppression (eFigure 2C, links.lww.com/NXI/A746). Activation markers, exhaustion markers, cytokines, and markers associated with Tregs were then analyzed in CD8⁺CD45RC^{low} (Figure 4C; eFigure 5E and F, eFigure 6A and B, links.lww.com/NXI/A746) and CD8⁺CD45RC^{neg} T cells (Figure 4D; eFigure 5G and H, eFigure 6C and D, links.lww.com/NXI/A746) of patients with MS at baseline according to clinical severity and compared with those of HVs. Consistent with our findings (Figure 3, B and C), we also observed here a significant downregulation of the inhibitory receptor 2B4 and of IFN-γ in patients with less severe MS (MSS <3.5) vs HVs for both CD8⁺ Treg subsets and a lower expression of IFN-γ in patients with less severe MS when compared with patients with severe MS (Figure 4, B and C; eFigure 5E–H, links.lww.com/NXI/A746).

To further confirm our results, we also analyzed the suppressive ability of CD8⁺CD45RC^{low} cells according to another severity ranking score: the age-related MS severity (ARMSS), which ranks the EDSS³⁰ based on patient's age during assessment³¹ (eFigure 7A, links.lww.com/NXI/A746). We also analyzed the data according to the EDSS, the duration of the disease, and the age of the patients (Table 1; eFigure 7B–D and eTable 3,

links.lww.com/NXI/A746). We observed that the suppressive ability of CD8⁺CD45RC^{low} T cells ex vivo was significantly lower in patients with MS with a moderate or severe disability (EDSS >2) than in patients with milder symptoms

Table 1 Correlation of CD4⁺ Effector T-Cell Proliferation in Mixed Lymphocyte Reaction Assays in the Presence of CD45RC^{low}CD8⁺ Tregs With Disease Severity Score MSSS, EDSS, Duration of Disease, and Age

Correlation	Ratio 1:4	MSSS	EDSS	ARMSS	Age
MSSS	Ns	ns	—	—	—
EDSS	Ns	r = 0.76 ^b	—	—	—
ARMSS	r = 0.55 ^a	r = 0.93 ^b	r = 0.85 ^b	—	—
Age	Ns	ns	r = 0.38 ^a	ns	—
Duration of disease	ns	ns	ns	ns	r = 0.712 ^b

Abbreviations: ARMSS = Age-Related MS Severity Score; EDSS = expanded disability status scale; MSSS = MS severity score.

r = Pearson correlation coefficient.

^a p < 0.05.

^b p < 0.001.

(EDSS ≤ 2) and HVs (eFigure 7A, links.lww.com/NXI/A746). CD8⁺CD45RC^{low} T cells from patients defined by an ARMSS less than 4.8, median of the cohort, are more potent suppressors than the one from patients with a higher ARMSS score and HVs (eFigure 7B, links.lww.com/NXI/A746), and suppressive capacity was significantly correlated with ARMSS (Table 1). To note, neither duration of disease using a threshold of 6 years corresponding to the median of disease length in the cohort (eFigure 7C, links.lww.com/NXI/A746) nor age using a threshold of 35 years, median of the cohort (eFigure 7D, links.lww.com/NXI/A746), by themselves affected CD8⁺CD45RC^{low} Treg-mediated suppression. Altogether, this shows a different signature of CD8⁺CD45RC^{low} and CD8⁺CD45RC^{neg} T cells in patients with MS depending on disease severity.

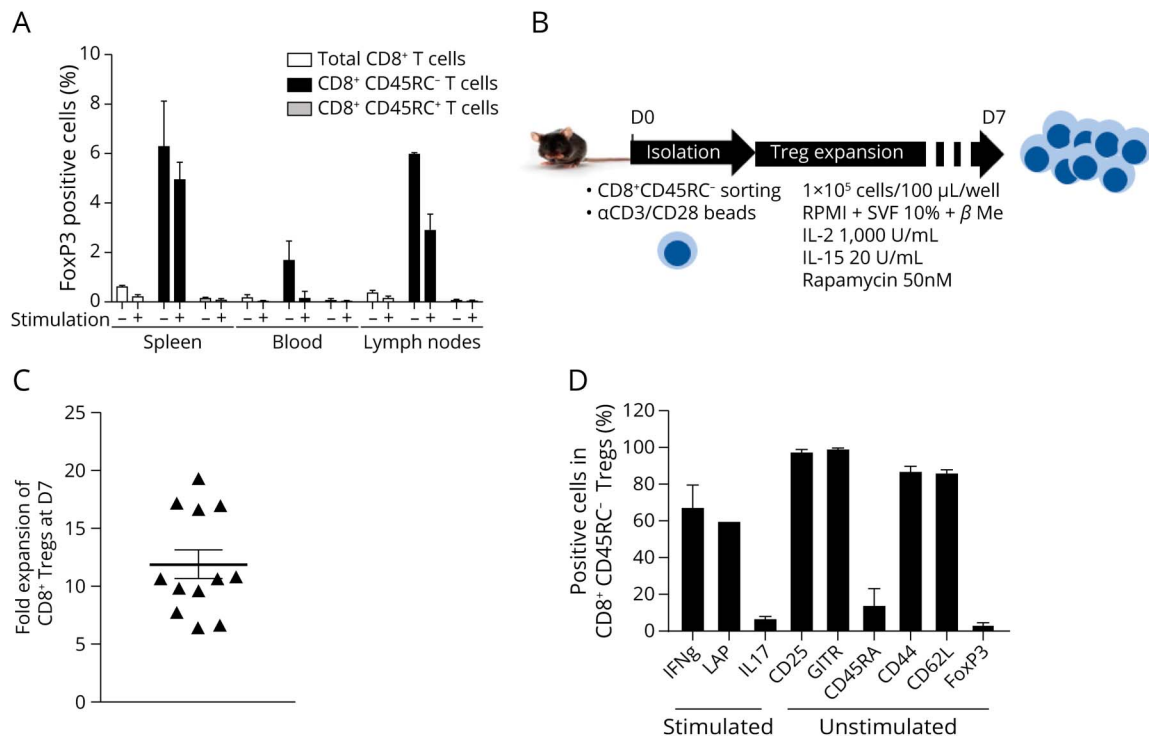
Finally, because a decreased functionality of CD8⁺CD45RC^{low} Tregs was associated with MS disease severity, we further investigated the transcriptome profiling of CD8⁺CD45RC^{low} Tregs by DGE sequencing (eFigure 8A and eTable 3, links.lww.com/NXI/A746). Transcriptomic analysis of FACS-sorted CD8⁺CD45RC^{low} from HVs and patients with MS with a MSSS <3.5 vs MSSS >3.5 demonstrated that autoimmune genes were overrepresented in CD45RC^{low} from patients with MS with a MSSS >3.5 vs MSSS <3.5, whereas signaling pathways related to the regulation of inflammatory responses and

IFN type-I response were overrepresented in CD45RC^{low} of less severe patients (eFigure 8B, links.lww.com/NXI/A746). In addition, gene set enrichment analysis of CD8⁺CD45RC^{low} MSSS >3.5 vs HVs showed a positive enrichment of genes involved in autoimmunity, regulation of IFN- γ signaling, major histocompatibility complex class I-mediated presentation and cross-presentation in MSSS >3.5 patients (eFigure 8C, links.lww.com/NXI/A746). Differential gene expression did not reveal significant differences of individual gene expression between MS groups for Treg subsets (data not shown). We then made use of linear regression model to analyze the correlation of transcripts with MS severity. Among transcripts correlating with MSSS in CD8⁺CD45RC^{low} T cells, genes associated with T-cell activation, recruitment, and CD4 Treg function such as *Nfkb1*,³² *DPP4* (CD26),³³ and *PDE8A*³⁴ showed lower expression levels in patients with more severe MS (eTable 3, links.lww.com/NXI/A746).

Expanded Mouse CD8⁺CD45RC^{neg} Tregs Showed a Selective Regulatory Gene Expression

To better understand whether CD8⁺ Tregs could modulate in vivo CNS autoimmune responses and offer potential therapeutic perspectives in MS, as we have previously shown for human transplant rejection and graft versus host disease (GVHD),^{21,22} we first characterized CD8⁺ Tregs in mouse based on CD45RC

Figure 5 Phenotype of Mouse CD8⁺ CD45RC⁻ Tregs Before and After Expansion



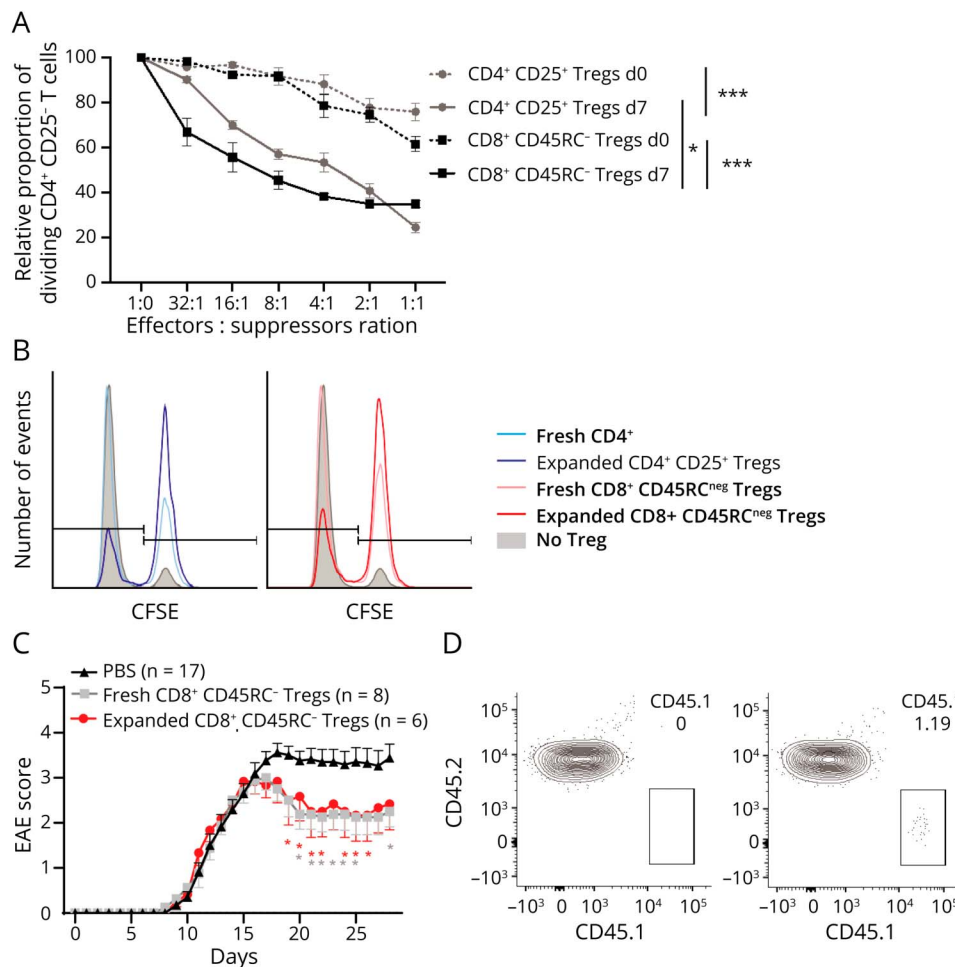
(A) Ex vivo CD8⁺, CD8⁺ CD45RC⁻, and CD45RC⁺ T cells were isolated from the spleen, blood, and lymph nodes of Foxp3-transgenic mice and analyzed for FoxP3 expression without or with PMA-ionomycin stimulation (n = 3–4). Left, mean \pm SEM. Right, representative dot plot of FoxP3 and CD45RC staining in CD8⁺ T cells. (B) Schematic depicting CD8⁺CD45RC⁻ T cell expansion. In brief, CD8⁺ Tregs were isolated, stimulated, and expanded with anti-CD3/CD28 mAb-coated beads, IL-2, IL-15, and rapamycin for 7 days. (C) Expansion fold of CD8⁺ Tregs from day 0 plating to day 7 harvesting (n = 12). Mean \pm SEM. (D) Expanded CD8⁺ CD45RC⁻ Tregs were analyzed for the expression of activation and exhaustion markers or cytokines and Treg-associated markers after a 4-hour PMA-ionomycin stimulation when indicated. The mean of percentage of expression \pm SEM. LAP = latency-associated peptide.

and FOXP3 expression. Unlike in humans, we observed that expression of FOXP3 in mouse was restricted to the CD8⁺CD45RC⁻ T-cell subset in the spleen, blood, and lymph nodes (Figure 5A; eFigure 9A and B, links.lww.com/NXI/A746). Thus, with the aim of cell therapy, we expanded mouse CD8⁺CD45RC⁻ T cells as previously described³⁵ (Figure 5B) and assessed their phenotype and suppressive property in comparison with CD4⁺CD25⁺ Tregs and fresh CD8⁺CD45RC⁻ T cells. Accordingly to our previous observation,³⁵ expansion of CD8⁺CD45RC⁻ T cells in the presence of high-dose IL-2, low-dose IL-15, and rapamycin led to approximately 12-fold expansion in 7 days (Figure 5C). We next performed flow cytometry analysis of various cell surface markers, intracellular cytokines, and transcription factor to determine the phenotype of expanded CD8⁺CD45RC⁻ Tregs (Figure 5D; eFigure 9C, links.lww.com/NXI/A746). Phenotypic analysis using CD44 and CD62L markers identifying naive and different memory subsets showed that expanded CD8⁺CD45RC⁻ Tregs comprised mostly memory cells (approximately 80% CD44⁺CD62L⁺), whereas fresh CD8⁺CD45RC⁻ Tregs were mostly naive and effectors cells (approximately 35% of CD44^{low}CD62L⁺ naive cells, 35% of CD44⁺CD62L^{low} effector cells, and 10% of CD44⁺CD62L⁺

memory cells) (data not shown). In addition, FoxP3 and CD25 expression were higher in expanded CD8⁺CD45RC⁻ Tregs compared with fresh CD8⁺CD45RC⁻ Tregs (although FoxP3 remained low in contrast to expanded human CD8⁺CD45RC^{low/neg} Tregs²¹). Expanded CD8⁺CD45RC^{neg} Tregs expressed key molecules involved in the function of Tregs cells such as IFN- γ and GITR. We then assessed in vitro fresh and expanded CD8⁺CD45RC⁻ Tregs suppressive function in comparison with fresh and expanded CD4⁺CD25⁺ Tregs in a coculture assay where increasing concentrations of Tregs were added to CFSE-labeled CD4⁺CD25⁻ effector T cells stimulated by allogeneic APCs (Figure 6, A and B). Of interest, we observed an equivalent suppressive capacity of fresh and expanded CD8⁺CD45RC⁻ Tregs in comparison with fresh and expanded CD4⁺CD25⁺ Tregs, respectively. In addition, as previously described with human Tregs, we also observed a significantly improved suppressive capacity of expanded Tregs vs fresh Tregs.

Finally, we assessed the potential of fresh or expanded CD8⁺CD45RC⁻ Tregs as cell therapy to decrease the EAE symptoms (Figure 6C). At day 0 postimmunization, 2.5 to 5 $\times 10^5$ of fresh or 2.5 $\times 10^5$ expanded CD8⁺CD45RC⁻ Tregs or PBS

Figure 6 In Vitro and In Vivo Function of Mouse CD8⁺ CD45RC⁻ Tregs Before and After Expansion



(A) Suppressive activity of fresh (d0) and expanded (d7) CD8⁺CD45RC⁻ T cells compared with CD4⁺CD25⁺ Tregs on proliferation of syngenic CFSE-labeled CD4⁺CD25⁻ T cells stimulated with allogeneic APCs, in a range of effectors: suppressors ratio. Proliferation was normalized to proliferation in the absence of Tregs. (B) Representative histograms of Teff proliferation in the absence (filled gray) or presence of fresh or expanded CD4⁺ or CD8⁺ Tregs at ratio Teff:Treg 1:1. (C) PBS (black triangle), fresh CD8⁺ Tregs (2.5–5 $\times 10^5$ cells/mouse, gray square) or 7-day expanded CD8⁺ Tregs (2.5 $\times 10^5$ cells/mouse, red circle) were injected i.v. into BL6 mice the day of immunization with MOG 35–55 peptide in CFA in the flank. Each mouse received 200 ng of pertussis toxin on days 0 and 2 post-immunization and were scored daily for 28 days. Data are shown as the mean clinical score of disease for each group \pm SEM. The Mann-Whitney test was applied for each day, * $p < 0.05$, red * for expanded, gray * for fresh Tregs compared with PBS condition. (D) In the EAE model, CD45.2 mice were injected with PBS or 2.5 $\times 10^5$ CD45.1 CD8⁺ Tregs. The CNS infiltrates were analyzed by flow cytometry for CD45.2 and CD45.1 markers. * $p < 0.05$; *** $p < 0.001$. CFA = complete Freund adjuvant; EAE = experimental autoimmune encephalomyelitis.

were administered intravenously to C57BL/6 mice. Although fresh or expanded CD8⁺CD45RC⁻ Tregs did not prevent EAE, we observed a significantly long-term lower cumulative EAE score in mice that received fresh or expanded CD8⁺CD45RC⁻ Tregs at day 0 compared with EAE mice injected with PBS, suggesting that adoptive transfer of Tregs by systemic injection reduced EAE severity. In addition, we were able to detect the CD8⁺CD45RC⁻ Tregs infiltrating the CNS (Figure 6D).

Discussion

In this study, we revealed that subsets of CD8⁺ T cells with regulatory abilities play a critical role in autoimmune neuroinflammation. Although a potential role for CD8⁺ Treg was evoked, our study demonstrates for the first time an association between the disease severity of patients with MS and the functionality of CD8⁺ Tregs. Despite that the lack of universal marker of CD8⁺ Tregs makes their study very challenging, several reports highlight a suspected contribution of CD8⁺ Tregs in EAE and MS. In active EAE murine models, mice develop a CD8-dependent resistance to subsequent immunization after induction.¹⁴ This vaccination effect has been linked to Qa-1 antigen-specific CD8⁺ T cell developing after the first immunization.¹⁴ Of interest, GA, a disease-modifying therapy used in MS, has been demonstrated to induce and potentiate human leucocyte antigen E- (the human counterpart to murine Qa-1) restricted CD8⁺ Treg.¹⁸ Their suppression was dependent of indoleamine 2,3-dioxygenase, IFN- γ , IL-10, and perforin in EAE.³⁶ A decrease in the frequency of CD25⁺FOXP3⁺CD8⁺ Tregs in the blood and CSF of patients with RRMS during exacerbation was reported. These cells were expressing CD122, glucocorticoid-induced TNFR family related protein (GITR), C-C chemokine receptor type 7, CD62L, and FOXP3 and exhibited suppressive abilities mediated through TGF- β , IL-10, and cytotoxic T-lymphocyte-associated protein 4 and induction of indoleamine 2,3-dioxygenase in dendritic cells.³⁷ A distinct population of CD8⁺ T cells regulating aberrant immune responses was identified based on their antigen specificity. Indeed, adoptive transfer of neuroantigen-specific CD8⁺ T cells was able to attenuate EAE through killing autoreactive CD4⁺ T cells in a major histocompatibility complex class I dependent way. However, these cells did not act through Qa-1 recognition, supporting a distinct cytotoxic-mediated suppressive role of CD8⁺ T cells associated with antigen specificity.³⁸⁻⁴⁰ Altogether, these previous findings support a role of CD8⁺ T cells in the regulation of EAE and MS responses, but if the above described subsets share some common features, their discrepancies render the identification of a unique subset of CD8⁺ Tregs challenging. We found that CD45RC^{low/neg} defined 2 distinct CD8⁺ Treg subsets playing a different role in MS. Indeed, we did not observe alteration of the distribution of CD8⁺CD45RC^{neg} Tregs in the blood and CSF of patients with MS when compared with controls during exacerbations and at baseline. Moreover, their suppressive function was enhanced during exacerbation and unaltered at baseline regardless of MS severity. Conversely, CD8⁺CD45RC^{low} Tregs blood proportion

was reduced during MS exacerbation, hence acute CNS inflammation, and unlike CD8⁺CD45RC^{neg} Tregs, their *in vitro* suppressive function was not enhanced. Besides, in severe forms of the disease, we observed a defect in CD8⁺CD45RC^{low} suppression compared with milder MS forms. These findings support first, the existence of 2 different CD8⁺ Treg subsets based on CD45RC expression in patients with MS and second, an alteration of the CD8⁺CD45RC^{low} Tregs at both the inflammatory and quiescent phases in MS. Although we observed a similar phenotype in patients with MS and controls in both CD8⁺ Treg subsets, transcriptomic analysis revealed an enrichment of genes associated with autoimmunity and an underrepresentation of genes associated with immune regulation in CD8⁺CD45RC^{low} Tregs of patients with severe MS. Whether the defect in CD8⁺CD45RC^{low} Tregs contributes to MS pathogenesis or results from severe immune dysfunction remains to be elucidated. Owing to a cross-sectional study design, we cannot rule out that observed differences could reflect patient specificity rather than disease state-specific. Yet we segregated patients in groups based on clinical activity, and we used a longitudinal severity scoring to minimize disease individual specificity. Longitudinal studies of recently diagnosed patients with untreated MS would help gaining more insights into CD8 Tregs dysfunction in MS. However, evidence of CD8⁺ Tregs capacities in regulating pathogenic immune responses in autoimmune neuroinflammation and other models underlines their putative contribution to suppress aberrant immune responses in the context of MS. In this regard, we demonstrate in this study that expanded CD8⁺ Tregs can be used as a cell therapy to dampen autoimmune neuroinflammation. *Ex vivo* amplification of CD8⁺ Tregs could restore/improve a deficient immune regulation, as we previously showed in an animal model of GVHD.²¹ Similarly, adoptive cell transfer of CD4⁺ Tregs has also been shown with a potential to reduce EAE severity.^{41,42} We did not select CD8⁺ Tregs based on antigen specificity before transfer, suggesting that transferred CD8⁺ Tregs acted through bystander effects or after epitope spreading if any antigen-specific mechanism was involved. Disease onset and maximal EAE score were unaffected by CD8⁺ Tregs infusion; however, we observed an improved recovery phase after d18. This raises the intriguing possibility that T-cell priming was not affected by CD8⁺ Tregs but that the latter contributed to precipitate the switch of immune balance toward a tolerogenic profile in the CNS.⁴³

Furthermore, recent findings revealed that active MOG-EAE induction elicits a burst of clonal CD8⁺ T-cell expansion outside the CNS, which are not myelin specific.¹⁶ These CD8⁺ T cells can suppress pathogenic Tconv in a perforin-dependent way *in vitro* and *in vivo*. In active MOG-induced EAE, this CD8⁺ T-cell burst in the blood and CNS synchronously matches CD4⁺ T-cell waves in both the blood and CNS. Of interest, none of these Tregs were Qa-1 restricted unlike CD8⁺ Tregs previously described in EAE mice, but were sharing the expression of CD44, CD122, and Ly49 with previously reported CD8⁺ Tregs. In recently diagnosed MS, a similar clonal expansion of CD8⁺ T cells has been found, whereas neither CD4⁺ T cells nor $\gamma\delta$ T cells showed a restricted TCR repertoire.¹⁶ The clonality of CD8⁺, but not CD4⁺ T cells in

MS, have been reported by several teams in both the blood and CNS.^{12,44} Yet a study brings new insights into one aspect of T-cell interaction in MS and autoimmune diseases and unveil a critical role of CD8⁺ T cells in regulating conventional T-cell responses.¹⁶ However, whether these CD8⁺ Tregs play a similar or complementary role within the CNS remains to be elucidated. Thus, preventing pathogenic T-cell migration before CNS entry⁴⁵ or within the CNS: direct pathogenic T-cell suppression, overcoming pathogenic T-cell resistance to Tregs,⁴⁶ controlling APC function and expansion,⁴⁷ and alleviating the destabilizing effects of inflammation on CD4⁺ Tregs functions⁴⁸ could be putative mechanisms of action of CD8⁺ Tregs that need to be further investigated, as some of these have been previously described for CD4⁺ Tregs. It is also possible that the number of cells injected was insufficient to control EAE symptoms in the early phase. An intrathecal injection and/or administration of higher numbers of cells might improve the efficacy of the therapy.

In addition, thanks to the development of engineering technologies, and based on the tremendous potential demonstrated for engineered chimeric antigen receptor-Treg, an improved targeting of CD8⁺ Tregs in MS could improve the therapeutic efficacy, as it has been demonstrated for CD4⁺ Tregs in EAE models^{49,50} and for CD8⁺ Treg in transplantation models.²² In this study, we demonstrated the existence of 2 CD8⁺ Treg subsets based on low and negative expression of CD45RC, respectively, which are unequally altered in MS. In addition, we showed that expanded CD45RC⁻CD8⁺ Tregs therapy can alleviate EAE severity underlying the potential of CD8⁺ Tregs as a therapeutic tool for autoimmune diseases including CNS dysimmunity.

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Disclosure

C. Guillonau, D. Laplaud, B. Nicol and S. Bézie have registered a patent. Go to [Neurology.org/NN](https://www.neurology.org/NN) for full disclosure.

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Continued

Appendix (continued)

Name	Location	Contribution
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