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Diminished Th17 (Not Th1) Responses Underlie Multiple Sclerosis Disease Abrogation after Hematopoietic Stem Cell Transplantation

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Objective: To define changes in phenotype and functional responses of reconstituting T cells in patients with aggressive multiple sclerosis (MS) treated with ablative chemotherapy and autologous hematopoietic stem cell transplantation (HSCT).

Methods: Clinical and brain magnetic resonance imaging measures of disease activity were monitored serially in patients participating in the Canadian MS HSCT Study. Reconstitution kinetics of immune-cell subsets were determined by flow cytometry, whereas thymic function was assessed using T-cell receptor excision circle analyses as well as flow cytometry measurements of CD31⁺ recent thymic emigrants (RTEs). Functional assays were performed to track central nervous system–autoreactive antigen-specific T-cell responses, and the relative capacity to generate Th1, Th17, or Th1/17 T-cell responses.

Results: Complete abrogation of new clinical relapses and new focal inflammatory brain lesions throughout the 2 years of immune monitoring following treatment was associated with sustained decrease in naive T cells, in spite of restoration of both thymic function and release of RTEs during reconstitution. Re-emergence as well as in vivo expansion of autoreactive T cells to multiple myelin targets was evident in all patients studied. The reconstituted myelin-specific T cells exhibited the same Th1 and Th2 responses as preablation myelin-reactive T cells. In contrast, the post-therapy T-cell repertoire exhibited a significantly diminished capacity for Th17 responses.

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Members of the Canadian MS/BMT Study Group are listed in the Appendix on page 12.

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Additional supporting information can be found in the online version of this article.

Interpretation: Our results indicate that diminished Th17 and Th1/17 responses, rather than Th1 responses, are particularly relevant to the abrogation of new relapsing disease activity observed in this cohort of patients with aggressive MS following chemoablation and HSCT.

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Powerful chemotherapy regimens followed by bone marrow reconstitution, such as chemoablation and autologous hematopoietic stem cell transplantation (HSCT), a form of bone marrow transplantation (BMT), have been applied to patients with severe autoimmune diseases including aggressive multiple sclerosis (MS).^{1–10} Associated risks of high-intensity regimens, however, preclude broader use of this approach in spite of its capacity to dramatically diminish new focal inflammatory disease activity in the central nervous system (CNS) of MS patients, who are otherwise treatment refractory.^{11–15} Hence, elucidating the immune mechanisms underlying the therapeutic effect of high-intensity HSCT could guide development of more selective treatments with a more favorable risk/benefit profile.

Surprisingly little is known, however, about immune reconstitution in humans following autologous HSCT, including the functional capacity of the reconstituted T-cell repertoire to participate in new autoimmune disease activity. Important questions relate to the impact of treatment on thymic function and the consequent release of recent thymic emigrants (RTEs) contributing to the reconstituting T-cell repertoire. Tracking CNS-directed antigen-specific T-cell responses from prior to ablative chemotherapy and during immune reconstitution is of special interest in the context of MS, where proinflammatory T cells, including those reactive to myelin self-antigens, have been considered pathogenic.^{8,16–18} Considerable work over the years in animal models of MS, principally experimental autoimmune encephalomyelitis (EAE), and studies of patients with MS, have implicated proinflammatory Th1 and Th17 responses as important mediators of new relapsing disease activity.^{17–23} More recently, a subset of T cells that express both Th1 and Th17 T-cell transcription factors and cytokines, denoted as Th1/17, have also been implicated in MS and EAE.^{24–26} The relative contributions of these different T-cell subsets to MS and EAE continue to be hotly debated.²⁷

Here, we report on the comprehensive immunomonitoring of both phenotypic and functional properties of the reconstituting T-cell repertoire, including CNS-reactive antigen-specific T-cell responses, in MS patients undergoing chemoablation and autologous HSCT. Complete abrogation of new MS disease activity was associated with a differential impact of HSCT on reconstituting Th1, Th17, and Th1/17 responses. These findings

provide novel and direct insights into the immune mechanisms that may underlie new relapsing disease activity in patients with MS.

Patients and Methods

Patients and Treatment Regimen

Fourteen MS patients (Table 1) were serially enrolled in the immunomonitoring substudy as part of the Canadian Collaborative MS/BMT Study, following informed consent as approved by the institutional ethics review boards. Detailed inclusion and exclusion criteria for trial participants are reported separately.²⁸ All patients had treatment-refractory, highly aggressive MS (details in the Supplementary Methods). Control MS patients were defined as those meeting all study criteria, who opted not to undergo the chemoablation and autologous HSCT regimen but were willing to participate in the comprehensive immunomonitoring, while treated according to standard medical practice (typically involving intravenous mitoxantrone, or cyclophosphamide). The baseline samples were collected before any therapeutic intervention (bone marrow harvest, mobilization chemotherapy, stem cell collection, or transplant conditioning). Details of the T-cell-depleted CD34⁺ hematopoietic stem cell mobilization, processing, and characterization, and of the high-intensity immunoablative regimen, are provided in the Supplementary Methods.

Sample Handling, Flow Cytometry, and T-Cell Receptor Spectratype Analysis

Whole venous blood samples for cell subset phenotyping by flow cytometry, and peripheral blood mononuclear cells (PBMC) isolated from whole blood by density centrifugation (Ficoll-Paque; Pharmacia Biotech, Uppsala, Sweden) and handled using rigorously standardized protocols as previously described,²⁹ were obtained at the time points indicated under specific experiments. Details of cell subset surface and intracellular staining, as well as acquisition by flow cytometry, are as published,²⁹ and as provided in the Supplementary Methods. For T-cell receptor spectratype analyses, a total of 24 polymerase chain reactions (PCRs; 1 for each T-cell receptor β variable [TCR β V] segment) were done for each PBMC sample, using V β -specific and C β in primers (see Supplementary Methods).

T-Cell Receptor Excision Circle Analyses

Highly sensitive nested quantitative PCR of serial PBMC samples was used to quantify signal-joint T-cell receptor excision circles (sjTRECs) as previously described^{30,31} and detailed in the Supplementary Methods and Supplementary Table, allowing detection of 1 copy of 10⁵ cells for each DNA circle. Parallel quantification of each deletion circle together with the CD3 γ amplicon was performed for each PBMC sample using

TABLE 1: Characteristics and Clinical Features of 14 MS Patients Treated with Immunoablation and Autologous HSCT

Patient	Age, yr	Sex	MS Duration, yr, Pre-Rx	Treatment prior to BMT	EDSS		Relapses, No.	
					Pre-Rx	2 Years Post-Rx	In 2 Years Pre-Rx	In 2 Years after Treatment
MS1	30	F	6	Rebif	6	6	3	0
MS2	40	M	10	Myloral study, Rebif, Betaseron	6	5.5	4	0
MS3	26	F	8	Rebif	6	6	3	0
MS4	33	M	7	Myloral study, Rebif, Betaseron	6	6	2	0
MS5	27	F	6	Betaseron, Rebif	4	1.5	2	0
MS6	33	M	10	Betaseron, Copaxone, Novantrone	4	4	2	0
MS7	37	F	3.5	Cyclophosphamide	5.5	6	2	0
MS8	26	F	5	Rebif, Copaxone	6.5	6.5	3	0
MS9	31	F	4.5	Rebif	6	6	2	0
MS10	31	M	7	Betaseron, Rebif	6	8	2	0
MS11	41	F	5	Rebif	3.5	3.5	2	0
MS12	29	M	4.5	Rebif	6.5	8	4	0
MS13	29	F	3.5	Copaxone, Rebif, Novantrone	6	4	4	0
MS14	28	F	5	Rebif, Novantrone	4	1.5	2	0
Mean	32		6.1				2.6	0
Median					6	6	2	0

BMT = bone marrow transplantation; EDSS = Expanded Disability Status Scale; F = female; HSCT = hematopoietic stem cell transplantation; M = male; MS = multiple sclerosis; Rx = treatment.

LightCycler technology (Roche Diagnostics, Mannheim, Germany). TRECs and CD3 γ LightCycler measurements were performed in independent experiments, but on the same first-round serially diluted standard curve. sjTRECs were quantified in triplicate, and duplicate experiments were performed for each individual diverse joining β TREC (DJ β TREC) for all studied samples. The sj/ β TREC ratio was determined as the sjTREC frequency divided by the sum of DJ β TREC frequencies (sj/ β TREC = sjTRECs/ \sum DJ β TRECs).

Antigen-Specific Stimulation Assays

Primary stimulation assays were performed as previously described³² and detailed in the Supplementary Methods. In brief, patient PBMC obtained at the indicated time points were stimulated in the presence of carboxy fluorescein succinimidyl ester (CFSE) with either anti-CD3, whole myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or peptide pools of MBP or proteolipid protein (PLP), with each peptide at 15 μ g/ml. Details of the peptide pools can be found in

the Supplementary Methods. CFSE dilution as a measure of proliferation, and intracellular cytokine staining for interferon (IFN) γ expression within CD4⁺ or CD8⁺ T cells, were determined on day 10 of culture using flow cytometry.

Secondary (split-well) antigen-specific assays for specificity confirmation were performed using a combination of an interleukin (IL)-7-based assay with a traditional split-well paradigm as previously described³³ and detailed in the Supplementary Methods. The antigens used were 20 μ g/ml of MBP, tetanus toxoid (TT), or glatiramer acetate (GA). On day 14, thymidine uptake was measured on equal portions of the cells. A positive well was stringently defined as an antigen-pulsed well with a stimulation index >3 compared to its corresponding no-antigen well, and with absolute proliferation of at least 3 standard deviations above the mean of all no-antigen wells. Supernatants from day 14 taken prior to the addition of the [³H]-thymidine were assayed by enzyme-linked immunosorbent assay (ELISA) to measure antigen-specific T-cell secretion of IFN γ and IL-5.

Assessing Th1, Th17, and Th1/17 T-Cell Responses

The capacity of the same PBMC samples to generate Th1, Th17, and Th1/17 responses was assessed as recently described³⁴ and detailed in the Supplementary Methods. Briefly, cryopreserved PBMC samples collected prior to and following treatment were activated with anti-CD3 antibodies (OKT3; 20ng/ml), with or without IL-23 (10ng/ml), anti-IFN γ (5 μ g/ml), and anti-IL-4 (5 μ g/ml) antibodies (R&D Systems, Minneapolis, MN). After 6 days in culture, cells were surface stained for CD3, CD4, and CD8, and intracellularly for IFN γ and IL-17A. Subset analyses were carried out by flow cytometry as described above. Culture supernatants were stored at -80°C and subsequently analyzed in a single batch for IFN γ and IL-17A.

Results

Abrogation of New Focal Inflammatory Disease Activity following Chemoablation and Autologous HSCT in Patients with Aggressive MS

Table 1 summarizes baseline demographics and measures of Expanded Disability Status Scale and relapse rates in the 2 years prior to and following treatment. In spite of previous treatment with immunomodulating and/or immunosuppressive treatments, patients experienced multiple clinical relapses and developed multiple new T2 and gadolinium-enhancing (Gd⁺) brain magnetic resonance imaging (MRI) lesions prior to autologous HSCT (see Table 1 and Fig 1). During the 2 years of immunomonitoring following the immune ablation and autologous HSCT, and while on no concurrent therapy, all patients exhibited complete absence of new T2 or Gd⁺ lesions on multiple serial brain MRIs, as well as complete absence of new clinical relapse activity (see Fig 1).

Highly Ablative Regimen Is Followed by Sustained Delay in Reconstitution of Naive T Cells

Treatment resulted in complete loss of circulating (CD3⁺CD4⁺ and CD3⁺CD8⁺) T cells in the days following ablation (Fig 2). During reconstitution, CD4 T cells failed to reach pretreatment levels even after 2 years, whereas CD8 T-cell levels returned more quickly toward baseline levels, resulting in a sustained inversion of the CD4/CD8 ratio. A sustained delay in the reconstitution of naive CD4 and CD8 T cells was seen, as the majority of reconstituting T cells were of the central memory or effector memory phenotypes. The proportion of cells with regulatory T-cell (Treg) phenotype, defined as CD3⁺CD4⁺CD25^{hi}CD127⁻Foxp3⁺, exhibited a trend toward an initial increase, although frequencies returned to pretreatment levels over the subsequent 18 months. B

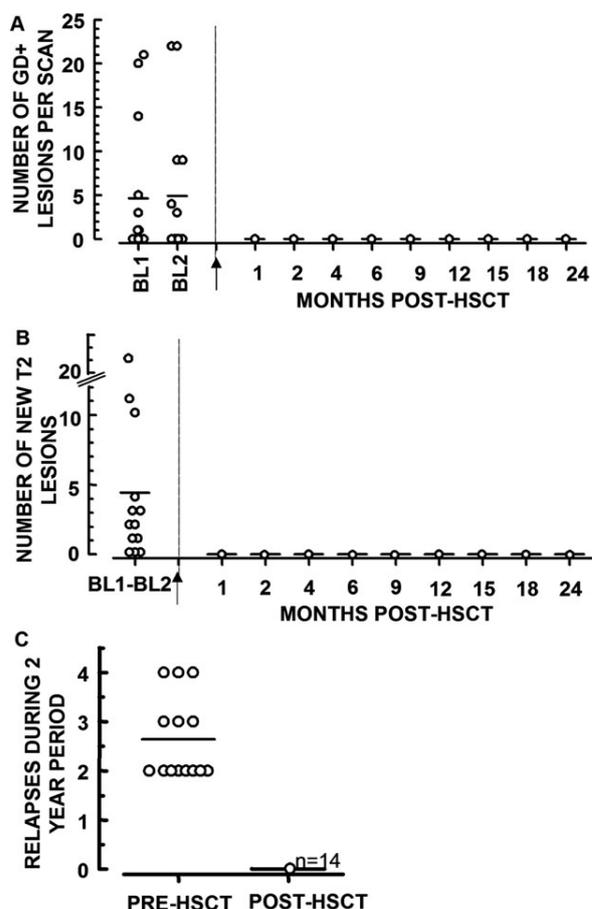


FIGURE 1: Abrogation of new focal inflammatory disease activity and clinical relapses following chemoablation and autologous hematopoietic stem cell transplantation (HSCT) in patients with aggressive multiple sclerosis (MS). (A) The number of new gadolinium-enhancing (Gd⁺) brain lesions before immunoablative chemotherapy and during immune reconstitution following autologous HSCT (arrow) in 14 patients serially treated in the Canadian MS/BMT Study. At least 2 baseline lead-in scans were done within 12 weeks of treatment initiation (BL1, BL2). Follow-up scans were performed at high frequency early after HSCT (4 scans within the initial 6 months), and then with decreasing frequency (every 3 months for the remainder until 18 months post-HSCT, every 6 months thereafter). An average of 5 Gd⁺ lesions were seen pretreatment, whereas no new Gd⁺ lesions were seen post-HSCT in any of the patients through 24 months of follow-up. This effect of treatment on decreasing the number of Gd⁺ lesions was statistically significant ($p < 0.001$; signed rank test). (B) The average number of new T2 lesions developing between the 2 screening brain magnetic resonance imaging scans (BL1–BL2) was 4.5, whereas no new T2 lesions developed across any of the patients through 24-months of follow-up. For 1 patient (MS2), baseline T2 lesion data were not available. (C) The average relapse rate in the 2 years prior to HSCT (PRE-HSCT) was 2.7, whereas treatment was associated with complete abrogation of new clinical relapses in the subsequent 2 years (POST-HSCT) in all patients.

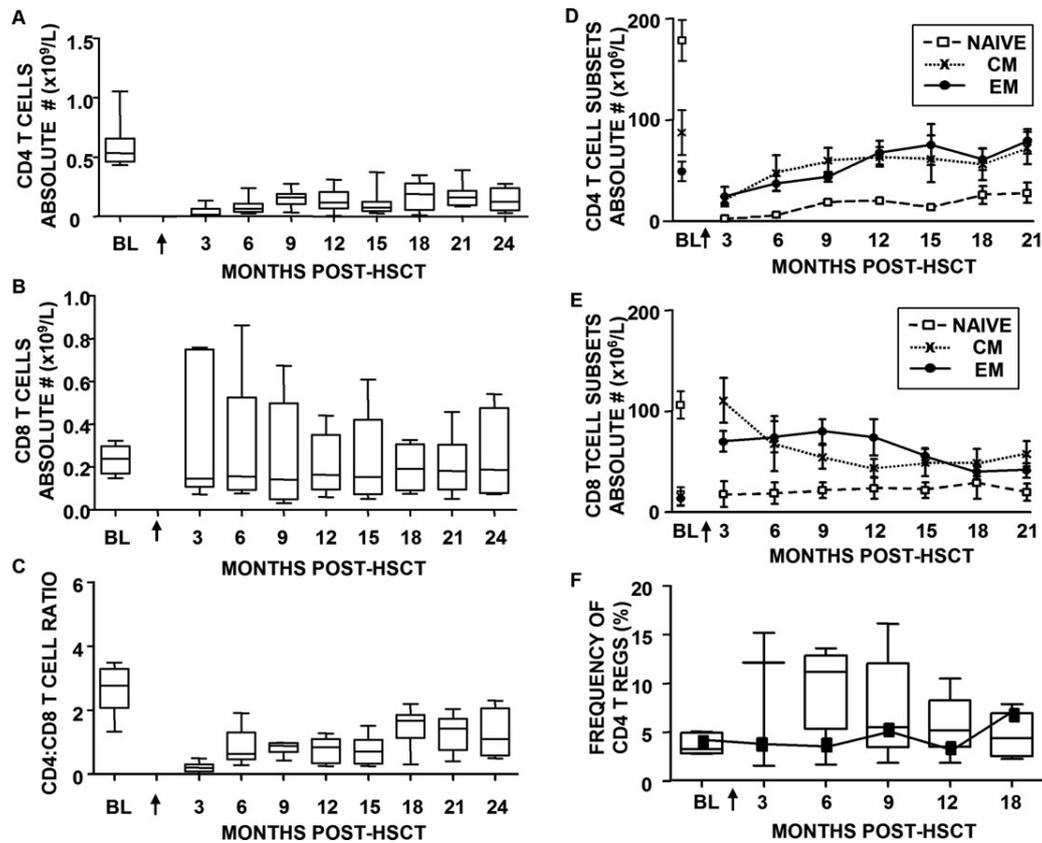


FIGURE 2: Kinetics of circulating immune cell subset reconstitution following immune ablation and autologous hematopoietic stem cell transplantation (HSCT). (A–C) Fresh whole blood samples were analyzed by flow cytometry before therapy (baseline [BL]), shortly after ablation (arrow), and every 3 months for up to 24 months. Immune ablation resulted in loss of circulating (A) $CD3^+CD4^+$ and (B) $CD3^+CD8^+$ T cells in all patients, which was confirmed by absence of CD3 amplification using polymerase chain reaction (data not shown). Functional loss was confirmed by the observation that delayed type hypersensitivity reactions to *Candida albicans*, present prior to ablation (15 ± 3 mm), were completely abrogated (0 mm; data not shown). During immune reconstitution, total CD4 T cells (A) failed to reach pretreatment levels even after 2 years, whereas total CD8 T cells (B) re-emerged more rapidly, resulting in a sustained reversal of the CD4/CD8 ratio (C). (D–F) CD4 and CD8 T-cell subsets were further identified as $CD45RA^-RO^+CD62L^+$ central memory (CM; crosses) or $CD45RA^-RO^+CD62L^-$ effector memory (EM; closed circles) T cells, or as $CD45RA^+RO^-CD62L^+$ -naive T cells (open squares). CM and EM T cells reconstituted with similar kinetics, whereas naive CD4 T cells exhibited long-standing delayed reconstitution (D). Sustained delay in the reconstitution of naive CD8 T cells was also observed, whereas CD8 memory subsets were increased (E). Cells with the phenotype of regulatory T cells (Tregs; $CD3^+CD4^+FoxP3^+CD25^+CD127^-$) exhibited a transient increase in frequency (relative to the total $CD4^+$ T-cell pool) above BL levels, whereas stable frequencies of circulating Tregs were observed from a control patient who did not undergo therapy (closed squares; F). Figures depict all available data from the first 7 patients in the study. In A–C and F, box and whisker plots depicting upper quartile, median, lower quartile, and range are shown. Error bars in D and E indicate standard error of the mean from at least 4 patient samples analyzed per point.

cells, NK cells, and monocytes emerged more quickly than T cells (by 3 months of reconstitution), and reached near or above preablation levels in most patients by 9 to 12 months (data not shown).

Thymic Function and the Release of RTEs Are Restored following Autologous HSCT, Leading to Reconstitution of a Normally Diverse T-Cell Repertoire

Serial measurements demonstrated significant reductions in levels of sjTREC within circulating T cells at 3 months post-HSCT, with levels subsequently normalizing

to pre-HSCT values by approximately 12 months in all patients (Fig 3). A similar reconstitution kinetic was seen when sjTREC levels were corrected for blood volume (sjTREC/ml), indicating that the initial decreases in sjTREC levels were unlikely to reflect the mere dilution of sjTREC due to enhanced extrathymic proliferation (a known phenomenon in lymphopenic patients^{35,36}). Reconstitution of $CD31^+CD4^+$ -naive T cells, recently shown to represent an early pool of RTEs,³⁷ mirrored the sjTREC kinetics, as did the sj/ β TREC ratio, a marker of functional intrathymic proliferation.^{31,38} Together, these data indicate that, although chemoablation

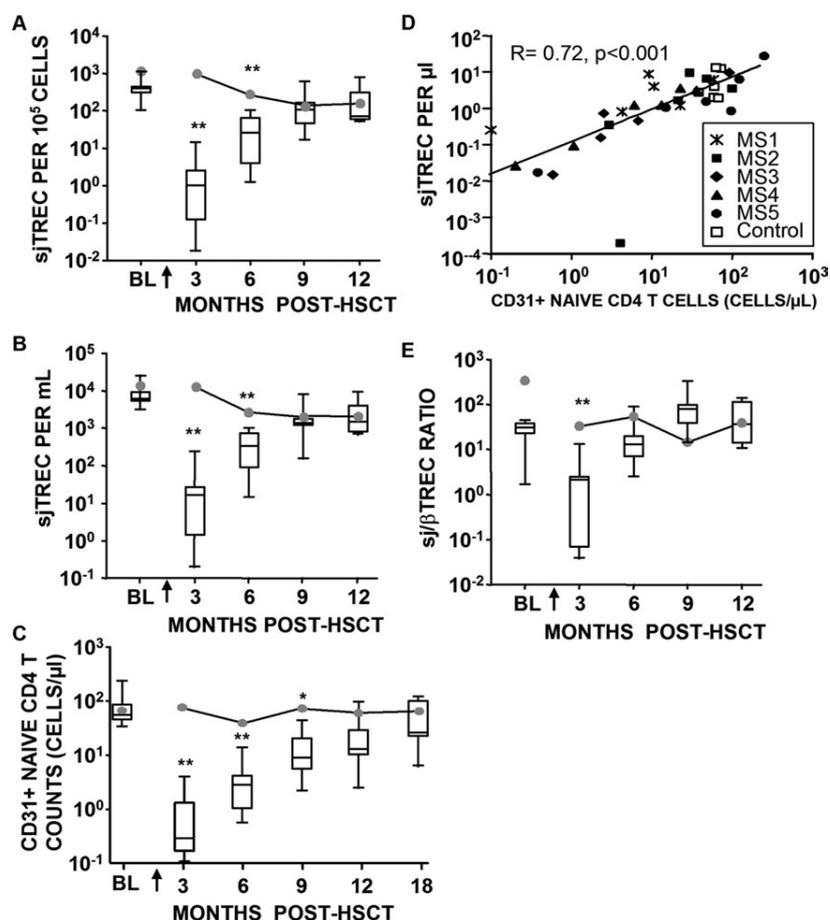


FIGURE 3: Restoration of intrathymic proliferation and normal levels of recent thymic emigrants (RTEs). To assess whether the prolonged delay in naive T-cell reconstitution reflected a persistently diminished capacity of the thymus to generate and release naive T cells (RTEs), serial quantification of levels of T-cell receptor excision circles (TRECs) in circulating T cells was initially carried out as an indirect measure of naive T cells released from the thymus. The grey dots on A,B,C E, and the open square symbol in D represent a control MS patient who was not treated. (A) Signal-joint TREC (sjTREC) levels were quantified in PBMCs as a marker of RTEs per 10⁵ cells at baseline (BL), and after ablation (arrow) at 3-month intervals thereafter. An early drop in sjTREC level was followed by a gradual return to BL levels (** $p < 0.006$ as compared to BL). (B) The number of sjTREC per milliliter of blood followed a similar pattern, indicating that the initial decreases in sjTREC levels were unlikely to reflect the mere dilution of sjTREC due to enhanced extrathymic proliferation (** $p < 0.006$). (C) Serial flow cytometry measures of whole blood samples enabled direct quantification of the CD31⁺ subset of circulating RTEs within the naive T-cell pool. The proportion of CD31⁺-naive T cells within the total circulating CD4 T-cell population declined significantly at 3 months, and recovered to near BL levels by 18 months of reconstitution (* $p = 0.02$; ** $p < 0.006$). (D) A strong correlation was observed between the absolute CD31⁺CD4⁺-naive T-cell counts and the sjTREC concentrations ($r = 0.72$; $p < 0.001$), establishing a strong relationship between these direct and indirect RTE measures, respectively. (E) The kinetics of the sj/βTREC ratio (calculated based on measurements of both signal-joint and diverse joining β TREC frequencies, as a marker of intrathymic proliferation) confirmed an initial decrease and a subsequent restoration of thymic function by 12 months (** $p < 0.006$). HSCT = hematopoietic stem cell transplantation.

and HSCT initially impair thymic function, thymopoiesis is subsequently restored in these MS patients to pre-treatment levels, allowing for normal generation and release of the RTE population. TCRBV spectratype analysis of all 24 TCRβV families (TCRβV-1 to TCRβV-24) in serial samples obtained prior to ablation and at month 12 (M12) of reconstitution (data not shown) confirmed the development of a normally diverse peripheral CD4 T-cell repertoire that was indistinguishable from baseline, in keeping with the prior reports of broad immune

reconstitution following other regimens of autologous HSCT in MS.¹⁴

Reconstituted T-Cell Repertoire following Autologous HSCT Is Notable for Re-emergence and In Vivo Expansion of CNS-Autoreactive T Cells

We next considered whether the re-emergence of an apparently broad T-cell repertoire, in the face of continued abrogation of new disease activity (see Fig 1),

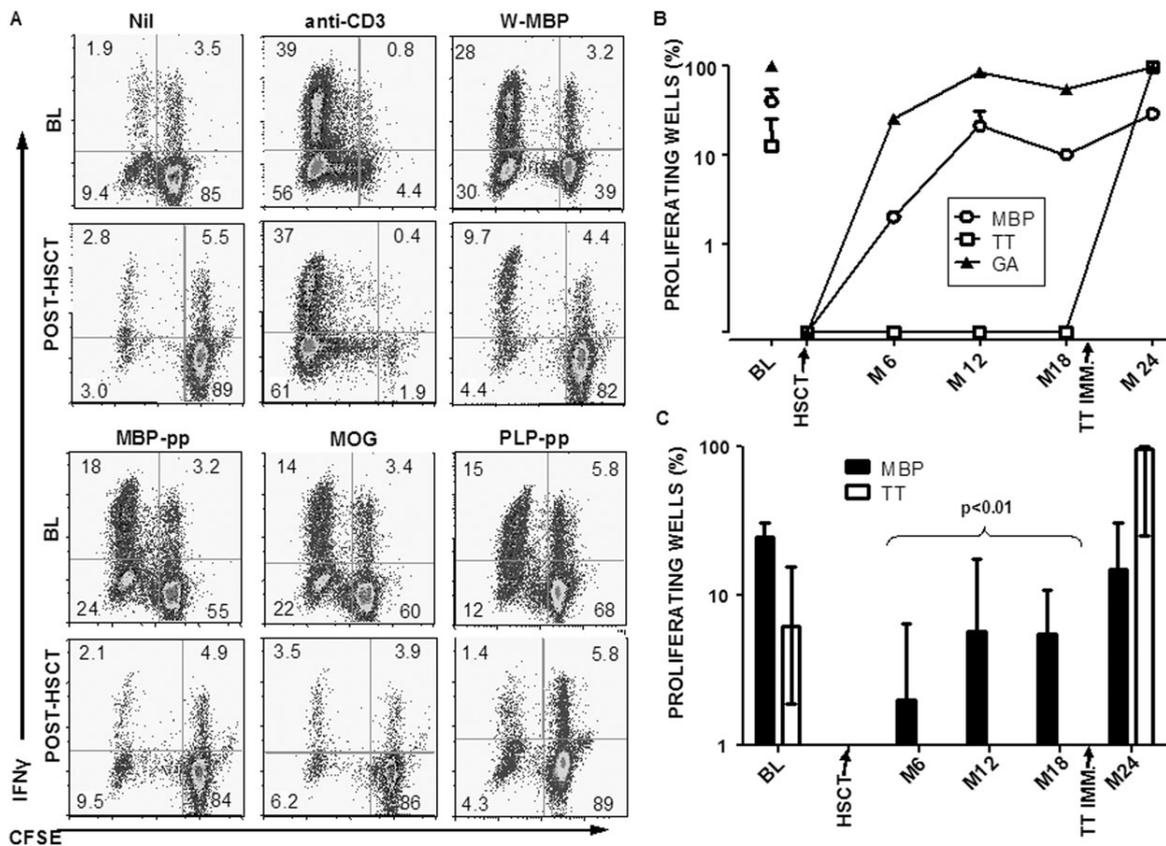


FIGURE 4: Re-emergence and in vivo priming of functional CD4⁺ T-cell responses to multiple central nervous system (CNS) epitopes during reconstitution. (A) T-cell reactivities to multiple CNS myelin epitopes were screened in a primary antigen-specific stimulation assay, using peripheral blood mononuclear cells (PBMC) isolated from the same patients at baseline (BL) and 12 months following chemoablation (post-hematopoietic stem cell transplantation [POST-HSCT]). Shown is a representative patient (of 4 assayed). Carboxy fluorescein succinimidyl ester (CFSE) dilution (as a measure of proliferation) and intracellular interferon (IFN)_γ expression within CD4⁺ T cells as determined by intracellular cytokine staining were analyzed on day 10 of culture using flow cytometry. Data are shown for CD4⁺ T-cell responses following stimulation with anti-CD3 (positive control), whole myelin basic protein (W-MBP), an MBP peptide pool (MBP-pp), myelin oligodendrocyte glycoprotein (MOG), and a proteolipid protein peptide pool (PLP-pp). Peptide pool details are in the Supplementary Methods. Similar results were seen with other peptide pools of both MBP and PLP (data not shown). (B, C) Antigen-specific T-cell-proliferative responses were measured by modified split-well assay, which was applied to serial PBMC samples obtained prior to chemoablation (BL) and at months 6 (M6), 12, 18, and 24 following HSCT. T-cell reactivities were assessed as the percentage of cultured wells demonstrating antigen-specific proliferative responses using strict predefined criteria (see Patients and Methods section). (B) Kinetics of reconstitution of T-cell reactivities to human brain-derived MBP (open circles), the recall antigen tetanus toxoid (TT; open squares), and the positive control antigen glatiramer acetate (GA; closed triangles), are shown for a representative patient. GA is a positive control to assess the functional breadth of the T-cell repertoire, as it induces T-cell receptor-mediated and major histocompatibility complex class II-restricted responses from a broad range of T-cell clones. Some time points were assayed twice over a 2-week period, providing error bars where indicated. (C) Comparison of T-cell reactivities to MBP (closed bars) and TT (open bars) for the first 6 patients in the study. Median values are shown; bars depict the interquartile range. T-cell responses to both antigens are abrogated ($F = 33.47$, $p < 0.0001$; 2-factor repeated measure analysis of variance [ANOVA] on ranks). Antigen-specific responses to TT were not detectable in the split-well assay at M6, M12, and M18 in all patients (B, representative patient; C, $n = 6$), and became detectable only after active immunization (TT IMM.) between M18 and M21. In contrast, re-emergence of MBP began as early as M6 post-treatment, and by M24 levels were indistinguishable from pretreatment (BL). Compared to TT reactivity, the reconstitution kinetic of T-cell reactivities to MBP in these patients was spontaneous and more rapid ($F = 37.42$, $p < 0.01$; 2-factor repeated measure ANOVA on ranks).

reflected selective lack of reconstitution of putatively pathogenic CNS-reactive T cells. Using a flow cytometry-based primary stimulation protocol that we optimized for use with cryopreserved samples, we initially screened for T-cell reactivities to multiple myelin antigens in PBMC obtained from the same patients prior to and

at 12 months following treatment. We observed the re-emergence of functional CD4⁺ T-cell reactivities (both proliferation and cytokine production) to multiple myelin epitopes, including whole MBP, MOG, and peptide pools derived from MBP and PLP, in all patients (Fig 4A; representative patient). Re-emergence of CD8⁺

TABLE 2: Cytokine Profiles of Antigen-Reactive T Cells

Patient	Pretreatment			12 Months Post-HSCT		
	T-Cell Lines, No.	Cytokine, pg/ml (SD)		T-Cell Lines, No.	Cytokine, pg/ml (SD)	
		IFN γ	IL-5		IFN γ	IL-5
MS-HSCT 1	63	5,864 (324)	464 (36)	47	5,374 (398)	408 (29)
MS-HSCT 2	39	4,463 (244)	54 (12)	33	4,076 (212)	48 (14)
MS-HSCT 3	18	1,794 (103)	0	11	2,158 (134)	0
MS-HSCT 4	57	6,248 (374)	348 (28)	38	5,756 (328)	321 (23)
MS-HSCT 5	26	3,284 (188)	589 (54)	21	3,476 (204)	0
MS-HSCT 1–5	203	4,331 (282)	291 (27)	150	4,168 (246), NS	155 (26), NS
MS-GA	46	5,245 (262)	588 (62)	34	1,420 (86) ^a	3,750 (172) ^a

Cytokine profiles of confirmed myelin basic protein–specific T-cell lines from the first 5 MS patients evaluated prior to immunoblation and 12 months into reconstitution from autologous hematopoietic stem cell transplantation (MS-HSCT 1 to MS-HSCT 5). IFN γ and IL-5 represent prototype Th1 and Th2 cytokines, respectively. Average cytokine levels (\pm standard error of the mean) produced by the indicated number of T-cell lines are shown for individual patients, and also pooled for all 5 patients (MS-HSCT 1–5). The Th1 predilection of the myelin-reactive T cells measured in these MS patients prior to immunoblative treatment was no different from their profile in the reconstituting immune system post-HSCT. To confirm that this assay is capable of measuring in vivo Th1 to Th2 cytokine deviation, GA-reactive T-cell lines were generated from an unrelated MS patient prior to and 12 months into treatment with GA (MS-GA). The assay was able to demonstrate that GA treatment resulted in significantly decreased production of IFN γ and enhanced production of IL-5 by GA-reactive T cells.

^a $p < 0.001$ from baseline by t test.

GA = gadolinium-enhancing; HSCT = hematopoietic stem cell transplantation; IFN = interferon; IL = interleukin; MS = multiple sclerosis; NS = not significant; SD = standard deviation.

T-cell reactivities to multiple myelin epitopes was also seen (data not shown). Although frequencies of T-cell–proliferative responses to myelin antigens following reconstitution (post-HSCT) appeared diminished compared to pretreatment levels, these primary stimulation assays do not reliably assess precursor frequencies and may also be confounded by bystander effects. We therefore next used a modified split-well restimulation assay to measure antigen-specific T-cell responses of confirmed specificity.³³ We applied this assay to serial samples from the same patients and tracked the kinetics of reconstitution of CNS reactivity, with direct comparison to the reconstitution kinetics of T-cell reactivity to the recall antigen TT. The immunoablative regimen resulted in complete loss of baseline antigen-specific proliferative responses to all antigens, which remained undetectable through M3 post-HSCT in all patients (see Fig 4B, representative patient; Fig 4C, summary of 6 patients for MBP and TT reactivities). T-cell reactivities to the recall antigen TT became detectable only after patients were actively immunized with tetanus (at M18, as part of the clinical trial protocol), resulting in robust proliferative responses to TT measured at M24. In contrast, antigen-specific T-cell responsiveness to MBP re-emerged spontaneously in all treated patients, and reached levels that were indistinguishable from pretreatment baseline. The

ability to detect reactivities to MBP in the same assay in which responses to TT-reactive precursor cells could not be detected establishes that naive CNS-autoreactive T cells not only re-emerged into the periphery of these patients during reconstitution, but also underwent in vivo priming.

Th1 and Th2 Responses Are Unchanged, Whereas Th17 Responses Are Significantly Diminished after HSCT

To address the possibility that the newly emerging myelin-reactive T cells secrete a less proinflammatory cytokine profile compared to pretreatment myelin-reactive T cells, we initially measured levels of the prototypic proinflammatory Th1 cytokine IFN γ and the Th2 cytokine IL-5, produced by the MBP-specific T cells of confirmed specificity in the split-well assay (Table 2). Prior to the immune ablation (pretreatment), MBP-reactive T cells secreted significantly more IFN γ than IL-5, consistent with prior reports of a Th1 predilection of these cells in patients with active MS.^{39–42} At 12 months post-treatment, the cytokine profiles of the reconstituted myelin-reactive T cells were essentially identical to pretreatment levels in all patients analyzed (see Table 2). These observations did not reflect a limitation of the assay, which was quite capable of detecting in vivo antigen-specific

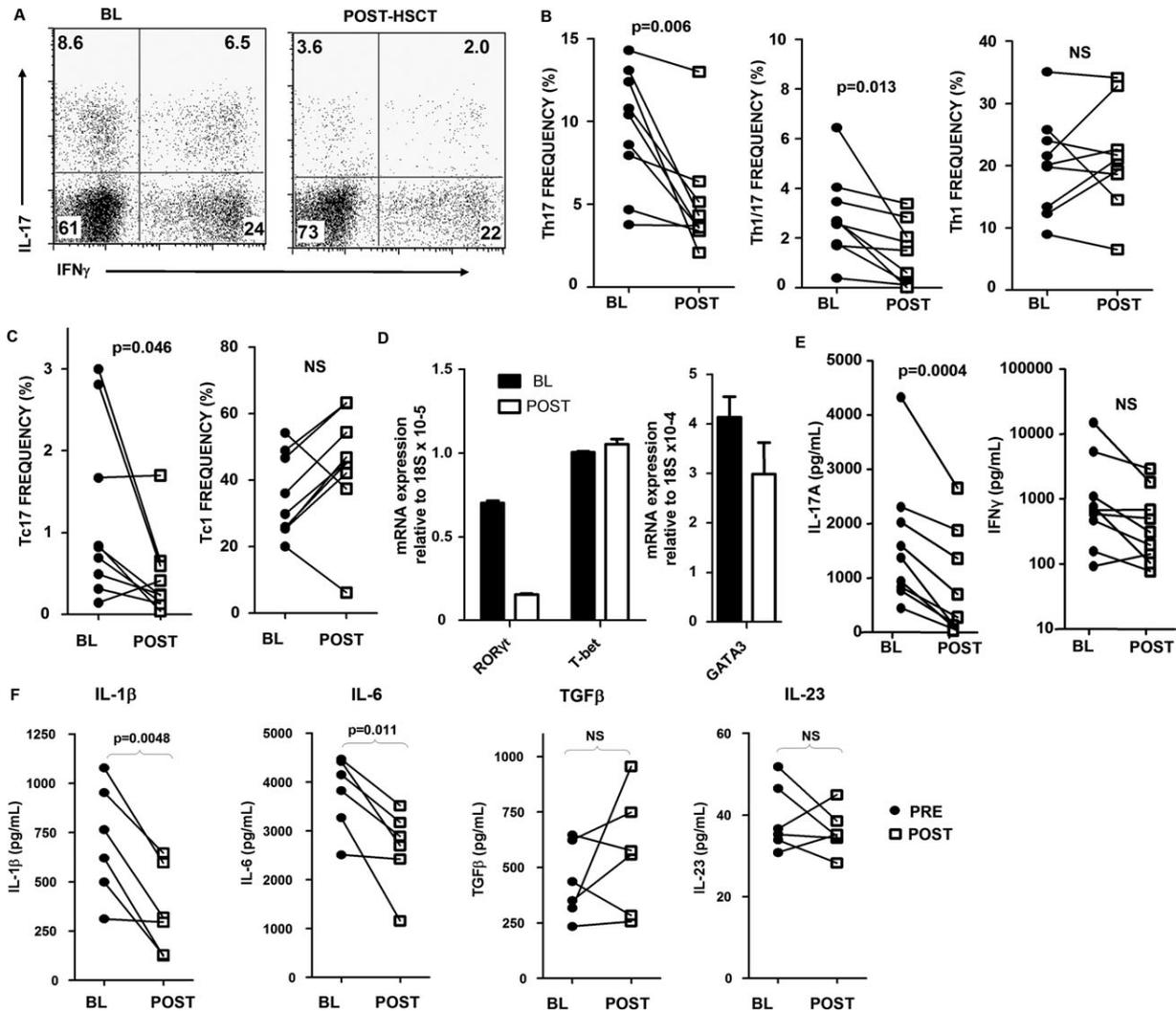


FIGURE 5: Significant reduction in interleukin (IL)-17 responses, but unchanged interferon (IFN) γ responses, in CD4 T cells reconstituting in multiple sclerosis (MS) patients after hematopoietic stem cell transplantation (HSCT). Cryopreserved peripheral blood mononuclear cell (PBMC) samples obtained from the same MS patients prior to ablative treatment (PRE) and at between 12 and 15 months of reconstitution (POST) were cultured with anti-CD3, recombinant human IL-23, anti-IL-4, and anti-IFN γ for 6 days, and cytokine expression was analyzed by flow cytometry using intracellular cytokine staining and by enzyme-linked immunosorbent assay (ELISA). (A) Flow cytometry dot plots from a representative patient depicting IL-17 and IFN γ expression in gated CD3⁺CD4⁺ T cells. Three populations of cytokine-expressing T cells can be distinguished: Th1 cells, expressing IFN γ only (IFN γ ⁺IL-17⁻); Th17 cells, expressing IL-17 only (IL-17⁺IFN γ ⁻); and double-positive (IL-17⁺IFN γ ⁺) Th1/17 cells expressing both cytokines. Numbers in plots indicate frequencies of gated cells within each quadrant. (B) Data from 9 patients studied serially demonstrates significantly reduced frequencies of Th17 T cells ($p = 0.006$; $n = 9$; Wilcoxon signed-rank test), and of Th1/17 T cells ($p = 0.013$), post-HSCT compared to baseline (BL) responses. In contrast, the frequencies of Th1 T cells were not reduced. (C) CD8 T cells were analyzed in parallel in the samples above; levels of IL-17⁺CD8 T-cell (Tc17) responses were low but detectable at BL, and appeared to be lower in post-HSCT samples, whereas levels of IFN γ ⁺CD8 T-cell responses were not significantly changed. (D) Quantitative polymerase chain reaction was performed on activated PBMC samples from pre-HSCT and 12 months post-HSCT. The levels of ROR γ t, T-bet, and GATA-3 were measured. (E) Analysis of the culture supernatants by ELISA confirmed these findings at the level of secreted proteins, revealing significantly lower production of IL-17 by the post-HSCT immune cells as compared to BL samples ($p = 0.0004$; $n = 9$; Wilcoxon signed-rank test), whereas levels of secreted IFN γ were not significantly reduced. (F) Analysis of selected Th17-polarizing cytokines. Cryopreserved PBMC samples were thawed and activated in Th17-polarizing conditions as described in the Patients and Methods section. The levels of the indicated cytokines were measured by ELISA. BL and post-HSCT samples from 6 patients were measured in parallel; the connecting line indicates a sample set. Paired t test was performed. NS = not significant; TGF = transforming growth factor.

Th2 cytokine immunodeviation when it occurred, as shown in a different therapeutic context for an unrelated MS patient treated with the approved therapy GA (Copaxone).

Because the antigen-specific split-well approach does not generate detectable Th17 responses, we developed a flow cytometry assay capable of simultaneously measuring Th1, Th17, and Th1/17 responses of T cells within cryopreserved PBMC. When directly comparing responses of PBMC obtained from the same patients prior to treatment (pre-HSCT) and following reconstitution (post-HSCT), we observed that post-HSCT samples exhibited significantly lower Th17 (IL-17A⁺IFN γ ⁻) responses ($p = 0.006$), as well as Th1/17 (IL-17A⁺IFN γ ⁺) responses ($p = 0.013$), as compared to pre-HSCT samples (Fig 5A, representative patient; Fig 5B, 9 serially studied patients). In contrast, post-HSCT Th1 (IFN γ ⁺IL-17A⁻) T-cell responses were not reduced, confirming our prior observations from the antigen-specific T-cell assays. Tc17 (IL-17⁺IFN γ ⁻ CD8⁻ T cell) responses, typically detectable at very low frequencies at baseline, also tended to be lower following reconstitution ($p = 0.046$), whereas Tc1 (IFN γ ⁺IL-17⁻ CD8⁺ T cell) responses did not, again reproducing the results of CD8 T-cell responses assessed in the antigen-specific assays. In keeping with these results, using quantitative PCR to evaluate expression of Tbet and ROR γ in activated PBMC obtained pre- and post-HSCT, we found that reconstituting cells expressed decreased levels of ROR γ , whereas levels of Tbet did not differ from pretreatment. Analysis of culture supernatants by ELISA confirmed a lower level of secretion of IL-17A in the post-HSCT samples ($n = 9$; $p = 0.0004$), whereas IFN γ secretion was not similarly reduced. Assessment of levels of known Th17 polarizing and stabilizing cytokines in activated PBMC supernatants revealed that post-HSCT PBMC secreted significantly decreased levels of IL-1 β ($p = 0.0048$) and IL-6 ($p = 0.011$), whereas levels of TGF β and IL-23 secretion were unchanged.

Discussion

In their elegant study, Muraro et al¹⁴ demonstrated that the diminished disease activity following chemoablation and autologous HSCT in patients with MS was not due to persistent immunosuppression, because a diverse and apparently functional immune repertoire emerged during immune reconstitution. The authors hypothesized that a lack of re-emergence of proinflammatory CNS-reactive T cells in the reconstituting immune repertoire was the basis for the robust and sustained therapeutic effect. This has been a challenging hypothesis to test rigorously, as most regimens to date have been subablative, making it

difficult to distinguish between newly developed autoreactive T cells and those that may have persisted in the host or the autologous graft.^{15,43} As part of the Canadian MS BMT Study, we had a unique opportunity to test this hypothesis, given the use of a high-intensity immunoblative chemotherapy regimen, as well as T-cell-depleted autologous stem cell grafts,²⁸ designed to most effectively eliminate residual T cells.

In keeping with prior reports,^{1-10,44} treatment in our cohort resulted in abrogation of clinical and brain imaging measures of new focal inflammatory MS disease activity. These effects were sustained in all patients throughout the 2 years of immunomonitoring, and in the absence of any ongoing treatment. The particularly robust treatment effects observed in our study may reflect the profile of patients recruited (relatively young, short disease duration, highly inflammatory disease activity), the high-intensity chemoablative conditioning regimen (including busulfan for improved CNS penetration), and the highly selected CD34⁺ hematopoietic stem cells, together designed to eliminate potentially disease-relevant autoreactive immune cells from the periphery, CNS, and graft. Prior animal studies had shown that chemoablation can be more effective than total body irradiation as preconditioning, and that T-cell depletion of the graft makes treatment more effective.^{45,46} We considered the impact of our ablative chemotherapy regimen on thymic function as well as phenotypic and functional properties of the T-cell repertoire reconstituting from the autologous stem cells. Our first observations confirmed the prior findings that a broad and functional T-cell repertoire re-emerges following high-intensity chemoablation in patients with MS. By tracking the kinetics of thymic function in these patients using several complementary approaches, we further documented that the high-intensity immunoblative treatment initially reduced the capacity of the thymus to sustain both precursor T-cell proliferation and release of RTEs into the circulation, likely reflecting a deleterious effect of the chemotherapy on thymic stroma. The subsequent rebound of the sj/ β TREC ratio to baseline values (or higher), and the return of sjTREC, sjTREC/ml, and CD31⁺CD4⁺-naive T-cell frequencies to baseline levels by 9 to 12 months of follow-up in all patients, indicate restoration of the capacity for intrathymic precursor T-cell proliferation, as well as the quantitative restoration of the thymic emigrant population. However, the absolute counts of circulating naive CD4 and CD8 T-cell subpopulations continued to remain well below pretreatment levels in all our patients. Together, these results demonstrate that thymic function as well as generation and release of RTEs can reach steady state in spite of incomplete restoration of

CD4- and CD8-naive T cells in the periphery, providing the fundamental insight that, *in vivo*, human RTEs represent an independently regulated T-cell subset. The failure to fully reconstitute naive T-cells following high-intensity immunoablative regimens has been previously reported, and attributed to the excessive propensity of the newly generated naive T cells to undergo apoptosis.⁴⁷

Contrary to our initial hypothesis that the sustained abrogation of new disease activity in our patients reflected failure of CNS-reactive T cells to develop in the reconstituting immune system, we documented the re-emergence as well as the *in vivo* expansion of functional autoreactive T cells capable of responding to multiple myelin epitopes in all treated patients. The apparently lower frequencies of myelin-reactive T cells measured post-HSCT when using our primary stimulation assay are consistent with a prior report using a similar activation assay.⁴⁸ In Sun et al,¹³ the authors reported re-emergence of MBP reactivity following their autologous transplant regimen in MS, although the early reconstitution kinetics they observed raised the possibility that this reflected the lack of complete ablation of pre-existing MBP-reactive T cells. We do not preclude the possibility that diminished disease activity in our patients reflects a lower burden of potentially encephalitogenic T cells; however, results of our secondary stimulation (split-well) assays indicate that when antigen-specific responses are confirmed upon restimulation, the responses of CNS-reactive T cells post-HSCT are similar to pre-HSCT responses. It is possible that circulating CNS-reactive T cells are simply not relevant to MS disease activity; however, a substantial body of literature continues to support the model that proinflammatory CNS-reactive T cells travel from the periphery into the CNS and contribute to new disease relapses. We therefore considered whether the balance between pro- and anti-inflammatory T-cell responses may have shifted with treatment. Our antigen-specific split-well assays allowed us to directly assess the balance of Th1 and Th2 cytokine responses of CNS-reactive T cells, and indicated that the reconstituted myelin-specific T-cell responses exhibited the same Th1 predilection as they did prior to treatment. Because these split-well assays do not generate measurable Th17 responses, we developed a novel assay enabling direct comparison of the propensity to generate Th1 and Th17 responses. Surprisingly, although Th1 responses of the reconstituting T cells appeared to be restored to baseline levels (as also seen in the split-well assay), the newly developed T-cell repertoire exhibited a significantly diminished capacity to mount Th17 responses, as corroborated at levels of both RNA expression and secreted protein. Our finding that Th1/17 double-positive

responses were also diminished may be of particular interest given recent reports that levels of circulating Th1/17 T cells better correlate with new disease activity in untreated MS patients than either Th1 or a Th17 cells, and that they may be preferentially recruited into the CNS of MS patients.²⁴ In this context, IL-17 has been shown to directly contribute to disruption of tight junctions of human blood-brain barrier (BBB) endothelial cells, and Th17 cells were found to very efficiently migrate across the BBB, where they promoted further CD4⁺ lymphocyte recruitment.²² Because the reduction in the ability to mount Th17 responses post-HSCT suggests that their activation and/or polarization is modified following treatment, we assessed levels of known Th17 polarizing and stabilizing cytokines (IL-1 β , IL-6, transforming growth factor [TGF] β , and IL-23). Although serum measurements did not reveal differences, activated PBMC obtained post-HSCT secreted significantly decreased levels of IL-1 β and IL-6, whereas levels of IL-23 and TGF β were unchanged. It is interesting to speculate whether the decreases in IL-1 β and IL-6 in the post-HSCT PBMC reflect functional changes in reconstituting antigen-presenting cells (APC) which in turn could result in decreased Th17 polarization. Dendritic cells are considered the major functional APC that return following BMT based on animal studies,⁴⁹ and in the context of autologous HSCT for treatment of B-cell lymphoma in humans.⁵⁰ Following autologous-HSCT in MS, we and others have seen that both B cells and monocytes, which can be precursors to DCs, return relatively rapidly (within 6 months) following treatment.¹⁴

Although our intracellular staining for IFN γ suggested that Th1 responses of the reconstituting T cells appeared to be no different than baseline levels (as noted above), one recognizes that several cell types other than Th1 cells can express IFN γ . Examples include IL-10⁺ Tregs⁵¹ and Foxp3⁺ Tregs.⁵² Several observations suggest that our intracellular measures of IFN γ are more likely to track with Th1 responses than with regulatory T-cell subsets. First, our serial flow-activated cell sorting analysis of Foxp3⁺ Tregs showed a transient increase in the frequency of these Tregs post-HSCT, such that their frequencies are back to baseline by the time we assess Th1 and Th17 responses. We also noted that our measures of secreted IFN γ (as opposed to intracellular IFN γ), both in bulk PBMC assays and in our myelin antigen-specific T-cell lines (which do not produce IL-10 nor exhibit differences in Foxp3 expression), demonstrated similar levels of secreted IFN γ in pre- and post-HSCT samples. Thus, although we do not preclude the possibility of different cell sources of IFN γ , our results are most consistent with the view that the similar levels of IFN γ measured pre-

and post-HSCT reflect reconstitution to basal levels of Th1-cell responses in the face of significantly decreased reconstitution of Th17 and Th1/17 responses.

Our observations that new disease activity is abrogated in MS patients in association with a significantly diminished Th17 response, and in spite of reconstitution to basal levels of primed CNS-reactive Th1 cells, fits very well with the concept that peripherally activated Th17 cells are required as gatekeepers to the CNS, and that their initial interaction with the BBB facilitates subsequent pathogenic Th1 cell entry. Such a mechanism, which has been defined in EAE studies,⁵³ could explain the implication over the years of both Th1 and Th17 responses in EAE and MS. Whether the newly described Th1/Th17 double-expressing cells are particularly efficient in this regard remains to be elucidated. Although we focus here on the reconstitution and implication of functional CD4 T-cell subsets, we noted a similar effect of treatment on CD8 T-cell responses. Whereas reconstituting CD8⁺IFN γ ⁺ (Tc1) T-cell responses were indistinguishable from baseline, essentially no CD8⁺IL-17⁺ (Tc17) T cells could be induced in the reconstituted patients. Pathologic roles of CD8 T cells in MS have long been suspected, and our results may be particularly relevant to the recently implicated CD8⁺CD161^{hi}IL-17-producing T cells.⁵⁴

The decreased new disease activity observed following HSCT in our patients could also be due to reduced capacity of migration of pathogenic T cells into the CNS. We found no treatment effect on the frequencies of circulating T-cell subsets expressing CCR6 (Supplementary Fig 1), a chemokine receptor implicated in mediating entry of Th17 cells into the CNS.^{53,55} Analysis of serum chemokines in pre- and post-HSCT samples revealed that treatment resulted in increased levels of circulating CXCL9, CXCL10 (IP-10) and CCL20 (MIP3 α), no changes in levels of chemokines including CXCL11 (ITAC), CXCL12 (SDF-1), CX3CL1 (fractalkine), CCL2, CCL3 (MIP-1 α), CCL4, and CXCL8 (IL-8), and a possible decrease in CCL5 (RANTES) levels in the second year post-HSCT (Supplementary Fig 2). These results indicate that the chemokine network of these patients is modified following HSCT. It is interesting to speculate as to the potential cellular sources responsible for these chemokine changes (eg, immune cells subsets, stromal cells, CNS as target organ), and whether these changes contribute to the HSCT treatment effect, or reflect a consequence of the diminished disease activity.

Other mechanisms that could contribute to the abrogation of new MS disease activity seen in our patients include an enhanced capacity to regulate otherwise pathogenic T cells. Although our phenotypic study

of Foxp3⁺ Treg kinetics identified only a transient increase in their frequencies during reconstitution, it is possible that an early window of enhanced regulation is sufficient for sustained disease control when a naive immune system is generated in MS patients. Our samples did not permit functional analysis of Tregs, and it is also possible that, although frequencies of Foxp3⁺ Tregs do not remain increased over time post-HSCT, they may have an enhanced regulatory capacity or, alternatively, that effector T cells emerging post-HSCT are more readily suppressed by Tregs. Potential involvement of additional regulatory cell subsets including induced CD4⁺ regulatory T cells and regulatory CD8⁺ T cells, as well as regulatory B cells and NK cells, is also of interest, and functional assays of the regulatory capacities of these cell subsets would ultimately be required to more fully assess their contribution.

In summary, our study provides a unique window into mechanisms that may underlie development of new inflammatory disease activity in patients with MS. Although additional mechanisms may apply, we demonstrate that the diminished capacity of peripheral T cells to mount IL-17 responses, but not their capacity to generate IFN γ responses, is associated with the dramatic and sustained abrogation of disease activity following high-intensity immunoablation and autologous HSCT in MS. Although this highly aggressive treatment paradigm does not provide direct insights into mechanisms underlying disease activity in the broad MS population, our findings suggest that Th17 and Th1/Th17 CD4 T cells, as well as Tc17 CD8 T cells (rather than Th1 or Tc1 T cells), may represent the peripheral effector T-cell subsets most relevant to the initiation of new relapsing disease activity in this cohort of patients with aggressive MS.

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Potential Conflicts of Interest

Nothing to report.

Appendix

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