Immunological correlates of favorable long-term clinical outcome in multiple sclerosis patients after autologous hematopoietic stem cell transplantation


A R T I C L E   I N F O

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High dose immunosuppression followed by autologous hematopoietic stem cell transplantation (AHSCT) induces prolonged clinical remission in multiple sclerosis (MS) patients. However, how patient immune profiles are associated with clinical outcomes has not yet been completely elucidated. In this study, 37 MS patients were assessed for neurological outcomes, thymic function and long-term immune reconstitution after AHSCT. Patients were followed for a mean (SD) of 68.5 (13.9) months post-transplantation and were retrospectively clustered into progression- and non-progression groups, based on Expanded Disease Status Scale (EDSS) outcomes at last visit. After AHSCT, both patient groups presented increased regulatory T-cell subset counts, early expansion of central- and effector-memory CD8+ T-cells and late thymic reactivation. However, the non-progression group presented early expansion of PD-1+CD8+ T-cells and of PD-1-expressing CD19+ B-cells. Here, we suggest that along with increased numbers of regulatory T-cell subsets, PD-1 inhibitory signaling is one possible immunoregulatory mechanism by which AHSCT restores immune tolerance in MS patients.

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1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by damage to myelin and axons, over time leading to progressive neuronal degeneration and microglial activation [1–3]. Several cells of the innate and adaptive immune system are involved in MS pathogenesis. During the demyelination process, CD4+ T-cell subsets are activated, such as Th1 and Th17 cells [4], but damage to the nervous tissue is mediated by autoreactive CD8+ T-cells and activated microglia [5–8]. B-cells also play significant role in CNS destruction, and may be associated with disease progression [9,10]. Furthermore, protective CD4+CD25+ regulatory T-cells have been shown numerically reduced and/or functionally defective in these patients [11–15].

High dose immunosuppression followed by autologous hematopoietic stem cell transplantation (AHSCT) has been investigated in the past years as treatment for MS patients that are refractory to first line therapy. Mechanistic studies have shown that AHSCT is able to control autoreactivity, thereby inducing prolonged clinical remission [16–18]. The procedure is based on the rationale that high dose immunosuppression is able to eliminate autoreactive T- and B-cells, therefore allowing installation of a new and tolerant immune system [19].

Knowledge about the immune mechanisms involved in AHSCT for autoimmune diseases has improved over the years [20–25]. However, their association with distinct clinical outcomes has not yet been completely elucidated. Muraro and collaborators described the generation of a new and diverse T-cell receptor (TCR) repertoire in MS patients after AHSCT [23]. Suppression of inflammatory activity after AHSCT was associated with regeneration of the T-cell compartment [26]. Increased numbers of...
regulatory T-cells (Treg) after transplantation indicate reestablishment of immune balance [27–29]. In addition, depletion of IL-17-producing mucosal-associated invariant T-cells [27] and diminished Th17 responses [28] have been described.

Increased expression of programmed cell death-1 protein (PD-1) in CD4+ and CD8+ T-cells in MS patients has been detected early after AHSC [29]. PD-1 is an inhibitory receptor expressed by T-cells, B-cells, natural killer T (NKT) cells, natural killer (NK) cells, dendritic cells (DC) and monocytes. It is involved with regulation of the immune response and maintenance of peripheral tolerance [30–32]. The interaction between PD-1 and its ligands (PD-L1 and PD-L2) lead to transduction of inhibitory intracellular signals, resulting in suppression of T-cell activation and effector responses [30,32]. In addition, the PD-1/PD-L pathway may induce regulatory T-cells in the periphery [33].

In the present study, we assessed long-term immune reconstitution in MS patients after treatment with AHSC. Here, we demonstrate transient increase of regulatory T CD4+ and of CD8+ cell subset, early expansion of central- and effector-memory CD8+ T-cells and late thymic reactivation. Importantly, we show that patients with better neurological outcome after AHSC present early expansion of CD8+ PD-1+ T-cells and of PD-1-expressing CD19+ B-cells.

2. Materials and methods

2.1. Patients and study design

Patients with relapsing–remitting, secondary progressive or primary progressive forms of MS were recruited for treatment with AHSC, according to previously described inclusion and exclusion criteria [34]. Autologous hematopoietic stem cells were mobilized from the bone marrow with 2 g/m2 cyclophosphamide plus G-CSF, subsequently collected by leukapheresis and cryopreserved. Subsequently, patients underwent transplant conditioning with 200 mg/kg cyclophosphamide plus rabbit anti-thymocyte globulin, followed by infusion of

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P: female; M: male; MS: multiple sclerosis; AHSC: autologous hematopoietic stem cell transplantation; EDSS: expanded disability status scale; PreTx: Pre-transplantation period; PP: primary progressive (n = 3, 9%); SP: secondary progressive (n = 28, 75%); RR: relapsing-remitting (n = 6, 16%); NA: Not available.

Patients were numbered according to transplantation date.

* Statistically significant (P < 0.05) when compared to other group.

a Evaluation post-AHSC: Non-Progression (stabilization or improvement of MS disability post-therapy); Progression (progression of MS disability post-therapy)

b Progression: raise of 0.5 or more in EDSS value (if initial EDSS ≤ 5.5), or raise of 1.0 or more (if initial EDSS > 5.0), between PreTx and last visit period; Stabilization: no one of previously alterations in EDSS value (According to Saccardi et al. [24]).

c Progression (15, 40%) and Non-Progression (22, 60%).

d Progression (15, 40%), stabilization (11, 30%) and clinical remission (11, 30%).
unmanipulated autologous hematopoietic stem cells. All procedures were approved by the Ethics Committee of the University Hospital of the School of Medicine of Ribeirão Preto (Process number 14105/06) and an informed consent was given by MS patients and controls before peripheral blood collection. MS patients were transplanted at the Bone Marrow Transplantation Unit of the School of Medicine of Ribeirão Preto, University of São Paulo, Brazil. Trial registration inclinicaltrials.gov identifier: NCT00273364.

Samples from 37 patients (26 females) with MS, who underwent AHSCT between May 2005 and March 2009, were included in the present study. Patients were refractory to first line treatment, having failed at least β-interferon and glatiramer acetate. Demographic and clinical characteristics of included patients are described in Table 1 and Fig. 1A. After transplantation, all patients were followed at planned intervals for clinical and laboratory assessments. Peripheral blood samples were collected for immune monitoring at the following time points: pre-AHSCT and at 2, 3, 6, 9, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 months after AHSCT.

2.2. Evaluation of clinical outcome

Pre and post-transplantation neurological disability was assessed by the Expanded Disability Status Scale (EDSS) [35]. Progression or improvement was considered when the EDSS scores respectively increased or decreased by 0.5 points when the initial score was ≥5.5, or by 1.0 or more points if the initial score was <5.0, remaining unchanged for at least 3 months.

Based on EDSS changes after AHSCT, from baseline to last visit, patients were retrospectively clustered into two groups: progression—patients who presented an increase in the EDSS scores; non-progression—patients whose EDSS scores either decreased or remained stable (Fig. 1A).

2.3. Immunophenotypic analyses

Whole peripheral blood samples (5 mL) were collected in ethylene diamine tetra acetic acid (EDTA) (8.55 mg/tube) for immunophenotyping by flow cytometry from MS patients. Peripheral blood samples (150 μL) were incubated with monoclonal antibodies, at room temperature for 15 min in the dark. After incubation, red cells were lysed with fluorescein activated cell sorter (FACS) lysing solution (Becton-Dickinson, San Diego, CA, USA) for 10 min at room temperature, in the dark. Cells were washed with 1 mL of FACS buffer (phosphate-buffered saline, 0.2% fetal bovine serum, 0.02% sodium azide), centrifuged twice for 5 min at 500 g, and then resuspended in 200 μL of FACS buffer and analyzed by flow cytometry. For FoxP3 and CTLA-4 assessment, cells were treated
with 300 μL of FACS permeabilization solution (Becton-Dickinson, San Diego, CA, USA) for 10 min at room temperature in the dark, washed and then analyzed by flow cytometry. To characterize lymphocyte sub-populations CD3+ (T-cells), CD3+CD4+ (T helper cells), CD3+CD8+ (cytotoxic T-cells), CD19+ (B-cells), CD4+ (or CD8+)CD27+ CD45RO+ (naive T-cells), CD4+ (or CD8+)CD27+CD45RO+ (central-memory T-cells), CD4+ (or CD8+)CD27−CD45RO+ (effector-memory T-cells), CD3+− CD4+ (or CD8+ )PD−1+, CD19+PD−1+, CD8+CD28−CD57+ (suppressor T-cells), CD4+CD25highFoxP3+ (or CTLA-4+, or GITR+) (regulatory T-cells), we used the following monoclonal antibodies: CD3-FITC, CD4-APC or -PE, CD8-PerCP-Cy5.5 or -APC, CD19-FITC, CD27-PE, CD31-PE, CD45RO-FITC, PD-1-PE, CD28-PE, CD25-APC, FoxP3-PE, CTLA-4-PE, GITR-PE (Becton-Dickinson, San Diego, CA, USA).

Isotype-matched controls were included and fifty thousand events/samples were acquired for each subset and hundred thousand events for regulatory T-cells. Samples were analyzed by a FACS Calibur Flow Cytometer (Becton-Dickinson, San Jose, CA, USA) and data were analyzed using FlowJo software (FLOWJO LLC, Oregon, USA). Blood cell counting was performed by the automated blood cell counter Mindray BC-2800 (Mindray Medical Instrumentation, China). Results were expressed as absolute cell numbers (cell/μL) using the following formula: Absolute lymphocytes (per μL) = total automated lymphocytes number (per μL) × percentage of specific lymphocyte subpopulation acquired by flow cytometry (%) /100. The gating strategy is available in Supplementary Table 1.

2.4. Absolute quantification of TREC by Real-time PCR

Peripheral blood mononuclear cells (PBMC) from MS patients were isolated by Ficoll-Hypaque™ density gradient centrifugation (Amersham-Pharmacia, Uppsala, Sweden). DNA was purified using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) according to manufacturer’s recommendations and diluted to concentration of 100 ng/μL.

TREC absolute quantification was performed by real-time PCR, using the TaqMan 5 nuclease assay (7500 Real Time PCR system). For each real-time PCR reaction, standard curves for TREC and albumin (endogenous control) were prepared using 1:10 dilution. DNA concentrations used were 2 × 10⁶, 2 × 10⁵, 2 × 10⁴, 2 × 10³, 2 × 10² molecules. In some cases, DNA concentration of 2 × 10¹ molecules was added to the standard curve. Plasmids containing TREC or albumin genes were kindly provided by Dr. Kenji Ikuta (Riken, Japan). For each PCR reaction, TaqMan probes (5′-VIC-CTT ACC CGA CTG AGG CAA-3′ for TREC, 5′-FAM-CTT ACC CGA CTG AGG CAA-3′ for albumin) were co-amplified with the DNA of interest.

A

B

C

D

E

F

Fig. 2. Long-term lymphocyte reconstitution after AHSCT. Reconstitution analysis of (A) total lymphocytes, (B) CD3+ T-cells, (C) CD3+CD4+ T-cells, (D) CD3+CD8+ T-cells, (E) CD4:CD8 ratio and (F) CD19+ B-cells following AHSCT. T- and B-lymphocytes were immunophenotyped by flow cytometry. Values are presented as absolute cell counts (cells/μL). *: P < 0.05 between marked time point vs pre-transplant. Boundaries of the boxes indicate the 25th and 75th percentiles, lines within the boxes indicate the medians, and whisker marks indicate the 10th and 90th percentiles. Statistical test performed was mixed linear regression model.
gifted by Dr. NgaiKa-Leung (Northwestern University, EUA). Standard curve was performed in triplicates.

Samples were prepared in duplicate using 8.85 μL of DEPC water, 12.5 μL of TaqMan® Universal PCR Master mix, 0.65 μL of probe (2.5 μM), 1 μL of forward primer (5 μM), 1 μL of primer reverse primer (5 μM) and 2 μL of target DNA. Real-time PCR was carried out at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 °C for 1 min. The number of TREC molecules in each sample was divided by the number of endogenous gene copies (for normalization), and results were expressed as TREC molecules/200 ng DNA.

2.5. Data analysis

For lymphocyte subpopulations, TREC and EDSS data comparison, we used the linear regression model with mixed effects (random and fixed effects) [36]. For variable frequency, we used a logarithmic transformation to make the data fit the proposed model. Adjustments were made with SAS® 9.0 software, using the PROC MIXED. For lymphocyte subpopulations (numbers and frequency) and TREC level comparisons, we used the post-test by orthogonal contrasts. EDSS score comparisons were performed by 2-way analysis of variance (ANOVA) with Bonferroni post-test to compare replicate means by row.

Lymphocyte subpopulations for each patient group were compared through quantification of the Area Under the Curve (AUC). The AUC of each lymphocyte subpopulation or marker was calculated by the trapezoid method, using as reference each individual baseline measurement. The trapezoidal areas between the baseline and 2, 3, 6, 9, 12, 18, 24, 30, 36, 42, 48, 54, 60 and 72 months post-transplantation time points corresponding to each patient were summed to obtain the AUC. Data were compared by two-tailed Mann-Whitney test. Results were reported as mean ± SD. Statistical significance was set at 5% (P < 0.05).

Fig. 3. Early expansion of memory CD8+ T cells after AHSCT. Reconstitution analysis of (A) CD4+CD27+CD45RO− naive T-cells, (B) CD8+CD27+CD45RO− naive T-cells, (C) CD4+CD27−CD45RO+ central memory T-cells, (D) CD8+CD27−CD45RO− central memory T-cells, (E) CD4+CD27−CD45RO+ effector memory T-cells and (F) CD8+CD27−CD45RO+ effector memory T-cells, following AHSCT. Lymphocytes were immuno phenotyped by flow cytometry. Values are presented as absolute cell counts (cells/μL). *: P < 0.05 between marked time point vs pre-transplant. Boundaries of the boxes indicate the 25th and 75th percentiles, lines within the boxes indicate the medians, and whisker marks indicate the 10th and 90th percentiles. Statistical test performed was mixed linear regression model.
3. Results

3.1. Neurological evaluations and disease progression following AH SCT

At enrollment for AH SCT, 28 (76%) patients had secondary progressive (SP-MS), 3 (8%) primary progressive (PP-MS) and 6 (16%) relapsing-remitting (RR-MS) forms of disease presentation. All patients were refractory to first line therapy. Disease duration ranged from 1 to 31 (8.9 ± 6.4) years and EDSS scores ranged from 3.0 to 7.0 (5.6 ± 1.2). Mean subject follow-up was 68.5 ± 13.9 months.

Combined analyses of all patients revealed that EDSS values (mean ± SD) did not significantly change after AH SCT, from 5.6 ± 1.2 at baseline, to 5.3 ± 1.4 at 1-year, 5.5 ± 1.5 at 2-years, 5.5 ± 1.5 at 3-years, 5.7 ± 1.6 at 4-years and 5.7 ± 1.5 at last clinical evaluation (Fig. 1B). However, when patients were clustered according to clinical outcomes after transplantation, comparing last visit EDSS scores with those from baseline, 40% (n = 15) presented an increase in the EDSS values (progression) and 60% (n = 22) showed a decrease or stabilization (non-progression). At baseline, groups were not different for EDSS scores, subject age and disease forms (Table 1), but patients from the non-progression group had shorter disease duration (mean ± SD) before AH SCT than those from the progression group (7.22 ± 4.38 vs 11.47 ± 8.03 years, p < 0.05). Furthermore, starting at one year after AH SCT and throughout the whole follow-up, EDSS scores were significantly higher (p < 0.05) in the progression group, compared with the non-progression group (Fig. 1C).

3.2. Persistent reduction of CD4:CD8 ratio after transplantation

High dose immunosuppression promoted a decrease in total lymphocyte numbers from 3 to 12 months after transplantation when compared to baseline (Figs. 2A and S1A). Furthermore, complete reconstitution of CD3+ T-cell counts was not detected up to 72-month follow-up, indicating that AH SCT induced long-term T-cell lymphopenia (Figs. 2B and S1B).

We also did not detect reconstitution of CD3+CD4+ T-cell counts to baseline levels in any of the patient groups until last follow-up (Figs. 2C and S1C). In contrast, normalized CD3+CD8+ T-cell counts were observed in all patients shortly after transplantation (2, 3, 18 and 24 months) (Fig. 2D), especially in the non-progression group at 2 months after AH SCT (Fig. S1D). As consequence, CD4:CD8 ratios were persistently reduced during the entire follow-up (Fig. 2E) in both groups (Fig. S1E).

Furthermore, while CD19+ B-cell counts were not altered in the early periods after AH SCT, their numbers increased at 18 and 24 months post-transplantation (Fig. 2F), mainly in the non-progression group (Fig. S1F). When AUC values from each group were compared, significantly higher CD19+ B-cell counts were detected in the non-progression group (P = 0.005) (Fig. S1F).

3.3. Early expansion of CD8+ memory T-cells after transplantation

All patients presented impaired CD4+ and CD8+ naive CD27+CD45RO+ T-cell reconstitution until 54 months post-transplantation (Figs. 3A and S1A). However, the CD8+CD27−CD45RO+ central-memory T-cells returned to baseline and remained stable for most of the time post-transplantation (Figs. 3D and S2D). CD4+ and CD8+CD27−CD45RO− effector memory T-cells counts minimally changed throughout follow-up (Figs. 3E, F, S2E and F).

3.4. Late thymic reactivation after transplantation

To assess thymic function, recent thymic emigrant (RTE) cells and T-cell receptor excision circles (TREC) were quantified in the peripheral blood [37]. TREC analysis evidenced thymic reactivation at two years post-transplantation (Fig. 4A) and no differences were found between patient groups (Fig. S3A). However, the number of CD3+CD4+CD45RA+CD31+ (RTE) cells remained below baseline until last follow-up in both groups (Figs. 4B and S3B).

3.5. Expansion of regulatory T-cells after transplantation

The population of CD8+CD28−CD57+ T-cells increased from 6 to 48 months post-transplantation (Figs. 5A and S4A). Likewise, FoxP3-expressing CD4+CD25hi Treg counts increased from 12 to 36 months after transplantation (Figs. 5B and S4B). To further evaluate the suppressive phenotype of reconstituted CD4+CD25hi Treg, we assessed expression of GITR and CTLA-4 (Table 2). GITR expression in CD4+CD25hi Treg increased in both groups at 24, 36, 42 and 48 months, while CTLA-4 expression increased at 6, 12, 24 and 30 months post-transplantation, mainly in the non-progression group. However, CTLA-4 expression was higher in the progression group at 24 months as compared to non-progression patients. (Table 2).
3.6. Expansion of CD8+ PD-1+ T-cells is related to improved neurological outcomes

The AUC of CD3+CD4+PD-1+ T-cell counts and PD-1 mean fluorescence intensity (MFI) in the CD3+CD4+ T-cells were not different when patient groups were compared (Fig. 6A, B and C). In contrast, the AUC of CD3+CD8+PD-1+ T-cell numbers in the non-progression group was higher than in the progression group (P = 0.032). PD-1 MFI in the CD3+CD8+ T-cells was not different between groups (Fig. 6A, B and C). In addition, PD-1 MFI in CD19+ B-cells increased only in patients from the non-progression group (P < 0.0007), but no statistical difference was observed between groups regarding CD19+PD-1+ B-cell counts (Fig. 6G, H and I).

4. Discussion

In the present study, we evaluated long-term immune reconstitution in MS patients after AHSCT, aiming to identify immune correlates of clinical response. Patients were clustered into two groups, according to neurological outcomes after AHSCT and were evaluated for immune reconstitution. Our findings corroborate previous studies, showing thymic reactivation and increased numbers of regulatory T-cells with higher expression of CTLA-4 and GITR after AHSCT. However, these results were not associated with different clinical outcomes.

Table 2: Expression of immunoregulatory molecules on CD4+ T cells of multiple sclerosis patients at baseline and after autologous hematopoietic stem cell transplantation.

<table>
<thead>
<tr>
<th>Time after AHSCT</th>
<th>Marker</th>
<th>Group</th>
<th>Before AHSCT</th>
<th>AUC 6 mo</th>
<th>AUC 12 mo</th>
<th>AUC 24 mo</th>
<th>AUC 36 mo</th>
<th>AUC 48 mo</th>
<th>AUC 60 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 mo</td>
<td>GITR (MFI)</td>
<td>Non-progression</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
</tr>
<tr>
<td>36 mo</td>
<td>GITR (MFI)</td>
<td>Non-progression</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
</tr>
<tr>
<td>60 mo</td>
<td>GITR (MFI)</td>
<td>Non-progression</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
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<td>24.22 ± 11.03 (18)</td>
<td>24.22 ± 11.03 (18)</td>
</tr>
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</table>

AHSCT: autologous hematopoietic stem cell transplantation. AUC: area under the curve. MFI: mean fluorescence intensity. Immunophenotype was performed by flow cytometry analysis. Data are presented as mean ± SD (number of patients). Statistical test performed was mixed linear regression model.

*P < 0.05: between all patients in marked time point vs before AHSCT.
†P < 0.05: between marked time points in non-progression or progression group when compared to the same group population before AHSCT.
#P < 0.05: between groups (non-progression vs progression) at marked period.
& The 30 and 42 m time points were omitted to improve table presentation.
Here, we demonstrate unprecedented findings. Firstly, thymic rebound is a late and transitory phenomenon. Second, numbers of regulatory T-cells and PD-1-expressing CD8+ T-cells increased early, but temporarily, after AHSCST. We ascribe these findings to the long-term follow-up of our MS patients. Moreover, higher expression levels of the PD-1 molecule were associated with better clinical outcomes.

Lymphopenia following high dose immunosuppression is considered a nonspecific effect of AHSCST, affecting most lymphocyte subpopulations. Indeed, in our patients, CD3+ T-cell counts dropped after AHSCST and did not return to baseline levels for the entire follow-up period. Interestingly, the non-progression group had longer lymphopenic periods when compared to the progression group, suggesting that long-term lymphopenia may be related to better clinical outcomes. These findings contradict a previously reported study, where seven MS patients that remained neurologically stable for 24 months after AHSCST did not present persistent lymphopenia. Nevertheless, proper comparisons between studies may be precluded by differences in the transplant procedure, such as conditioning regimens and graft selection.[23,26,27].

We also assessed thymic function by quantification of recent-thymic emigrants (RTE) CD3+CD4-CD45RA-CD31+ cells and TREC levels in the peripheral blood from MS transplanted patients. While RTE counts did not reach baseline values at any time point after transplantation, TREC levels increased between 24 and 30 months after AHSCST, indicating a late and transient thymic rebound in transplanted MS patients. Comparably, previous studies have already reported that TREC levels return to baseline values at 12 and 24 months after transplantation, respectively.[23,39]. In accordance to other studies, our results demonstrate that thymic function is not completely eliminated with age and that thymic reactivation after AHSCST contributes to the immune recovery of MS patients.[37,40–43].

Several short follow-up studies have demonstrated expansion of regulatory T-cells (Treg) after AHSCST.[27,29,44–46]. We show that AHSCST promotes significant, albeit short-lived, expansion of peripheral CD4+CD25hiFoxp3+ Tregs from 6 to 36 months post-transplantation. Moreover, we observed higher expression of the immune regulatory molecules CTLA-4 and GITR after transplantation, which are associated with the immunosuppressive potential of Tregs.[47,48]. Although additional suppression assays should be further performed to fully determine transplant-induced regulatory function changes, our results are underscored by a recent study that demonstrates effective recovery of Treg suppressive function following AHSCST in patients with systemic sclerosis.[44].

We likewise observed sustained expansion of CD8+CD28−CD57+ suppressor T-cells in our MS patients, also from 6 to 36 months post-AHSCST. Expansion of this CD8+ T-cell suppressor subpopulation has already been observed in MS patients after AHSCST.[23,27,29]. This lymphocyte subpopulation has immunoregulatory properties mediated via soluble factors and possibly contributes to restoration of immune tolerance after AHSCST.[49–52].

Transient increase of PD-1+ T-cells has already been described in a small MS patient cohort that underwent AHSCST.[29]. Here, we were able to demonstrate early increase of CD8+PD-1+ T-cell numbers and of PD-1-expressing CD19+ B-cells in a group of MS patients with better neurological outcomes (non-progression group). These findings indicate that PD-1 may be a marker for good clinical response after AHSCST.

Interestingly, increased CD3+CD8+PD-1+ T-cell frequency was found concomitant with inverted CD4:CD8 ratios and with expansion of CD8+CD27−CD45RO+ effector-memory T-cells and CD8+CD28−CD57+ suppressor T-cells, suggesting that these lymphocyte subpopulations could be expressing PD-1 molecules. Indeed, other studies demonstrate that PD-1 is mainly expressed by effector-memory T cells.[31,53–55].

The PD-1:PDL1 pathway is essential for regulation of immune responses in several neurological diseases, such as MS, stroke and Alzheimer’s disease.[56,57]. In a mouse model of experimental allergic encephalomyelitis (EAE), blockade of PDL-1 or PDL-2 accelerates disease course and severity, as well as the number of lymphocytes found in the CNS.[58]. Clinical studies have also demonstrated an association of PD-1 deficiency with progression of MS.[57,59,60]. Additionally, patients with stable MS have higher expression of PD-1 in myelin basic protein (MBP)-specific CD4+ and CD8+ T-cells when compared to patients with acute relapses.[57,60].

Absence of PD-1 is associated with development of a multi-organ autoimmune after lymphopenia-induced proliferation (LIP).[61]. Interestingly, the disorder was only developed by mice that were treated with PD-1−/− hematopoietic stem cells (HSC), and not by those reconstituted with mature PD-1−/− lymphocytes, indicating that the PD-1 expression is critical to establish self-tolerance of the recent-thymic emigrants.[61]. Additionally, the autoimmune disease driven by LIP in this disease model was not associated with abnormal function or expansion of Treg.[62]. Altogether, these results suggest that PD-1 may play a negative regulatory role to control rapidly proliferating and potentially pathogenic autoreactive CD8+ T-cells during homeostatic reconstitution of lymphopenic environments.[63].

In addition, transcriptional signatures revealed that CD8+ T-cell exhaustion (CD8+ T-cells with high PD-1 expression) predict poor clinical outcomes in viral diseases but, conversely, better prognosis in autoimmune diseases. These observations suggest that induction of cell exhaustion may be a new therapeutic strategy for autoimmune and inflammatory diseases.[64].

5. Conclusions

Here, we demonstrate that the non-progression and progression MS patient groups present similar profiles of long-term immune reconstitution after AHSCST. Altogether, we show that long-term immune recovery is driven by early homeostatic expansion of central- and effector-memory CD8+ T-cell subpopulations in the absence of complete CD3+ T-cell reconstitution to baseline levels, resulting in prolonged inversion of the CD4:CD8 ratio. Late and transient thymic rebound and expansion of CD8+CD28−CD57+ and of CD4+CD25hiFoxp3+ regulatory T-cell subsets, with increased expression of both GITR and CTLA-4, were detected in both groups and did not influence distinct clinical responses.

We suggest early expansion of CD8+PD-1+ T-cells and of PD-1-expressing CD19+ B-cells as immunological correlates of favorable clinical outcome in MS patients treated with AHSCST. We believe that improved PD-1 inhibitory signaling is one possible immunoregulatory mechanism by which AHSCST controls autoimmunity and restores immune tolerance in MS patients, along with increased regulatory T-cell numbers. These findings may stimulate future therapeutic interventions in MS patients by manipulation of the PD-1/PDL1 pathway.

Fig. 6. Early expansion of CD8+PD-1+ T-cells in multiple sclerosis patients with better neurological outcome after AHSCST. Reconstitution analysis of all MS patients together (A/D/G) or patient groups according to neurological outcomes (B/C/E/F/H/I). CD3+CD4+PD1− T-cell (A/B/C); CD3+CD8+PD1− T-cell (D/E/F) and CD19+PD1− B-cell (G/H/I) counts following AHSCST. Lymphocytes were immunophenotyped by flow cytometry. Values are presented as absolute counts (cells/μl), PD-1 mean fluorescent intensity (MFI) or area under curve (AUC) analysis. *: P < 0.05 between marked time point vs pre-transplant in the non-progression group. #: P < 0.05 between marked time point vs pre-transplant in the progression group. #: #: P < 0.05 between groups at identified period. (Left) Boundaries of the boxes indicate the 25th and 75th percentiles, lines within the boxes indicate the medians, and whisker marks indicate the 10th and 90th percentiles. (Right) Data is shown as mean ± SD for patient groups according to clinical outcome. Statistical test performed was mixed linear regression model. For AUC comparison Mann-Whitney test was performed. n.s.: non significant.
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Conflict of interest
The authors declare no commercial or financial conflict of interest.

Author contributions
Malamgrim and MC Oliveira are the guarantor of this work, i.e., had full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: JCV, BPS, KCRM, MCO.

Study supervision: MCO, KCRM.

Acquisition of clinical data: AAB, DGB, CTG, VDM, BPS, MCO.

Acquisition of laboratory data: LCMA, JTCA, GLVO, GTS, ESR, PRVPB.

KCRM. Analysis and interpretation of data: LCMA, JTCA, KCRM, MCO.

Administrative, technical, or material support: DTC.

Obtained funding: JCV, DTC, MCO, KCRM.

Drafting of the manuscript: LCMA, JTCA, KCRM, MCO.

Critical revision of the manuscript for important intellectual content: LCMA, JTCA, KCRM, MCO, AAB, DGB, CTG, VDM, BPS, DTC, GLVO, GTS, ESR, PRVPB.

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References


