

ORIGINAL ARTICLE

Autologous hematopoietic SCT normalizes miR-16, -155 and -142-3p expression in multiple sclerosis patients

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Autologous hematopoietic SCT (AHSCT) has been investigated in the past as a therapeutic alternative for multiple sclerosis (MS). Despite advances in clinical management, knowledge about mechanisms involved with clinical remission post transplantation is still limited. Abnormal microRNA and gene expression patterns were described in MS and have been suggested as disease biomarkers and potential therapeutic targets. Here we assessed T- and B-cell reconstitution, microRNAs and immunoregulatory gene expression after AHSCT. Early immune reconstitution was mainly driven by peripheral homeostatic proliferation. AHSCT increased CD4⁺CD25^{hi}FoxP3⁺ regulatory T-cell counts and expression of CTLA-4 and GITR (glucocorticoid-induced TNFR) on CD4⁺CD25^{hi} T cells. We found transient increase in exhausted PD-1⁺ T cells and of suppressive CD8⁺CD28⁻CD57⁺ T cells. At baseline, CD4⁺ and CD8⁺ T cells from MS patients presented upregulated miR-16, and miR-155 and miR-142-3p and downregulated *FOXP3*, *FOXO1*, *PDCD1* and *IRF2BP2*. After transplantation, the expression of *FOXP3*, *FOXO1*, *PDCD1* and *IRF2BP2* increased, reaching control levels at 2 years. Expression of miR-16, miR-155 and miR-142-3p decreased towards normal levels at 6 months post therapy, remaining downregulated until the end of follow-up. These data strongly suggest that AHSCT normalizes microRNA and gene expression, thereby improving the immunoregulatory network. These mechanisms may be important for disease control in the early periods after AHSCT.

Bone Marrow Transplantation (2015) 50, 380–389; doi:10.1038/bmt.2014.277; published online 8 December 2014

INTRODUCTION

In recent years, autologous hematopoietic SCT (AHSCT) has been found to be able to suppress inflammatory activity in patients with multiple sclerosis (MS) and induce sustained disease control.^{1–3} Clinical remission after AHSCT depends on qualitative immunological changes that promote immune tolerance. Restoration of the CD4⁺CD25⁺ regulatory T-cell (Treg) immune compartment^{4,5} and profound resetting of the adaptive immune system⁶ have been described. Suppression of inflammatory activity in MS patients undergoing AHSCT does not depend on persisting lymphopenia and is associated with *de novo* regeneration of the T-cell repertoire.^{7,8} Additionally, decreased T-helper type 17 response⁹ and depletion of IL-17-producing mucosal-associated invariant T cells have been shown after AHSCT in MS patients.¹⁰

Autoimmune diseases such as MS result from failure of tolerance mechanisms to prevent the expansion of pathogenic T cells. Dysfunctional CD4⁺CD25^{hi} Tregs¹¹ and abnormalities in *FOXP3* mRNA and protein expressions in peripheral T cells¹² are described as part of this scenario. In recent years, microRNAs (miRNAs/miRs) have emerged as an important gene expression controller in health and disease.

MiRNAs are small noncoding RNA molecules that modulate the expression of multiple protein-encoding genes at the

posttranscriptional level and are involved in the regulation of immunity, among other functions.^{13,14} Loss or dysregulation of certain individual miRNAs severely compromises immune development and response, and may lead to disorders such as autoimmunity and cancer.¹⁵ Abnormal miRNA function has been detected in several human diseases, including MS,¹⁶ and is suggested as a disease biomarker and a potential therapeutic target.¹⁵ In MS patients, aberrant expression of miR-16,¹⁷ miR-155¹⁸ and miR-142-3p¹⁹ has been described. These miRNAs are involved in the regulation of T-cell activation, and their overexpression is related to T-cell-mediated autoimmune inflammation.^{13,14} Moreover, they control Treg function and the expression of *FOXP3*,^{20,21} an important gene involved with self-tolerance.

In the context of AHSCT, molecular mechanisms underlying clinical effectiveness are poorly understood.²² In our study, we demonstrate, for the first time, that AHSCT promotes persistent downregulation of disease-related miRs, while increasing the expression of their predicted target genes. Moreover, we detected increased Treg counts, with higher expression of immunoregulatory molecules. In the context of homeostatic expansion and absence of thymic reactivation in the two first years that follow AHSCT, we propose that regulatory cells are in fact non-regulatory

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Received 15 August 2014; revised 17 October 2014; accepted 21 October 2014; published online 8 December 2014

Table 1. Demographic and clinical data of the MS patients

Patient code	Age at AHSCT (years)	Gender	MS duration ^a (years), PreTx	MRI in preceding 1 year	MS course	Previous therapy	MRI enhancing lesions after HSCT	EDSS at inclusion, baseline	EDSS score, 6 mo nth after AHSCT	EDSS score, 1 year after AHSCT	EDSS score, 2 years after AHSCT
MS 01	48	M	31	Gd ⁻	RR	CST, IFN, GA	Gd ⁻	3.0	4.5	4.5	4.5
MS 02	49	M	5	Gd ⁻	RR	CST, IFN, GA	Gd ⁻	4.0	4.0	4.0	4.0
MS 03	38	F	1	Gd ⁺	RR	CST, IFN, GA	NA	4.5	4.5	4.5	4.5
MS 04	40	F	2	Gd ⁺	RR	CST, IFN, GA	Gd ⁻	3.0	3.0	3.0	3.0
MS 05	22	F	4	Gd ⁻	RR	CST, IFN, GA	NA	3.0	3.0	3.0	3.0
MS 06	39	M	4	Gd ⁺	SP	CST, IFN, GA	Gd ⁻	5.5	5.0	5.0	5.0
MS 07	55	M	12	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	3.5	6.0	4.0	4.0
MS 08	42	F	11	Gd ⁺	SP	CST, IFN, GA	Gd ⁻	5.0	5.5	5.5	6.0
MS 09	41	F	5	Gd ⁺	SP	CST, IFN, GA	Gd ⁻	6.5	6.0	6.0	6.0
MS 10	43	M	9	Gd ⁺	SP	CST, IFN, GA	Gd ⁻	6.5	6.5	6.5	6.5
MS 11	26	F	4	Gd ⁺	SP	CST, IFN, GA	Gd ⁺	6.5	6.5	6.5	7.0
MS 12	33	F	17	Gd ⁺	SP	CST, IFN, GA	Gd ⁻	6.5	5.0	4.0	4.0
MS 13	44	M	10	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	5.5	3.5	3.5	3.5
MS 14	19	F	8	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	6.0	6.0	6.0	6.0
MS 15	31	F	6	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	6.0	6.0	6.0	6.5
MS 16	40	F	8	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	6.5	6.5	6.5	6.5
MS 17	41	F	10	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	6.0	4.5	5.0	6.0
MS 18	43	M	4	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	6.0	4.0	4.5	4.0
MS 19	47	F	6	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	6.0	6.0	6.0	6.0
MS 20	20	F	7	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	6.5	6.5	6.5	6.5
MS 21	43	F	15	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	6.5	4.0	4.0	4.0
MS 22	53	F	5	Gd ⁻	SP	CST, IFN, GA	Gd ⁻	6.5	4.5	4.5	4.5
MS 23	21	M	2	Gd ⁻	SP	CST, IFN, GA	NA	6.0	4.5	NA	4.5
MS 24	44	F	9	Gd ⁻	PP	CST, IFN, GA	Gd ⁻	6.5	6.0	6.0	6.0
Mean	38.4	—	8.1	—	—	—	—	5.4	5.1	5.1	5.1
Median	41	—	6.5	—	—	—	—	6.0	5.25	5	5.5

Abbreviations: AHSCT = autologous hematopoietic SCT; CST = i.v. corticosteroids; EDSS = expanded disability status scale; F = female; GA = glatiramer acetate; Gd⁻ = absence of gadolinium-enhancing lesions; Gd⁺ = presence of gadolinium-enhancing lesions; M = male; MRI = magnetic resonance imaging; MS = multiple sclerosis; NA = not available; preTx = pretransplantation period; PP = primary progressive; RR = relapsing-remitting; SP = secondary progressive. ^aDuration of MS from the time of diagnosis.

cells that express *FOXP3* under the influence of normalized miR expression.

Our results thus suggest that clinical remission in MS patients after AHSCT may be associated with improvement of immunoregulatory mechanisms.

PATIENTS AND METHODS

MS patients and transplant protocol

Twenty-four MS patients refractory to first-line therapy were selected for AHSCT. The protocol was approved by the National Ethics Committee and all patients signed informed consent. Detailed inclusion and exclusion criteria, mobilization regimens and clinical evaluations have been reported previously.²³ Briefly, the immunoablative transplant conditioning included four doses of CY (50 mg/kg on days -5, -4, -3 and -2) and rabbit antithymocyte globulin (thymoglobulin; Genzyme, Cambridge, MA, USA) (4.5 mg/kg divided in days -5 to -1) followed by infusion of unmanipulated peripherally collected autologous CD34⁺ cells, mobilized with 2 g/m² CY and granulocyte-colony-stimulating factor. Demographic, clinical and follow-up data from all patients are listed in Table 1.

After AHSCT, patient follow-up included clinical assessments of general health and of neurological disability through the Expanded Disability Status Scale (EDSS) and magnetic resonance imaging (MRI). Blood was drawn at each appointment for immunological monitoring. For this present study, clinical and immunological data were obtained within the first 2 years after AHSCT.

Blood samples from nine age- and sex-matched healthy controls were analyzed for gene and miRNA expression.

Immunophenotypic analyses

Patients had whole PB samples analyzed for cell phenotyping by flow cytometry (FACSCalibur Flow Cytometer; Becton-Dickinson, San Jose, CA, USA), before and at 6 months and 1 and 2 years after transplantation. Immunophenotypical analyses of lymphocyte subsets are provided in the Supplementary Data. Data were analyzed by the FlowJo software (FlowJo, TreeStar, OR, USA). Results are expressed as absolute cell numbers (cell per μ L) or relative expression (%).

PBMC isolation and miRNA/gene analyses

Cell purification. PBMCs were isolated from all MS patients and healthy controls by Ficoll density gradient centrifugation (Sigma-Aldrich, St Louis,

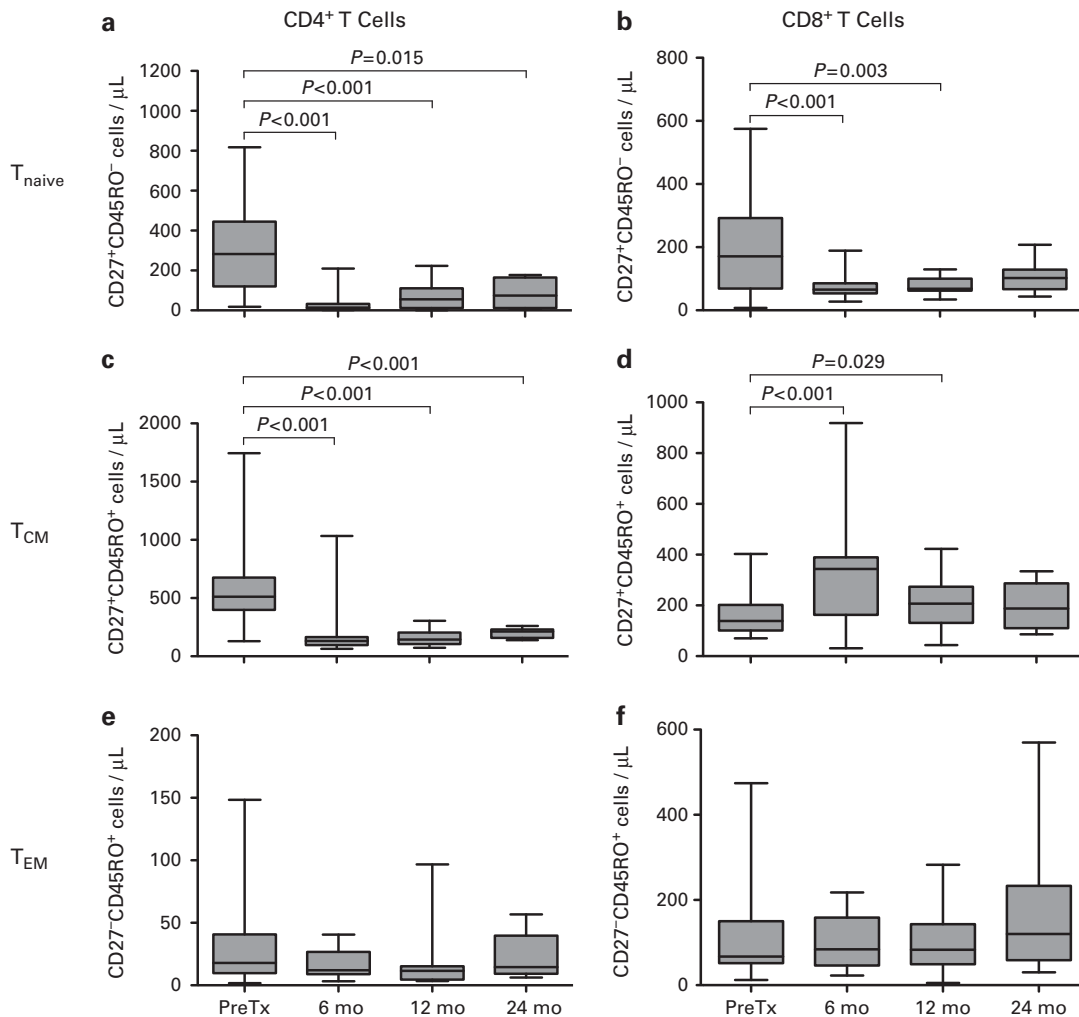


Figure 1. Naive and memory T-cell reconstitution. AHSCT promotes reduction of CD4⁺, CD8⁺ T_{naive} and CD4⁺ T_{CM}, but CD8⁺ T_{CM} increase. Numbers are presented as absolute values (cells per μ L) of T-cell sub-populations at pretransplantation (PreTx) and follow-up time points. Subsets of flow cytometry immunophenotyped CD4⁺ T-cell (left) and CD8⁺ T-cell (right) counts, classified as T_{naive}: CD27⁺CD45RO⁻ naive T cells (a and b); T_{CM}: CD27⁺CD45RO⁺ central-memory T cells (c and d); and T_{EM}: CD27⁻CD45RO⁺ effector-memory T cells (e and f). The boundaries of the boxes indicate the 25th and 75th percentiles, the lines within the boxes indicate the median and the whiskers mark the 10th and the 90th percentiles. Mixed linear regression model was used as the statistical test. PreTx, *n* = 23; 6 months (6 mo), *n* = 16; 12 months (12 mo), *n* = 15; 24 months (24 mo), *n* = 15.

MO, USA). CD4⁺ and CD8⁺ T lymphocytes were isolated by positive selection using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the obtained cell subsets was checked by flow cytometry and was found to be >95%.

RNA extraction and real-time PCR. Total RNA was extracted from CD4⁺ and CD8⁺ T cells by Trizol (Invitrogen, Carlsbad, CA, USA) method, according to the manufacturer's instructions. RNA quantity and quality were verified using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis.

TaqMan assays were used for quantitative analysis of mature miRNA and gene expression, as well as endogenous controls. Further information is

provided as Supplementary Data. Expression values were calculated using the $2^{-\Delta\text{Ct}}$ method.²⁴

Statistical analysis

Immune reconstitution data were compared by linear regression model with mixed effects (random and fixed effects) using the post test by orthogonal contrasts. For variable frequency, we used a logarithmic transformation to make the data fit the proposed model. Adjustments were made using the PROC MIXED (SAS 9.0 software, Cary, NC, USA). To compare EDSS values and gene and miRNA expressions, we used one-way analysis of variance followed by Tukey's multiple test. For statistical

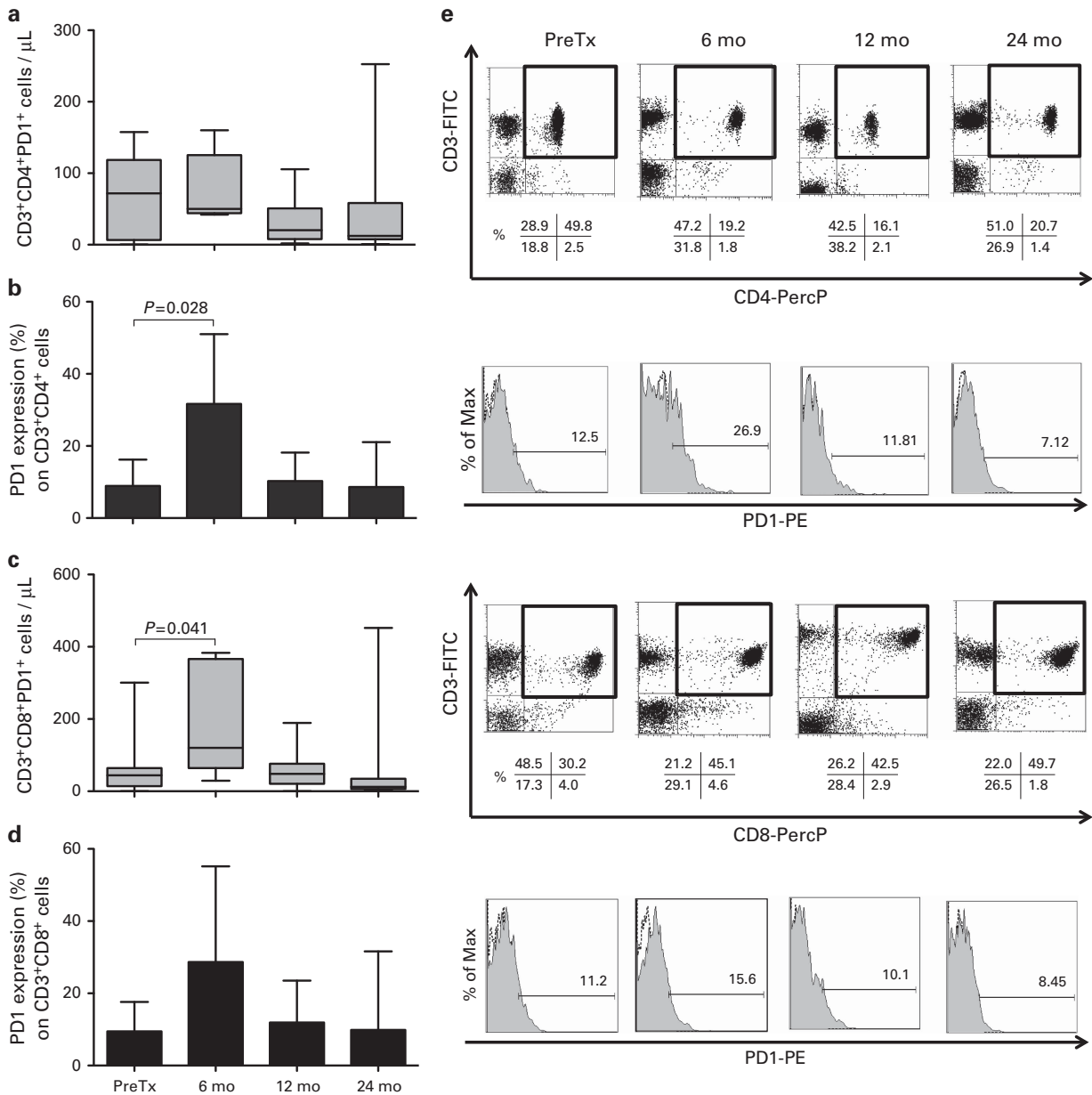


Figure 2. Transient increase of exhausted T cells. Increase of exhausted CD3⁺CD8⁺ T-cell counts and higher PD-1 expression on CD3⁺CD4⁺ T cells at 6 months after AHSCT. Numbers are presented as absolute values (cells per μL) of T-cell sub-populations or PD-1 expression (%) at pretransplantation (PreTx) and follow-up time points. Flow cytometry immunophenotyped CD3⁺CD4⁺PD-1⁺ (a) and CD3⁺CD8⁺PD-1⁺ (c) exhausted T-cells counts, and PD-1 expression (%) on CD3⁺CD4⁺ (b) and CD3⁺CD8⁺ T cells (d). Gating strategy and expression of PD-1 on CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells of one representative patient (e). The boundaries of the boxes indicate the 25th and 75th percentiles, the lines within the boxes indicate the median and the whiskers mark the 10th and the 90th percentiles. Bars represent mean \pm s.d. Mixed linear regression model was used as statistical test. PreTx, $n = 14$; 6 months (6 mo), $n = 7$; 12 months (12 mo), $n = 9$; 24 months (24 mo), $n = 12$.

analyses and graphical presentations, we used GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA) software for Mac OS X.

RESULTS

Clinical evaluations after AHSCT

All twenty-four patients successfully completed the proposed treatment protocol. Before and after AHSCT clinical data are summarized in Table 1. Only one patient (MS 12) presented acute inflammatory lesions (gadolinium-enhancing lesions) on MRI at 1.5 years post transplantation, with subsequent neurological worsening, quantified by EDSS, at the 2 years post therapy time point. The remaining patients were free of relapses for mean follow-up of 22.25 months (range: 6–24 months) after AHSCT, in the absence of any immunosuppressive or modulatory treatment.

Leukocyte recovery

Total and T lymphocytes recovered at 6 months and 1 year post transplantation, respectively (Supplementary Figures 1A and B). No reconstitution of CD4⁺ T-cell counts to pretransplant levels was observed until the end of the 2-year follow-up. In contrast, CD8⁺ T-cell counts did not change during the first year, becoming higher than baseline only at 2 years after AHSCT, leading to reduction of CD4/CD8 ratios (Supplementary Figures 1C–E). Additionally, B-cell counts reconstituted to baseline values at 6 months, achieving higher levels at 2 years posttransplantation (Supplementary Figure 1F).

Immune reconstitution by peripheral T-cell expansion

To determine changes in specific CD4 and CD8 sub-populations, we evaluated the naive (T_{naive}), central-memory (T_{CM}) and effector-memory (T_{EM}) T-cell reconstitution after AHSCT. As expected, high-dose immunosuppression reduced CD4⁺ T_{CM} cell counts during the entire follow-up (Figure 1c). Conversely, CD8⁺ T_{CM} counts increased within the first year of follow-up (Figure 1d). The immunosuppressive regimen did not ablate the CD4⁺ and CD8⁺ T_{EM} compartments (Figures 1e and f) but decreased naive T cells until the end of follow-up (Figures 1a and b). We also verified that CD3⁺CD4⁺CD45RA⁺CD31⁺ recent thymic emigrant counts were

reduced until 2 years after AHSCT (Supplementary Figure 2A), while the recent thymic emigrant/CD3⁺CD4⁺ proportion did not change (Supplementary Figure 2B).

Collectively, these results show that during the first 2 years after AHSCT, reconstitution of circulating T-cell pool is mainly due to expansion of remaining peripheral T cells.

Transient increase in exhausted T cells and higher PD-1 expression PD-1 is a costimulatory molecule expressed in exhausted dysfunctional T cells and has an important role in the regulation of immune response.²⁵ PD-1 increases IL-10 production, promoting lower proliferation and increased apoptosis of myelin basic protein-specific cells. Therefore, PD-1 is associated with disease remission in MS patients.²⁶ We observed that CD3⁺CD4⁺PD-1⁺ T-cell counts increased from (mean ± s.d.) 68.76 ± 57.63 cells per µL at baseline to 77.86 ± 49.98 cells per µL at 6 months post transplantation, which is not statistically significant (P=0.251; Figure 2a). Additionally, at 6 months post transplantation, we observed a 3.38-fold increase (P=0.028) of PD-1 expression on CD3⁺CD4⁺ T cells (Figures 2b–e) and a 3.26-fold increase (P=0.041; Figure 2c) in CD3⁺CD8⁺PD-1⁺ T-cell counts, when compared with pretransplant levels. A nonsignificant 2.96-fold increase of PD-1 expression was detected on CD3⁺CD8⁺ T cells from (mean ± s.d.) 9.66 ± 8.47% at baseline to 28.69 ± 26.51% at 6 months posttherapy (P=0.081; Figures 2d and e). No changes were observed in the CD19⁺PD-1⁺ B cells (Supplementary Figure 3).

These results show transient increase of exhausted T cells and higher PD-1 expression at 6 months post transplantation, probably as result of persistent self-Ag stimulation in the lymphopenic environment that immediately follows transplantation.²⁵ In this context, Thangavelu *et al.*²⁷ describe that PD-1 expression is required for systemic self-tolerance in newly generated T cells during the establishment of immune homeostasis after AHSCT, and may contribute to clinical remission.

Immunoregulatory cell kinetics

The CD8⁺CD28⁻CD57⁺ T cells, a senescent oligoclonal expanded sub-population with immunosuppressive properties,^{28,29} have

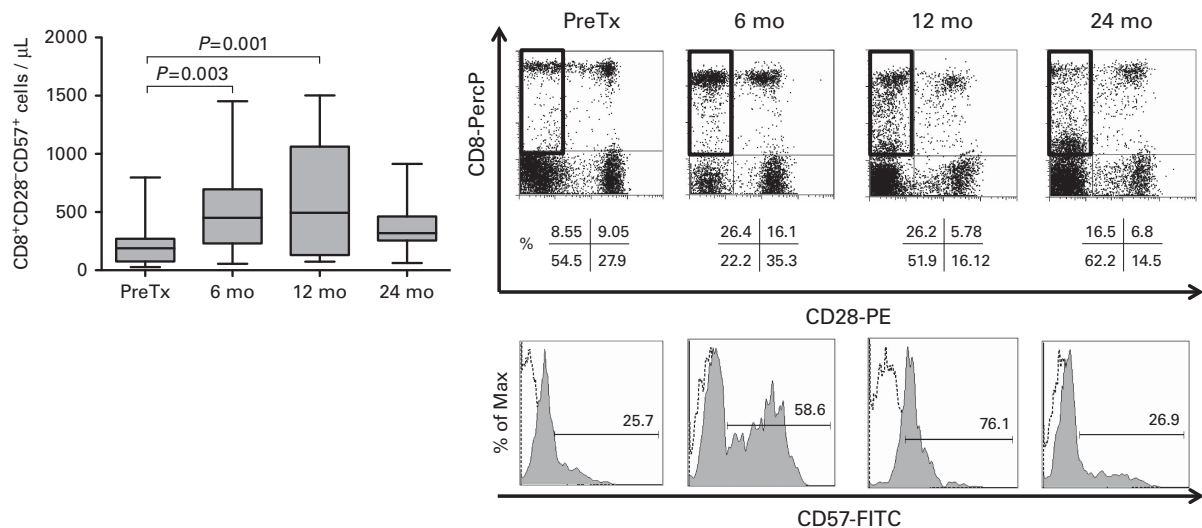


Figure 3. Expansion of regulatory CD8⁺CD28⁻CD57⁺ T cells after AHSCT. Numbers are presented as absolute values (cells per µL) of CD8⁺CD28⁻CD57⁺ suppressor T cells immunophenotyped by flow cytometry at pretransplantation (PreTx) and follow-up time points. (Left) The boundaries of the boxes indicate the 25th and 75th percentiles, the lines within the boxes indicate the median and the whiskers mark the 10th and the 90th percentiles. (Right) Gating strategy and expression of CD57 on CD8⁺CD28⁻ cells of one representative patient. Statistical test performed was mixed linear regression model. PreTx, n = 21; 6 months (6 mo), n = 13; 12 months (12 mo), n = 10; 24 months (24 mo), n = 13.

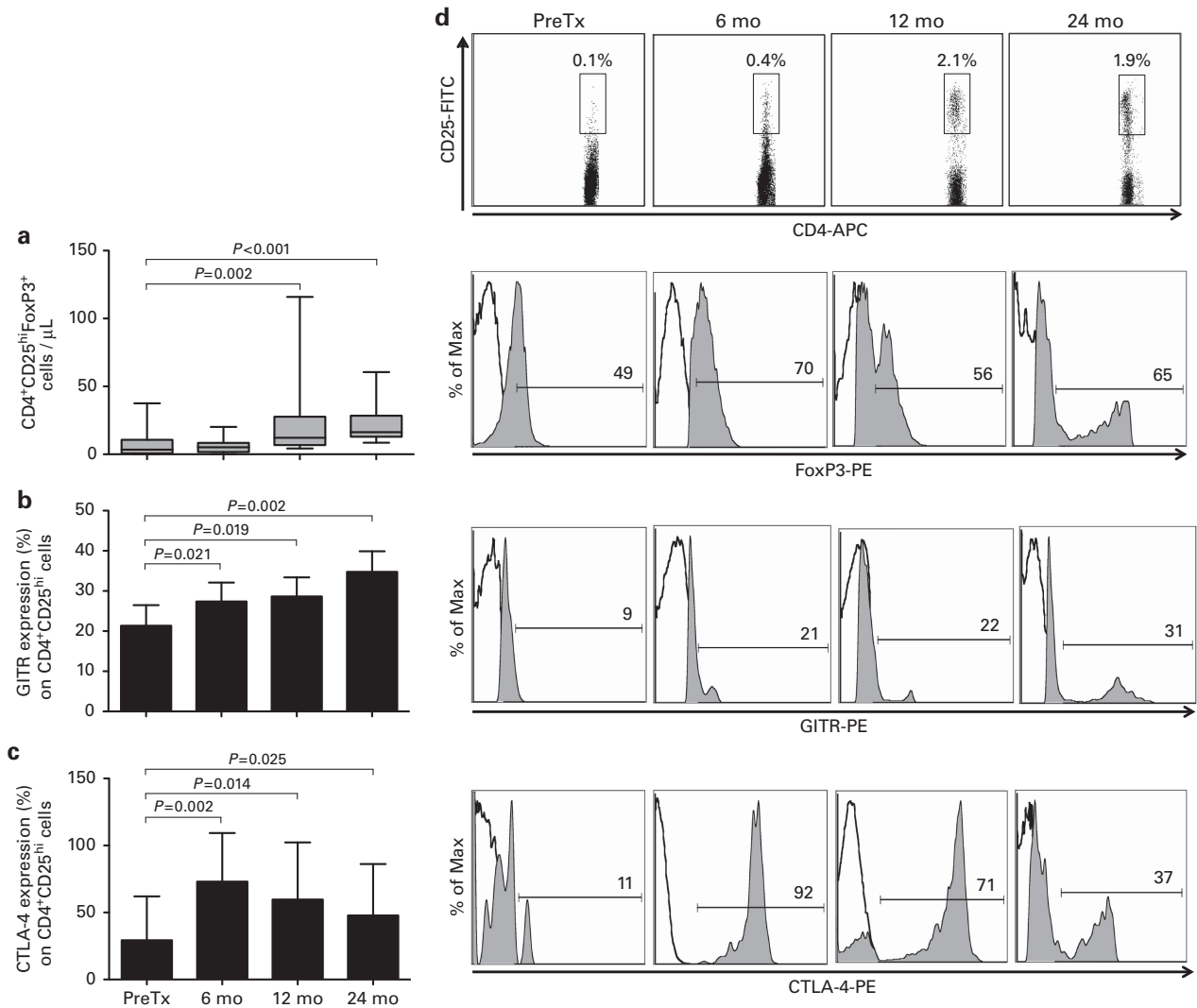


Figure 4. Increase of Tregs and higher immunosuppressive phenotype. Increase of Tregs counts and higher GITR/CTLA-4 expression after AHSCT. Numbers are presented as absolute values (cells per μ L) of Tregs or CTLA-4/GITR expression (%) at pretransplantation (PreTx) and follow-up time points. Flow cytometry immunophenotyped CD4⁺CD25^{hi}FOXP3⁺ Tregs (a), GITR (b) and CTLA-4 (c) expression on CD4⁺CD25^{hi} T cells. Gating strategy and expression of FOXP3, GITR and CTLA-4 on CD4⁺CD25^{hi} T cells of one representative patient (d). The boundaries of the boxes indicate the 25th and 75th percentiles, the lines within the boxes indicate the median and the whiskers mark the 10th and the 90th percentiles. Bars represent mean \pm s.d. Statistical test performed was mixed linear regression model. PreTx, $n = 21$; 6 months (6 mo), $n = 13$; 12 months (12 mo), $n = 10$; 24 months (24 mo), $n = 13$.

been shown to increase in the PB of MS patients after AHSCT.^{8,10} Indeed, in our study, we observed significantly increased values of CD8⁺CD28⁻CD57⁺ suppressor T cells at 6 and 12 months post transplantation when compared with baseline, possibly indicating that the lymphopenic environment promotes an oligoclonal expansion of these cells (Figure 3).

Treg numbers were elevated at 1 and 2 years after AHSCT (Figure 4a), with 160% ($P = 0.002$) and 192% ($P < 0.001$) increase of absolute numbers, respectively. When analyzed in the context of diminished CD3⁺CD4⁺ T-cell counts (Supplementary Figure 1C), Treg/CD4⁺ ratios (%) increased from (mean \pm s.d.) $0.92 \pm 1.2\%$ at baseline to $2.64 \pm 2.98\%$ at 6 months ($P = 0.025$), $3.64 \pm 2.02\%$ at 12 months ($P < 0.001$) and $5.33 \pm 3.73\%$ at 24 months ($P < 0.001$) after AHSCT (Supplementary Figure 4). Furthermore, GITR (glucocorticoid-induced TNFR) expression on CD4⁺CD25^{hi} T cells also increased (Figure 4b), achieving maximum values at 2 years after AHSCT. In parallel, we also observed a significant increase in

CTLA-4 expression on CD4⁺CD25^{hi} T cells, starting at 6 months until the end of follow-up (Figure 4c).

Altogether, these results demonstrate that AHSCT leads to the expansion of peripheral immunoregulatory cells and to a shift towards a more immunosuppressive phenotype of CD4⁺CD25^{hi} T cells. These immunological changes may be associated with the restoration of self-tolerance and clinical remission promoted by the procedure.

Upregulated miR-16, miR-155 and miR-142-3p expression normalized after AHSCT

Aberrant expression of miRNAs can lead to autoinflammatory events and contribute to MS development.¹⁶ Altered miRNA expressions have been proposed as biomarkers of the disease, and conventional treatment has been shown to restore the expression of dysregulated miRNAs in MS.¹⁹ As aberrant expression of miR-16,¹⁷ miR-155¹⁸ and miR-142-3p¹⁹ has been previously

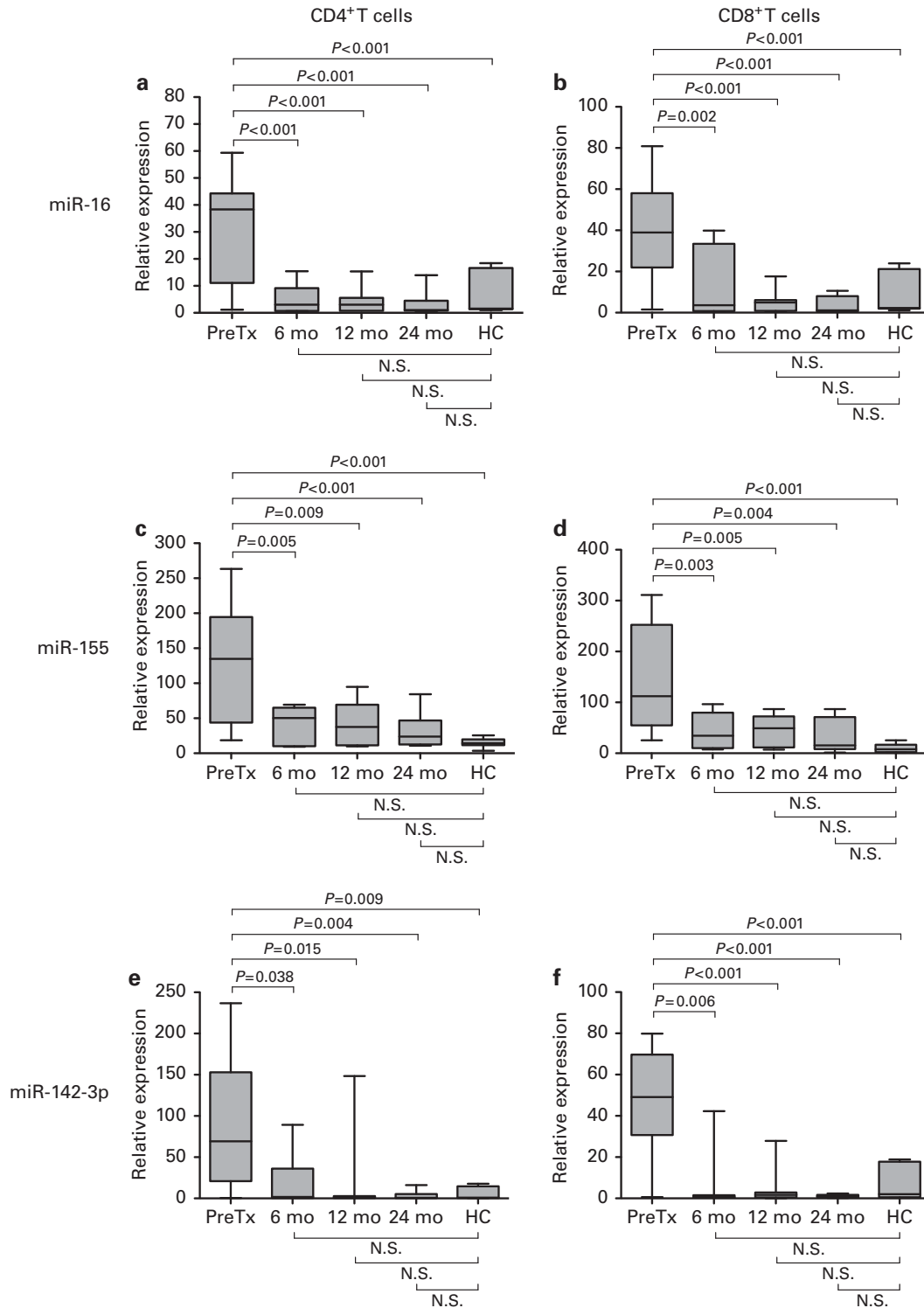


Figure 5. Expression of miR-16, miR-155 and miR-142-3p was upregulated in MS patients and normalized after AHST. Compared with healthy donors, MS patients showed higher miRNA expression, which decreased and normalized after AHST. Relative expression of miRNAs: miR-16 (a and b), miR-155 (c and d) and miR-142-3p (e and f) on CD4⁺ (left) and CD8⁺ T cells (right) by real-time PCR. The boundaries of the boxes indicate the 25th and 75th percentiles, the lines within the boxes indicate the median and the whiskers mark the 10th and the 90th percentiles. Statistical test performed was one-way analysis of variance. Pretransplantation (PreTx), $n = 9$; 6 months (6 mo), $n = 9$; 12 months (12 mo), $n = 9$; 24 months (24 mo), $n = 9$; healthy controls (HC), $n = 9$. NS, nonsignificant.

reported in MS, we decided to evaluate the expression of these miRNAs in our transplanted patients.

At baseline, MS patients presented higher expression of miR-16, miR-155 and miR-142-3p in CD4⁺ and CD8⁺ T cells, when

compared with healthy donors ($P < 0.001$; Figure 5). After AHST, the expression of these miRNAs normalized at 6 months and remained stable until the end of the evaluation period ($P < 0.001$; Figure 5).

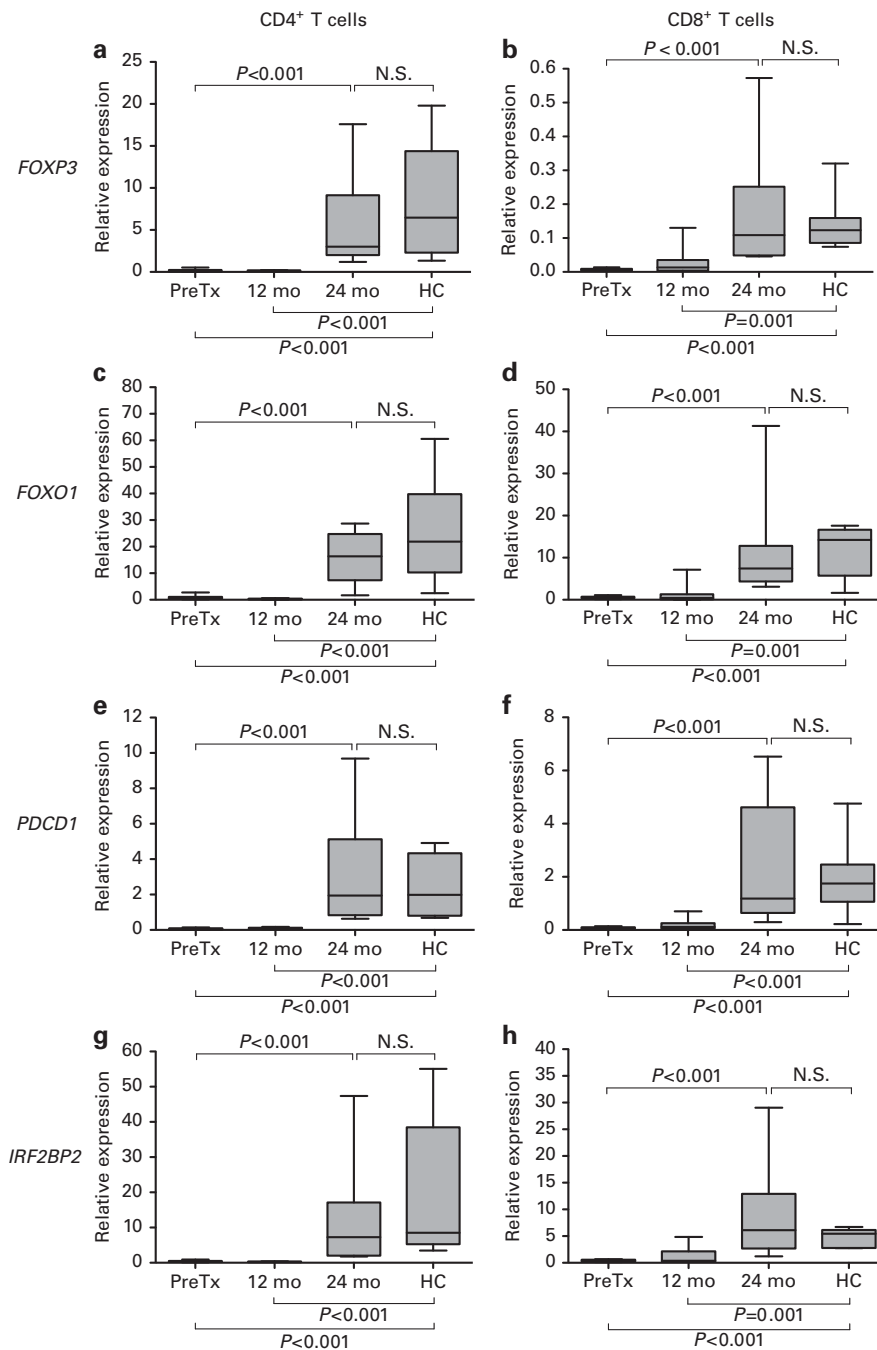


Figure 6. Immunoregulatory gene dysregulation in MS patients and normalization 2 years after AHSCT. When compared with healthy controls, MS patients presented lower immunoregulatory gene expression that increased and normalized at 2 years after AHSCT. Relative expression of immunoregulatory genes *FOXP3* (a and b), *FOXO1* (c and d), *PDCD1* (e and f) and *IRF2BP2* (g and h) on CD4⁺ (left) and CD8⁺ T cells (right) by real-time PCR. The boundaries of the boxes indicate the 25th and 75th percentiles, the lines within the boxes indicate the median and the whiskers mark the 10th and the 90th percentiles. Statistical test performed was one-way analysis of variance. Pretransplantation (PreTx), $n = 12$; 12 months (12 mo), $n = 11$; 24 months (24 mo), $n = 11$; healthy controls (HC), $n = 9$. NS, nonsignificant.

Immunoregulatory *FOXP3*, *FOXO1*, *PDCD1* and *IRF2BP2* gene expression normalized after AHSCT

Once downregulation and normalization of miR-16, miR-155 and miR-142-3p expressions were detected after transplantation, we investigated changes in expression of their predicted target genes, identified according to available electronic databases (<http://www.targetscan.org> and <http://www.mirbase.org>). *FOXP3* (miR-155 predicted target; expression controlled by miR-16),²⁰

FOXO1 (miR-142-3p predicted target), *PDCD1* (miR-16 predicted target) and *IRF2BP2* (miR-155 predicted target) were selected for quantification of expression by RT-PCR. Other genes were not analyzed owing to limitations in sample size.

MS patients presented low expression of *FOXP3*, *FOXO1*, *PDCD1* and *IRF2BP2* in CD4⁺ and CD8⁺ T cells at baseline ($P < 0.001$; Figure 6). At 2 years after AHSCT, these genes had their expression increased ($P < 0.001$; Figure 6) to healthy control levels.

DISCUSSION

Elimination of autoreactive cells, thymic reactivation and restoration of regulatory processes are described as the main therapeutic mechanisms of AHSCT for autoimmune disease, including MS.^{4–10} Combined, they promote control of autoimmunity and clinical amelioration.^{30,31} In MS, Muraro *et al.*^{7,8} showed installation of a new and diverse T-cell receptor (TCR) repertoire and thymic reactivation after AHSCT. Other groups have reported similar findings, including in different autoimmune diseases.^{6,9,10,32} To further explore immunological mechanisms of AHSCT and improve clinical applications of this therapy, we studied Treg reconstitution, miR and gene expression.

Aberrant expression of miR-155,¹⁸ miR-142-3p¹⁹ and miR-16¹⁷ has been previously reported in MS patients. However, only two studies have accessed the effects of MS therapies upon miRNA expression.^{19,33} Here, we report, for the first time, that AHSCT induces normalization of miR-155, miR-142-3p and miR-16 expression, sustained for at least 2 years of follow-up.

Paraboschi *et al.*¹⁸ described higher levels of miR-155 in MS patients when compared with healthy controls, showing the importance of this miRNA in MS pathogenesis. Additionally, miR-155-knockout mice do not develop experimental autoimmune encephalomyelitis.³⁴ In our study, expression of miR-155 was steadily downregulated after AHSCT, reaching healthy control levels. *FOXP3* and *IRF2BP2* are predicted targets of miR-155, and consequently modulated by its expression. As expected, we detected increased expression of miR-155 target genes after transplantation, indicating that AHSCT might contribute to improve the immunoregulatory network.

Similarly, aberrant expression of miR-142-3p has been reported in MS patients.¹⁹ miR-142-3p inhibits Treg-suppressive activity²¹ and can also modulate the expression of its target gene, *FOXO1*. Here, we were able to show strong downregulation of miR-142-3p expression after AHSCT, followed by increased expression of *FOXO1*, both reaching healthy control levels.

Upregulation of miR-16 has been shown in MS patients.¹⁷ Moreover, miR-16 overexpression promoted inhibition of *FOXP3* in Tregs, thereby modifying them to a more conventional T-cell-like function.²⁰ On the other hand, silencing of miR-16 pathway in conventional T cells may lead to an inducible Treg-like function by stimulating *FOXP3* and CTLA-4 expression.²⁰ This dynamic behavior corroborates our results, once we observed a switch from 'high-miR-16 low-*FOXP3*' to 'low-miR-16 high-*FOXP3*' expression.

PD-1/miR-155-double-knockout mice are more susceptible to experimental autoimmune encephalomyelitis, whereas PD-1-knockout mice accumulate CD4⁺ T cells in the brain.³⁵ We believe that increased *PDCD1* expression after AHSCT may be due to the downregulation of miR-16, as *PDCD1* is one its predicted target genes.

Reduced expression of IRF-1 and IRF-2 mRNAs has been reported in untreated patients with active MS.³⁶ Low levels of these regulatory factors could affect the feedback control of multiple IFN-stimulated genes, leading to chaotic cytokine response and inflammation. Therefore, normalization of *IRF2BP2* gene expression after AHSCT may be associated with the clinical remission achieved by most patients post therapy. However, this hypothesis must be verified by studies with longer follow-up.

We also show, for the first time, transient increase of exhausted CD3⁺CD8⁺PD-1⁺ T cells, and higher expression of PD-1 on CD3⁺CD4⁺ T cells after transplantation. The PD-1/PD-L1 costimulatory pathway controls lymphocyte proliferation and activation,^{37,38} and has a critical role in regulating autoimmune damage in experimental autoimmune encephalomyelitis models.³⁹ In MS patients, PD-1 and PD-L1 expression is associated with different patterns of disease activity.²⁶ Our data suggest that PD-1/PD-L1-directed

strategies could be envisioned as the future therapeutic approaches for MS.

Intrinsic defects in function of CD4⁺CD25^{hi} Tregs have already been described in MS patients,¹¹ suggesting that impairment of the immunoregulation may be associated with MS pathophysiology. Improvement of regulatory function is already established as part of the beneficial effects of autologous transplantation.^{4,5,40} Indeed, in our MS patients AHSCT increased Treg counts from the first year until the 2-year follow-up. Furthermore, CD4⁺CD25^{hi} Tregs presented higher expression of immunoregulatory molecules after AHSCT, which may have contributed to restore self-tolerance and to control autoimmunity. CTLA-4 expression regulates Treg function and is clearly associated with the immunosuppressive potential.⁴¹ The same is reported for GITR expression, which modulates Treg-suppressive capacity in autoimmunity and contributes to peripheral tolerance.⁴² Additionally, expansion of CD8⁺CD28⁻CD57⁺ suppressor T cells has been previously reported in transplanted MS patients.^{8,10} Here, we confirm that these cells undergo oligoclonal expansion in the initial periods of lymphopenic environment.

Current discussions about the use of more or less myeloablative conditioning regimens are still unsettled.⁴³ Different conditioning regimens may diversely affect the immune system.^{8–10} Mechanistic evaluations on the effects of AHSCT for MS are limited to thymic recovery and regulatory T-cell reconstitution. To our knowledge, this is the first study to address gene and miR expression in MS after transplantation. Therefore, whether the chosen conditioning regimen affects genes and miRNAs differently than others remains still to be answered.

Altogether, we demonstrate that AHSCT can induce epigenetic changes, thereby improving the immunoregulatory network in MS. Longer follow-up is warranted to further correlate immunological alterations with clinical outcomes. Nevertheless, our results indicate that targeting miR-16, miR-155 and miR-142-3p could represent a valid future therapeutic option for MS and perhaps for other autoimmune diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We acknowledge, thank and honor the memory of Professor Julio César Voltarelli, who led the HSCT studies for autoimmune disorders in Brazil. He prematurely passed away on 21 March 2012. We also thank the multiprofessional team of the Bone Marrow Transplantation Unit of the University Hospital at the Ribeirão Preto Medical School, University of São Paulo, Brazil, for their outstanding job with the patients. We furthermore acknowledge the laboratory personnel of the Hemotherapy Regional Blood Center of Ribeirão Preto, University of São Paulo, Brazil owing to their importance to this work. This work was supported by grants from CNPq and FAPESP.

AUTHOR CONTRIBUTIONS

KCRM and MCO are the principal investigators and takes primary responsibility for the paper; LCMA, JCCL, APS, DLZ, PVBP and RAP performed the laboratory work for this study; MCO, DSB, BPS and AAB recruited the patients; DTC and WASJ coordinated the research; and LCMA, JCCL, KCRM and MCO wrote the paper.

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