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Original Paper

Autologous haematopoietic stem cell transplantation reduces abnormalities in the expression of immune genes in multiple sclerosis

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Abstract

Autologous haematopoietic stem-cell transplantation (AHSCT) has been experimented as a treatment in patients affected by severe forms of multiple sclerosis (MS) who failed to respond to standard immunotherapy. The rationale of AHSCT is to 'reboot' the immune system and reconstitute a new adaptive immunity. The aim of our study was to identify, through a robust and unbiased transcriptomic analysis, any changes of gene expression in T-cells potentially underlying the treatment effect in patients who underwent non-myeloablative AHSCT for treatment of MS. We evaluated by microarray DNA-chip technology the gene expression of peripheral $CD4^+$ and $CD8^+$ T-cell subsets sorted from patients with MS patients before AHSCT, at 6 months, 1 year and 2 years after AHSCT and from healthy control subjects. Hierarchical clustering analysis revealed that reconstituted $CD8^+$ T-cells of MS patients at 2 years post-transplantation, aggregated together with healthy controls, suggesting a normalization of gene expression in $CD8^+$ cells post-therapy. When we compared the gene expression in MS patients before and after therapy, we detected a large number of differentially expressed genes (DEG) in both $CD8^+$ and $CD4^+$ T-cell subsets at all time points after transplantation. We catalogued the biological function of DEG and we selected 27 genes known to be involved in immune function for accurate quantification of gene expression by real-time PCR. The analysis confirmed and extended with quantitative data, a number of significant changes in both the $CD4^+$ and $CD8^+$ T-cells subsets from MS post-transplant. Notably, $CD8^+$ T-cells revealed more extensive changes in the expression of genes involved in effector immune responses.

Key words: autologous haematopoietic stem cell transplantation, gene expression, immune reconstitution, multiple sclerosis, T-cell subset

INTRODUCTION

Multiple sclerosis (MS) is the most common demyelinating disease in adults and impacts the lives of over 2.5 million individuals worldwide. The aetiology of MS is unknown, but available data support the notion that abnormalities in $CD4^+$ and $CD8^+$ T-cell activation and/or regulation lead to loss of immune homoeostasis and to detrimental inflammatory

Abbreviations: AHSCT, autologous haematopoietic stem-cell transplantation; AID, autoimmune disease; AZA, azathioprine; CNS, central nervous system; CY, cyclophosphamide; DEG, differentially expressed genes; EDSS, expanded disability status scale; GA, glatiramer acetate; IFN, interferon; MS, multiple sclerosis; MTX, mitoxantrone; PBMC, peripheral blood mononuclear cell; qRT-PCR, quantitative reverse transcription–PCR; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; TCR, T-cell receptor; TNF, tumour necrosis factor.

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responses against central nervous system (CNS) myelin and axons [1].

Genome-wide association studies further support the notion that MS is an immune-mediated disease. Indeed, most of the products of genes implicated in susceptibility to MS are immune-related, including molecules involved in antigen presentation, cytokine receptors, cell survival, cell signalling, co-stimulation and cell adhesion [2–4].

Immunotherapies for MS are increasingly effective at attenuating inflammatory manifestations in relapsing-remitting multiple sclerosis (RRMS) [5], yet patients affected by aggressive forms of MS often fail to respond to approved treatments and develop progressive unremitting disability. High dose immunosuppression followed by autologous haematopoietic stem-cell transplantation (AHSCT) has been explored as a radical treatment strategy in such severe treatment refractory forms of MS [6]. Although most of the patients who underwent AHSCT had secondary progressive multiple sclerosis (SPMS) with moderate to advanced disability and were treated in uncontrolled clinical trials, stabilization or improvement has been observed in approximately 75 % of patients, suggesting a beneficial treatment effect [7]. Immunological studies suggest that AHSCT induces an extensive renewal of the immunological repertoire. Muraro et al. [8,9] showed a significant expansion of the naïve $CD4^+$ T-cell pool, a profound renewal and broader clonal specificity of the T-cell receptor (TCR) repertoire after AHSCT utilizing myeloablative conditioning regimes. These data suggested that T-cell reconstitution after transplantation could play a role in the observed stabilization of disease. The use of a lympho-depleting but non-myeloablative conditioning regimen has been advocated to improve safety and tolerability. The immunological effects of non-myeloablative AHSCT are likely to differ from those of myeloablative treatment because of the lower intensity of the cytotoxic chemotherapy. Focused studies of non-myeloablative AHSCT have shown depletion of pro-inflammatory ($CCR6^+$ Th17) $CD8^+$ T-cells [10] and expansion of regulatory T-cells and natural killer (NK) cells [11], but a more comprehensive understanding of the effects of treatment on T-cell function is lacking.

Gene microarray technology allows to measure the expression of thousands of genes simultaneously and reproducibly, allowing new insights into the molecular mechanisms of pathogenesis and treatments in several diseases, including MS [12]. As a step in understanding the mechanisms involved in immune reconstitution after non-myeloablative AHSCT, we evaluated by microarray analysis the gene expression profiles of $CD4^+$ and $CD8^+$ T-cells subsets of peripheral blood from MS patients before and at three-time points after transplantation and compared them with the gene expression profiles of healthy controls. Extensive transcriptional changes of $CD4^+$ and $CD8^+$ T-cells subsets were detected after immune reconstitution. Notably, the gene expression profiles of $CD8^+$ T-cells from patients with MS at 2 years post-therapy clustered together with the profiles from healthy controls. To validate and accurately quantify the changes affecting T-cell function post-transplantation, we measured by real-time PCR in $CD4^+$ and $CD8^+$ T-cells the relative expression of genes encoding for immune-related receptors, chemokine and adhesion molecule, intracellular signalling molecules, transcription factors and tumour necrosis factor (TNF) signalling.

The results suggest that the extensive transcriptional changes in immune-related genes on peripheral T-cells subsets (CD8 > CD4) observed after non-myeloablative AHSCT in patients with MS, with relative normalization or renewed of the gene expression profile in $CD4^+$ and $CD8^+$ T-cells, take part in the reprogramming of immune homoeostasis and in the resolution of CNS-targeted inflammation.

MATERIALS AND METHODS

Patients with MS and AHSCT protocol

Sixteen patients with MS, who had been refractory to conventional immunomodulatory and immunosuppressive treatments (demographic and treatment details in Table 1), were selected for a clinical protocol of AHSCT, approved by the Brazilian National Institutional Review Board (CONEP), and gave their written informed consent. Autologous $CD34^+$ stem cells were mobilized from the bone marrow to the peripheral blood with two doses of cyclophosphamide (CY; 2 g/m^2) administered within a 12-h interval and with granulocyte/colony-stimulating factor (10 g/kg/day) given 24 h after the last CY dose. The autologous stem-cell product from peripheral blood was collected by leucoapheresis to obtain $3.4–25.1 \times 10^6$ cells $CD34^+$/kg body weight (mean 8.5×10^6/kg). The cells were cryopreserved until the autologous transplantation date. The patients received a conditioning regime consisting of four doses of CY (50 mg/kg at days -6, -5, -4 and -3) and rabbit antithymocyte globulin (0.5 mg/kg at day -6 and 1 mg/kg at days -5, -4, -3 and -2) to ablate peripheral immune cells, followed by infusion of the autologous haematopoietic cell product. The conditioning regime used in the protocol is considered to be non-myeloablative.

All patients were followed at planned intervals after AHSCT with standardized clinical assessments of neurological disability that included the expanded disability status scale (EDSS) and with MRI.

Human blood samples and RNA isolation

Peripheral blood from MS patients was collected in EDTA tubes at baseline [immediately before the haematopoietic stem cell (HSC) mobilization treatment] and, in those subjects who consented to longitudinal blood sampling, at 6 months, 1 year and 2 years after AHSCT. Additionally, blood samples were collected for the present study from eight healthy donors (four females and four males, median age 34 and 31 years, range 30–40 and 30–35 years respectively; overall median age 33 years). Peripheral blood mononuclear cells (PBMCs) were purified from blood by gradient centrifugation (Ficoll-HyPaque^{TM} plus, $d = 1077$) and $CD4^+$ and $CD8^+$ T-cells were isolated by positive selection using an immunomagnetic system (Miltenyi Biotec-MACS). Purity of sorted $CD4^+$ and $CD8^+$ T-cells was checked by immunostaining and FACS analysis (FACScalibur, Becton Dickinson). Only cellular samples with a purity of $\geqslant$90 % in a total gate of 10 000 events were used in the present study.

Table 1 **Clinical and MRI evolution of patients with MS before and after AHSCT**
CS, cyclosporine; F, female; M, male.

Patient	Sex	Age at HSCT (years)	Disease duration (years)	Disease course	Prior treatment	EDSS −1 year before AHSCT	EDSS baseline pre-AHSCT mobilization	EDSS 6 months post-HSCT	EDSS 1 year post-HSCT	EDSS 2 years post-HSCT	Relapses in 12 months before AHSCT	Relapses in 24 months post-AHSCT	Gad^+ lesions in 12 months before AHSCT	Gad^+ lesions in 24 months post-AHSCT	T2 volume lesions pre-AHSCT	T2 volume lesions post-AHSCT
MS1	M	39	4	SP	CS, IFN	5.0	5.5	5.0	5.0	5.0	1	1	>1	0		
MS2	M	57	32	SP	CS, IFN	3.0	3.0	4.5	4.5	4.5	0	0	0	0		
MS3	F	32	7	RR	CS, IFN	6.0	6.0	6.0	4.5	4.5	0	0	0	0	7.87	5.54
MS4	M	38	7	RR	CS, IFN	3.5	4.5	4.5	4.5	4.5	0	0	0	0		
MS5	M	55	12	RR	CS, IFN, AZA	3.0	3.5	6.0	4.0	4.0	0	0	0	0	33.37	14.40
MS6	M	49	5	RR	CS, IFN, AZA	3.0	4.0	4.0	4.0	4.0	1	0	0	0	23.95	7.53
MS7	F	40	8	PP	CS, IFN	6.0	6.0	6.0	6.0	6.5	0	0	0	0	45.84	17.30
MS8	M	33	4	PP	CS, IFN, MTX	4.5	6.0	4.5	5.0	6.0	0	0	0	0	47.33	15.71
MS9	M	35	10	SP	CS, IFN	5.5	5.5	5.0	4.0	4.0	0	0	0	0	12.04	8.53
MS10	F	40	3	RR	CS, IFN	3.5	3.0	3.0	3.0	3.0	1	0	>1	0	105.19	32.08
MS11	M	44	9	SP	CS, IFN, GA	7.0	6.5	7.0	7.0	7.0	1	0	>1	0	12.90	11.17
MS12	F	22	4	RR	CS, IFN, GA	3.0	3.0	3.0	3.0	3.0	0	0	0	0		
MS13	F	26	4	SP	CS, IFN, GA	6.0	6.5	6.5	6.5	6.5	2	0	>1	0	38.22	37.34
MS14	F	33	18	SP	CY	6.0	6.5	6.5	6.5	7.0	3	0	>1	0		
MS15	F	47	7	SP	CS, IFN	5.5	6.0	4.0	4.5	4.0	1	1	0	0		
MS16	F	21	7	SP	CS, IFN	5.0	6.0	6.0	6.0	6.0	1	0	0	0		

$CD4^+$ and $CD8^+$ T-cells were immediately lysed in TRIzol® (Invitrogen) after sorting and total RNA was extracted and precipitated according to the manufacturer's protocol. RNA quality was checked by electrophoresis in 1% agarose gel and by using Nanodrop ND-1000 spectrophotometer and samples showing insufficient amount or quality were discarded. RNAs from $CD4^+$ and $CD8^+$ T-cells from eight MS patients before AHSCT (pre-mobilization), as well as from four patients at 6 months, 1 year and 2 years after AHSCT were used for gene expression studies by microarray analysis. Our strict requirements of high purity of immunomagnetic sorting of CD4 and CD8 subsets ($\geqslant$ 95%) and of high quality and quantity of total RNA from each sample to be included in the gene expression profiling forced us to reject some post-therapy samples. For this reason, data from longitudinal samples from the same four patients could not be generated at all-time-points and we included in the analysis samples from other patients in the study with adequate RNA yield and purity. Even though this prevented a strictly longitudinal gene expression profiling study within the same group, two out of four patients were studied by gene microarray at all three-time-points post-transplant; and one patient at 6 months and 2 years post-transplant. In addition, RNA aliquots of $CD4^+$ and $CD8^+$ T-cells from 12 patients before AHSCT and eight patients at one or more post-AHSCT time-points (among which three patients at all four time points) were reserved for validation and accurate quantification of gene expression by quantitative reverse transcription–PCR (qRT-PCR). The specific patient samples included in the gene microarray and in the qRT-PCR analyses are specified in Supplementary Table S1 (http://www.clinsci.org/cs/128/cs1280111add.htm).

Gene microarray hybridization and analysis

The gene expression profile of peripheral $CD4^+$ and $CD8^+$T-cells was analysed using one-colour microarray-based gene expression analysis from Agilent Technologies following the manufacturer's instructions. The cDNA product was hybridized in a microarray chip containing 44 000 human transcripts of 60-mer oligomers. The GeneChips were scanned using GenePix 4000B (Axon Instruments) and images were analysed through Agilent Feature Extraction software v.7.5. All parameters available were evaluated using extraction software in order to determine the quality of RNA hybridization of all arrays and control probes. The gene expression measurements made in the present study are available on the internet from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE 32988).

All analyses were done using GeneSpring version 10 software (Agilent Technologies). The gene expression profile of peripheral $CD4^+$ and $CD8^+$ T-cells from MS patients before transplantation was compared with 6 months, 1 year and 2 years after transplantation, as well as with healthy controls. Briefly, a normalization using the 75th percentile value of intensity fluorescence signal was done in all arrays to compare the differences between median expression levels among all samples. Parametric analyses (unpaired Student's t test) were performed on the data with a Benjamini–Hochberg correction for multiple testing to identify gene sets differentially expressed among the study groups with a false discovery rate of <0.05 and >2-fold modulation (either up or down). Furthermore, the gene expression profile of $CD4^+$ and $CD8^+$ T-cells was evaluated by hierarchical cluster analysis using one-way ANOVA test with significance set at $P < 0.05$. A molecular characterization employing DAVID (Database for Annotation, Visualization and Integrated Discovery), a database from National Institutes of Health, U.S.A., was performed by using the lists of differentially expressed genes (DEG) in order to identify the genes related to immune function.

Quantitative RT-PCR analysis

Each RNA sample from peripheral $CD4^+$ and $CD8^+$ T-cells was used to produce cDNA for validation by qRT-PCR. A total of 500 ng of total RNA was reverse transcribed to double-stranded cDNA using random primers, SuperScript II Reverse Transcriptase (Invitrogen) and the supplied protocol. Twenty-six experimental genes and one control gene were assayed by qRT-PCR. All reactions were performed with the following thermal cycling conditions: step 1: 15 min at 95°C, step 2: 40 cycles of 15 s at 95°C and step 3: one cycle to determine the dissociation curve. The assays were realized using QuantiTect™ SYBR Green PCR master mix and 10× QuantiTect primers (Qiagen) and all qRT-PCRs were run through Mx3000p Stratagene system and MxPro™ QPCR software. All cDNA samples were assayed in duplicate for each gene of interest and the control and the dissociation curve for each gene was established.

Relative gene expression was calculated by the comparative C_T method described in the 'Guide to Performing Relative Quantitation of Gene Expression using Real-Time Quantitative PCR' from Applied Biosystems. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as 'housekeeping' control gene.

RESULTS

Absence of inflammatory disease activity in MS patients following AHSCT

Sixteen individuals with MS (eight females, eight males, median age 38 years, range 21–57 years) participated in the present study. Six had RRMS, eight had SPMS, two had primary-progressive multiple sclerosis (PPMS) and the median disease duration from onset of symptoms was 8.8 years (range 4–32 years). Previously, all patients had received at least one and up to three prior lines of immune-modulatory or -suppressive treatment [including interferon (IFN) β, azathioprine (AZA), mitoxantrone (MTX), glatiramer acetate (GA) and CY], as well as corticosteroids (CSs). During the 12 months preceding HSCT, all patients met one or more criteria for disease activity, including worsening of their disability, occurrence of clinical exacerbations and presence of brain gadolinium-enhancing lesions or new T2 lesions detected in their brain MRI. Therefore, the clinical course before transplantation suggested that conventional immunotherapies had failed to control MS activity in these patients.

The 16 patients underwent AHSCT between April 2006 and 2008 and were followed as described above in the Materials and methods section. Their demographic and clinical characteristics, as well as their pre-and post-AHSCT disease evolution are

presented in Table 1. In six out of 16 patients, the EDSS score had increased (i.e. their disability had worsened) during the previous 12 months and a clinical relapse was reported in a total of eight patients at this interval of time (−12 months to pre-mobilization's day). The disability outcome was classified by comparing the patients' EDSS score at baseline (pre-transplant) and at 2 years post-therapy as (i) improved: EDSS score decreased by $\geqslant 0.5$ point, or (ii) worse: increased by $\leqslant 0.5$ point when baseline EDSS score was $\geqslant 5.0$ (or respectively decreased or increased by at least 1.0 point when baseline EDSS was < 5), or (iii) stable when not meeting the improvement or worsening criteria. At the 2-year post-transplant follow-up, 12 out of 16 MS patients (75%) showed either improvement (four patients) or stabilization (eight patients) of disability compared with pre-AHSCT baseline. Worsening from progressive disability at the 2-year follow-up was observed in four patients (MS2, MS7, MS11 and MS14; three SPMS and one PPMS). All 16 patients, however, showed absence of MRI gadolinium enhancing lesions and absence of new white matter lesions post-transplant. Quantitative T2 lesion volume (T2LV) MRI analysis performed in a subset of nine patients (Table 1) demonstrated a 44.27% mean reduction in lesion volumes at 2 years post-transplant when compared with pre-mobilization baseline. By conservatively classifying as having stable T2LV those patients with post-transplant T2LV within −20% of the pre-transplant value to allow for volume quantification error, two patients had stable T2LV (MS11, MS13) and seven patients (MS3, MS5, MS6, MS7, MS8, MS9 and MS10) had decreased T2LV post-transplant.

Taken together, these results showed that AHSCT was followed by a remission of MRI detectable CNS inflammatory disease activity in all patients, with reduction in existing lesion load in some and by improvement or arrest of neurological deterioration in the majority of patients over the duration of a 2-year follow-up.

Normalization of gene expression profiles in CD4$^+$ and CD8$^+$ T-cells after immune reconstitution

We hypothesized that the study of gene expression in T-cell subsets could reveal changes involved in the remission of inflammatory MS activity post-transplant. To this end, we used microarrays to analyse the gene expression profile in peripheral CD4$^+$ and CD8$^+$ T-cells from MS patients before transplantation at 6 months, 1 year and 2 years after transplantation and also healthy controls. A hierarchical clustering analysis was performed on a dataset comprising 13038 and 8876 genes that were shown by ANOVA to be differentially expressed in CD4$^+$ and CD8$^+$ T-cells respectively. GeneSpring GX's clustering module was used to organize the samples according to a group hierarchy based on the similarity of the gene expression profiles. The resulting dendrograms (gene trees), where samples with high similarity arise from the same branch and segregate together on the most distal ramifications, are shown in Figure 1. This unsupervised analysis yielded three or four large clusters for each (CD4 or CD8) T-cell subset and post-AHSCT samples showed different transcriptional profiles compared with pre-transplantation samples. For both CD4$^+$ and CD8$^+$ T-cells, samples from healthy controls, from MS patients pre-transplantation and from MS patients at 6 months and at

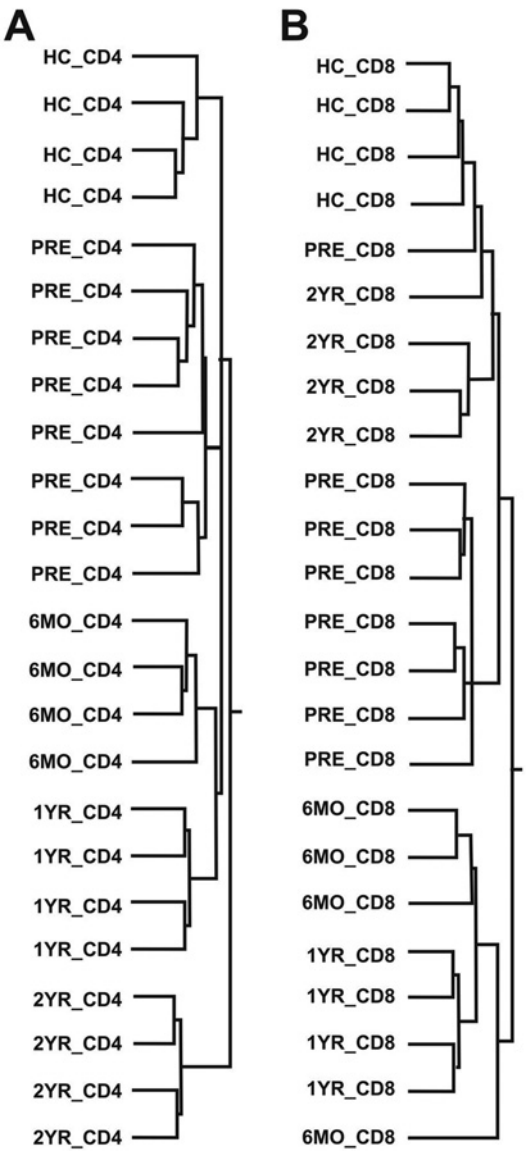


Figure 1 Hierarchical clustering of gene expression profiling in CD4$^+$ (A) and CD8$^+$ (B) T-cells from MS patients before (pre) and at 6 months (6MO), 1 year (1YR) and 2 years (2YR) after AHSCT

The cluster analysis was performed using the numbers of DEG (corrected $P \leqslant 0.05$, one-way ANOVA test) and showed different transcription profile in CD4$^+$ and CD8$^+$ T-cells of MS patients at post-transplantation follow-up. For CD4+ T-cells, all groups aggregated in distinct clusters. Of note, for CD8$^+$ T-cells, MS patients 2 years post-transplant and healthy controls (HC) samples clustered together.

1 year post-transplantation formed distinct clusters. In noteworthy contrast, all CD8$^+$ samples obtained 2 years after transplantation were grouped in the same cluster with healthy controls' samples. Although one CD8$^+$ sample from pre-transplantation was also grouped together with the healthy controls cluster, the overall transcriptional profiles of the reconstituted CD8$^+$ T-cells from MS patients at 2 years after transplantation shared more similarities with healthy controls than with pre-therapy or earlier post-therapy samples from the patients. Interestingly, CD4$^+$ cells from 2 years post-transplant were grouped in a distinct cluster,

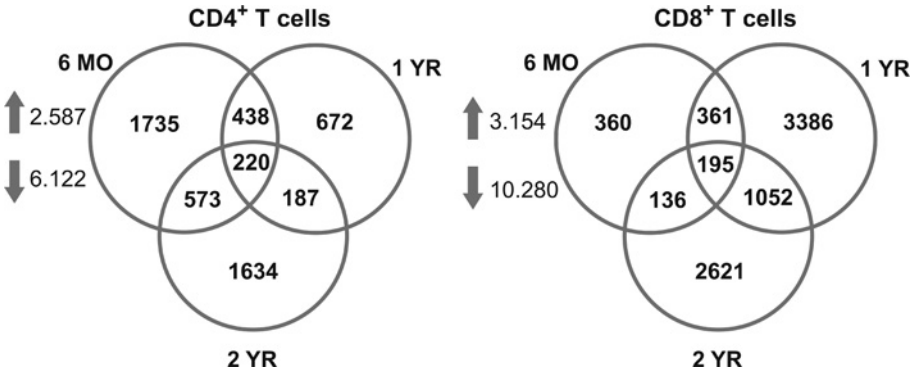


Figure 2 Number of DEG in CD4$^+$ and CD8$^+$ T-cells from MS patients after AHSCT compared with pre-therapy

The level of expression obtained from CD4$^+$ or CD8$^+$ T-cells of the MS patients group before transplantation was compared with the groups at 6 months, 1 year or 2 years after transplantation. The number of DEG was determined by unpaired Student's *t* test with a Benjamini–Hochberg multiple testing correction yielding $P < 0.05$ and fold change > 2.0 as significant. Overlapping sections of the Venn diagram show the number of common genes, whose expression levels meeting the above criteria, were shared among the indicated post-transplantation time-points. The largest numbers of significantly modulated genes were found in CD8$^+$ T-cells at 1 year (1YR) and 2 year (2YR) post-transplant.

possibly indicating that a substantial renewal of gene expression profiles was reached at 2 years post-therapy, although the profiles for this T-cell subset differed from those of healthy controls. Taken together, these results suggested that the gene expression profile of peripheral CD4$^+$ and CD8$^+$ T-cells is radically modified before and after AHSCT and it normalizes in CD8$^+$ cells.

Differentially expressed genes after AHSCT

We employed statistical criteria to define DEG in CD4$^+$ and CD8$^+$ T-cell subsets post-transplantation compared with pre-transplant. The number of DEG found at each of the three time points post-transplantation, as well as the numbers of common DEG among two or all three post-therapy time points are represented as Venn diagrams in Figure 2. The number of genes that changed significantly at the post-transplant time-points differed between CD4$^+$ and CD8$^+$ T-cells. At 6 months post-transplant there were more DEG in CD4$^+$ T-cells (1735 genes) than in CD8$^+$ T-cells (360 genes). This was reversed at 1 year (672 genes in CD4$^+$ T-cells compared with 3386 in CD8$^+$ T-cells) and at 2 years post-transplant (1634 genes in CD4$^+$ T-cells compared with 2621 in CD8$^+$ T-cells). The majority of DEG were down-regulated post-transplantation both in the CD4$^+$ (6122 down/2587 up) and in the CD8$^+$ (10280 down/3154 up) T-cell subsets. The number of genes that were differentially expressed in MS patients pre-transplantation compared with healthy controls was similar to CD4$^+$ T-cells (2157 DEG) and to CD8$^+$ T-cells (2300 DEG).

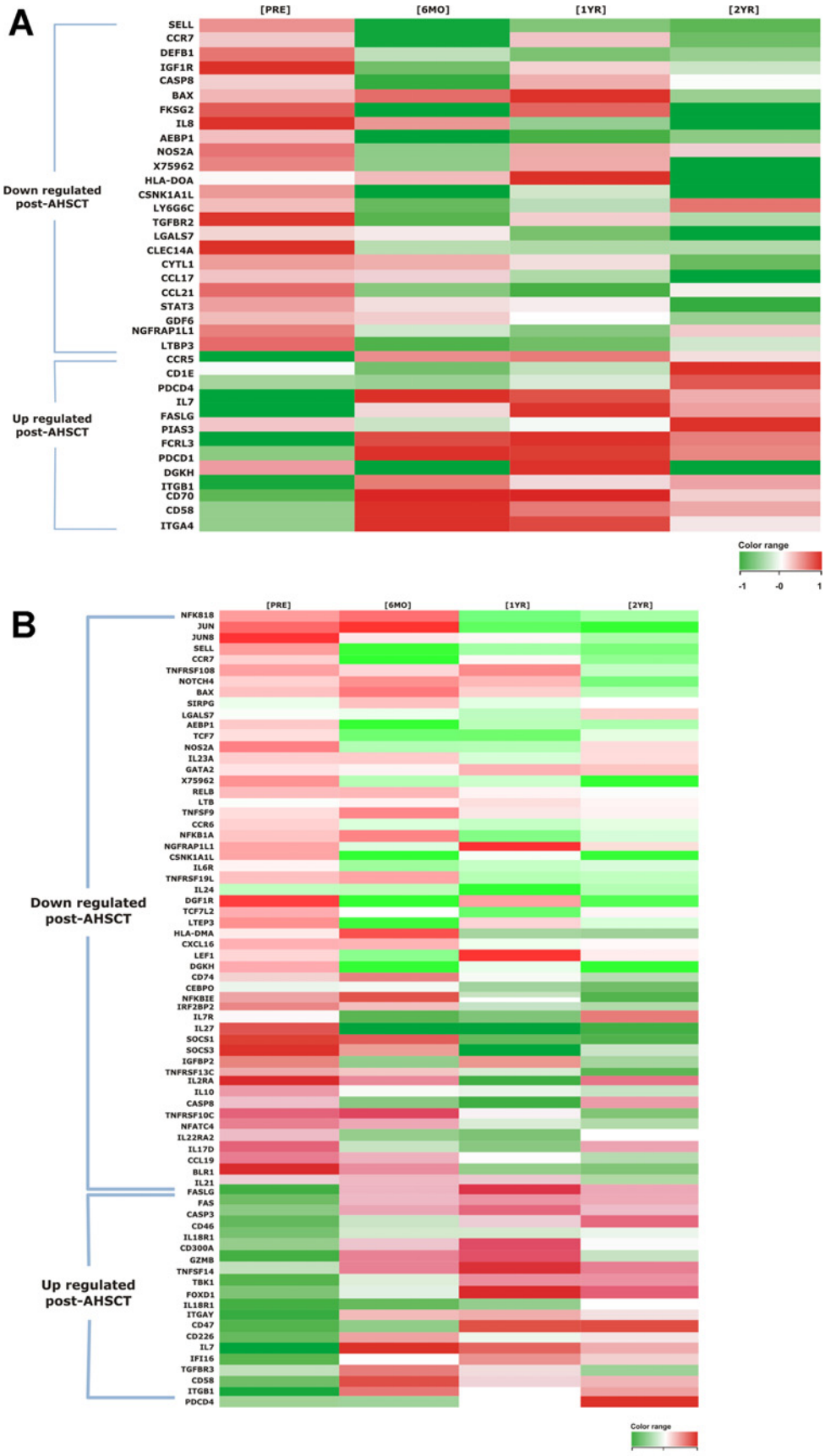
Modulation of immune genes after AHSCT

We surveyed DEG obtained from the comparative analysis of peripheral CD4$^+$ and CD8$^+$ T-cells from MS patients before transplantation and 6 months, 1 year and 2 years after transplantation and we selected genes implicated in immune tolerance, inflammatory responses or autoimmune disease (AID). Within this category of genes, in CD4$^+$ and CD8$^+$ T-cells, we identified a total of 37 and 73 DEG respectively, which were modulated after transplantation in at least two of the three post-transplantation time-points (6 months, 1 year and 2 years) compared with pre-transplant (Figure 3A for CD4 T-cells, and Figure 3B for CD8 T-cells). A subset of 27 immune-related genes was selected for validation and accurate quantification by qRT-PCR based on their relevance to CD4 or CD8 T-cell function and/or evidence in the literature of relevance of these genes in MS or other AIDs (Supplementary Table S2 at http://www.clinsci.org/cs/128/cs1280111add.htm). We quantified 18 down- and five up-regulated genes in CD8$^+$ T-cells; and six down- and three up-regulated genes in CD4$^+$ T-cells isolated from eight MS patients pre- and post-AHSCT. The values of genes demonstrating and confirming a statistically significant difference in their relative expression post-AHSCT are shown in Supplementary Table S3 (http://www.clinsci.org/cs/128/cs1280111add.htm). The results are grouped according to the functional gene classification of their products as follows: (i) TNF signalling; (ii) chemokines, adhesion molecules and other immune cell receptors; (iii) intracellular signalling molecules; and (iv) transcription and translation factors.

We also compared the subset of immune-related genes in MS patients pre-transplantation and in healthy controls and we detected 29 DEG in CD4$^+$ T-cells and 22 genes in CD8$^+$ T-cells (Supplementary Figure S1 at http://www.clinsci.org/cs/128/cs1280111add.htm). These genes, however, did not display significant changes in the patients after AHSCT and, therefore, we did not include them in the qRT-PCR analysis.

DISCUSSION

We have examined the RNA expression profiles using whole-genome microarray analysis in CD4 and CD8 T-cells from patients with MS undergoing non-myeloablative AHSCT and from healthy controls. Four relevant findings from this analysis should be highlighted: (i) MS patients pre-treatment and healthy controls exhibit nearly completely distinct profiles; (ii) the expression profiles in patients with MS change post-AHSCT, more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STAT3
GDF6
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LTBP3
CCR5
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FASLG
PIAS3
FCRL3
PDCD1
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TCF7
NOS2A
IL23A
GATA2
X75962
RELB
LTB
TNFSF9
CCR6
NFKB1A
NGFRAP1L1
CSNK1A1L
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TNFRSF19L
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DGF1R
TCF7L2
LTEP3
HLA-DMA
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CD58
ITGB1
PDCD4
Down regulated post-AHSCT
Up regulated post-AHSCT
Color range
-1
-0
1

extensively in $CD8^+$ than in $CD4^+$ T-cells; (iii) the profiles of $CD4^+$ T-cells from MS patients at 2 years post-transplant are more distinct than others post-transplant groups and also healthy controls; and notably, (iv) the profiles of $CD8^+$ T-cells from patients with MS at 2 years post-transplant are more similar to unrelated healthy controls than to the pre- or early post-treatment MS patients.

AHSCT has been used as a therapy to treat patients with severe AIDs. Several small clinical trials have reported suppression of disease activity and stabilization of clinical course in patients with poor prognosis MS, and in some cases improvement of neurological function [13–15]. Immunological studies support the notion that AHSCT induces qualitative changes in the immune system in patients with MS [8], idiopathic juvenile arthritis [16] and systemic lupus erythematosus (SLE) [17,18]. In our study, the absence of new CNS lesions in all patients after AHSCT, associated with clinical stabilization or improvement in the majority, may mirror the potent effects on T-cell homoeostasis induced by the treatment. Although the neurological disability continued to worsen in four patients (patients MS2, MS7, MS11, MS14; three with SPMS and one with PPMS), with a maximum increase in EDSS score of 1.5 point at 2 years post-transplantation compared with baseline, none of them developed new or enhancing brain MRI lesions or any clinical relapse after AHSCT, suggesting that the persistence of a chronic neurodegenerative process in patients with SPMS, rather than new inflammatory disease activity was the probable reason of the neurological worsening, i.e. that the AHSCT may have been effective immunologically but not against neural degeneration in these patients. Alternatively, one may speculate that the non-myeloablative AHSCT regime may have been efficacious at suppressing the peripheral immune system and blood-born inflammatory lymphocytes but ineffective on non-lymphoid or any disease-mediating cell sequestered or residing in the CNS. Whether or not the clinical evolution was due to failure or incomplete efficacy of AHSCT in these patients, the clinical results did not allow parsing different clinical outcomes in groups of patients of large enough size for correlation or stratification analysis of the gene expression results and we therefore analysed the patient data together.

To investigate the hypothesis that suppression of inflammation observed after AHSCT in MS may involve the modulation of RNA expression in T-cells, we evaluated the transcriptional profile in T-cells subsets of MS patients before and after therapy and in healthy controls. First, the gene expression profiles from patient's pre-transplant showed extensive differences from healthy controls (> 2000 DEG both in $CD4^+$ and in $CD8^+$ T-cells) as well as from post-transplant. Although at 6 months post-AHSCT the number of DEG was greater in $CD4^+$ than in $CD8^+$ cells, at the subsequent time-points the number of DEG was larger in $CD8^+$ cells, suggesting that a more extensive modulation of gene expression had taken place in the $CD8^+$ subset in the later stages of immune reconstitution. The majority of DEG at any post-transplant time-point was down-regulated (70% in $CD4^+$ and 77% in $CD8^+$). The most remarkable consequence of all these changes was that $CD8^+$ T-cell samples from MS patients at 2 years post-AHSCT and healthy controls shared similar profiles suggest a relative normalization of gene RNA expression. A similar observation, to our knowledge, has not been reported.

Since MS is considered a multi-factorial and heterogeneous disease, evaluating gene expression by microarray technology has been used as a strategy to better understand the pathogenesis and the mechanisms of therapeutic intervention. Gene expression profiling has allowed the identification of responders compared with non-responders to treatment with IFN-β [19], as well as the differentiation of profiles segregating treated or untreated MS patients from healthy controls [20,21]. The same technology has been used to describe variation of gene expression in MS-concordant and discordant twins [22]; changes underlying the evolution from pre-MS to clinically defined MS [23]; and profiles associated with occurrence of disease exacerbations [24–26]. Two main aspects uniquely differentiate our study from previous reports using DNA microarray for gene expression analysis of blood cells in MS: first, we investigated patients undergoing AHSCT; and secondly, to achieve greater specificity of gene expression profiling we analysed sorted $CD4^+$ and $CD8^+$ T-cell subsets instead of the whole blood or PBMCs investigated in all previous studies, except Satoh et al. [22] who purified $CD3^+$ T-cells and Zastepa et al. [27] who purified $CD4^+$ $CD45RA^+$ cells since their focus was on naïve CD4 T-cells. Given the radical differences in the biology of the two main T-cell lineages, CD4 and CD8 cells and the large variation of CD4/CD8 ratio during immune reconstitution, we decided to sort these two subsets for gene expression profiling. We decided, though, not to sort more selectively, cell subpopulations with specialized function (e.g. effector and regulatory subsets) because our goal was to detect potential transcriptomic changes that might result from a rearrangement in the frequency or from changes in gene expression of several T-cell subpopulations, but might not be present or significant within the few individual cell subpopulations that can practically be included in this type of analysis.

DNA microarray analysis offers high throughput, yet the gold standard for quantitative analysis remains qPCR [28]. Since a large number of DEG was found and further investigating by qRT-PCR, all genes individually was unfeasible, we focused on a subset of genes showing robust changes and known to be critically involved in immune responses. Therefore, we quantified

Figure 3 Genes down- and up-regulated in $CD4^+$ T-cells (A) and $CD8^+$ T-cells (B) from MS patients at any time-point of 6 months (6MO), 1 year (1YR) and 2 years (2YR) after AHSCT as compared with MS before transplantation (pre)

The heat map illustration was generated by including differentially expressed immune-related genes in $CD4^+$ or $CD8^+$ T-cells which were previously identified by unpaired Student's *t* test, with significance level of $P < 0.05$ after Benjamini–Hochberg multiple testing correction. Green colour indicates low expression, white colour indicates intermediate level and red colour signifies high expression. For both $CD4^+$ and $CD8^+$ T-cell, the majority of genes that varied significantly according to the stated criteria were down-modulated.

the expression of 27 genes encoding for molecules implicated in TNF signalling, chemokine, adhesion and other immune receptors interactions and in gene transcription and translation, all molecular pathways potentially involved in causing or counteracting MS disease activity. The observed changes in the expression of immune-related genes suggest that several pathways controlling T-cell activation, migration and effector functions are modulated in response to homoeostatic requirements after AHSCT in a way that is likely to exert a net anti-inflammatory effect on the course of MS.

In conclusion, our results demonstrate that non-myeloablative AHSCT induces a significant reprogramming of transcriptional expression in peripheral $CD4^+$ and $CD8^+$ T-cells of MS patients. The normalization of genes that are crucially involved in T-cell signalling, activation and differentiation as well as transcription factors controlling immune regulatory networks could contribute to the clinical remission of MS patients through re-establishment of physiological homoeostasis of auto-reactive T-cells. Our data provide the first evidence at the molecular level supporting the concept that this therapy induces profound changes in the gene expression profiles of patients with MS and among these, in the expression of genes controlling T-cell activation that are probably involved in the long-term suppression of inflammation observed post-therapy.

CLINICAL PERSPECTIVES

- AHSCT has emerged as a potential immune-resetting therapy aimed at arresting CNS inflammation in MS.
- Our clinical results are in line with other studies, which reported that around 75% of MS patients undergoing this therapy remain stable during a 2-year post-therapy follow-up, suggesting the abrogation or attenuation of the inflammatory process detectable within the CNS.
- Our gene expression studies demonstrate transcriptional changes in peripheral $CD4^+$ and $CD8^+$ T-cells subsets of the MS patients after immune reconstitution, which suggest a renewal and a relative normalization of gene expression profiles post-transplantation, more evident in $CD8^+$ cells.

AUTHOR CONTRIBUTION

Alessandra Sousa designed the research, performed research, analysed data and wrote the paper. Kelen Malmegrim designed the research. Rodrigo Panepucci and Amélia Araujo contributed to the microarray assay. Dimas Covas contributed to the cellular assays. Doralina Brum, Amilton Bareira and Antonio Carlos Dos Santos contributed to the recruitment and neurological assessment of MS patients. Maria Oliveira, Daniela Moraes, Fabiano Pieroni, George Barros and Belinda Simões are all staff members of Blood Marrow Transplantation Unit at Hospital das Clinicas, who contributed to the clinical care of the patients involved in the present study. Richard Nicholas contributed to the critical analysis of data and manuscript writing. Richard Burt developed the clinical protocol of AHCST for MS. Júlio Voltarelli designed the research, discussed the data and developed the clinical protocol of AHCST for MS. Paolo Muraro designed the research, discussed the data and wrote the paper.

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