Defective expression of apoptosis-related molecules in multiple sclerosis patients is normalized early after autologous haematopoietic stem cell transplantation

G. L. V. de Oliveira,*[†]

A. F. Ferreira,*[†] E. P. L. Gasparotto,*

S. Kashima,[†] D. T. Covas,[†§]

C. T. Guerreiro,[§] D. G Brum,[‡]

A. A. Barreira,[§] J. C. Voltarelli,*[§2]

B. P. Simões,[†§] M. C. Oliveira,[†§]

F. A. de Castro*[1] and

K. C. R. Malmegrim*[†1]

*Department of Clinical, Toxicological and Bromatological Analysis, Faculty of Pharmaceutical Sciences of Ribeirão Preto, São Paulo, Brazil, [†]Center for Cell-Based Research, Regional Blood Center of Ribeirão Preto, School of Medicine of Ribeirão Preto, São Paulo, Brazil, University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil, [‡]Department of Neurology, Psicology and Psiquiatry, School of Medicine of Botucatu, University of State of São Paulo (UNESP), Botucatu, and [§]Department of Clinical Medicine, School of Medicine of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil

[1]These authors contributed equally to this work.
[2]Júlio César Voltarelli deceased.

Summary

Defective apoptosis might be involved in the pathogenesis of multiple sclerosis (MS). We evaluated apoptosis-related molecules in MS patients before and after autologous haematopoietic stem cell transplantation (AHSCT) using BCNU, Etoposide, AraC and Melphalan (BEAM) or cyclophosphamide (CY)-based conditioning regimens. Patients were followed for clinical and immunological parameters for 2 years after AHSCT. At baseline, MS patients had decreased proapoptotic *BAD*, *BAX* and *FASL* and increased *A1* gene expression when compared with healthy counterparts. In the BEAM group, *BAK*, *BIK*, *BIM*$_{EL}$, *FAS*, *FASL*, *A1*, *BCL2*, *BCLX*$_L$, *CFLIP*$_L$ and *CIAP2* genes were up-regulated after AHSCT. With the exception of *BIK*, *BIM*$_{EL}$ and *A1*, all genes reached levels similar to controls at day + 720 post-transplantation. Furthermore, in these patients, we observed increased CD8^{+} Fas^{+} T cell frequencies after AHSCT when compared to baseline. In the CY group, we observed increased *BAX*, *BCLW*, *CFLIP*$_L$ and *CIAP1* and decreased *BIK* and *BID* gene expressions after transplantation. At day + 720 post-AHSCT, the expression of *BAX*, *FAS*, *FASL*, *BCL2*, *BCLX*$_L$ and *CIAP1* was similar to that of controls. Protein analyses showed increased Bcl-2 expression before transplantation. At 1 year post-AHSCT, expression of Bak, Bim, Bcl-2, Bcl-xL and cFlip-L was decreased when compared to baseline values. In summary, our findings suggest that normalization of apoptosis-related molecules is associated with the early therapeutic effects of AHSCT in MS patients. These mechanisms may be involved in the re-establishment of immune tolerance during the first 2 years post-transplantation.

Keywords: apoptosis-related molecules, autologous haematopoietic stem cell transplantation, autoreactive cells, immune tolerance, multiple sclerosis

Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease caused by autoreactive lymphocytes that cross the blood–brain barrier, thereby promoting inflammation, degeneration and axonal damage [1,2]. More than 2·5 million people worldwide are affected with MS, which is currently the most common cause of neurological disability in young adults [3]. The pathogenesis of MS has been linked to immunogenetic susceptibility [4], environmental factors [5,6], immunoregulatory defects, including loss of tolerance [7,8], and decreased numbers of regulatory T cells [9].

Some studies have implicated defective apoptosis during central and peripheral tolerance processes in autoimmune disease pathogenesis [7,10]. Apoptosis plays an important role during lymphocyte development and, when functionally impaired, may predispose to defective tolerance

processes [11,12]. Central tolerance is determined by mitochondria-dependent apoptosis (intrinsic apoptotic pathway), which involves components of the Bcl-2 family (Bad, Bak, Bax, Bid, Bik, Bim, Bok, Boo, BMF, Noxa, Puma, A1, Bcl-2, Bcl-w, Bcl-x_L and Mcl-1) [13,14]. In the periphery, autoreactive T cells can be silenced by anergy or deleted by apoptosis through activation-induced cell death (AICD), with the participation of death receptor family proteins of the extrinsic apoptotic pathway (Fas, FasL, c-Flip, Faim, Dr4, Dr5 and TRAIL) [13,15].

Abnormal apoptosis has already been implicated in the pathogenesis of several autoimmune diseases, including systemic lupus erythematosus [16,17], rheumatoid arthritis [18,19], type 1 diabetes [20–22] and MS [23–25]. Abnormal apoptosis processes in immune cells, with decreased pro-apoptotic and increased anti-apoptotic gene expression, are implicated in the development of autoimmunity [19,22,26].

Studies in the experimental autoimmune encephalomyelitis (EAE) mouse model have demonstrated the association of deregulated apoptosis with maintenance of autoreactive T cells and demyelination in the central nervous system [27–30]. Defective apoptosis has been also associated with MS pathophysiology in humans [31,32]. Furthermore, autoreactive T cells from MS patients are more resistant to apoptosis due to deregulation of cell death regulators Fas and FasL and Bcl-2 family molecules [33–35].

Conventional therapies used for MS treatment include immunomodulators [interferon (IFN)-β, co-polymers], immunosuppressive drugs (cyclosporin, cyclophosphamide, azathioprine, mitoxantrone) and corticosteroids for acute relapses [36]. More recently, monoclonal antibodies and other therapeutic agents targeting specific immune components and adhesion molecules of the blood–brain barrier, such as natalizumab and fingolimod, among others, have been added to the list [37,38]. However, a number of MS patients do not respond to conventional treatments, showing disease progression. Since 1997, high-dose immunosuppression followed by autologous hematopoietic stem cell transplantation (AHSCT) has been implemented as a new investigational therapeutic strategy for refractory patients with autoimmune diseases, including MS [39–42].

Immune monitoring studies have indicated that AHSCT may reset the immune system, decrease lymphocyte autoreactivity and restore immune tolerance, thereby inducing disease remission in MS patients [43–45]. Nevertheless, although the therapeutic efficacy of AHSCT in MS patients has been demonstrated clearly, the immune mechanisms involved in this therapy are not yet understood completely, especially those associated with diverse clinical outcomes.

Here, we evaluated the expression of apoptosis-related molecules in MS pathogenesis and their modulation during the 2-year follow-up after AHSCT in MS patients conditioned with two different regimens, BCNU, Etoposide, AraC and Melphalan (BEAM)/horse anti-human thymocyte globulin (hATG) or cyclophosphamide (CY)/rabbit ATG.

Material and methods

Patients and controls

A total of 18 patients (10 females), with median age of 42 (ranging from 21–54 years), and progressive MS forms, previously refractory to first-line therapy (intravenous corticosteroids, β-IFN, glatiramer acetate and immunosuppressive drugs), were recruited for treatment with BCNU, Etoposide, AraC and Melphalan (BEAM)/hATG (BEAM group; $n = 7$) or cyclophosphamide (CY)/rATG (CY group; $n = 11$) conditioning regimens, followed by infusion of autologous hematopoietic stem cells (HSCs) at the Bone Marrow Transplantation Unit of the Ribeirão Preto Medical School, University of São Paulo, Brazil (trial registration in clinicaltrials.gov identifier: NCT00273364) (Table 1). Diagnosis of MS was confirmed by physicians from the Neurology Department of the same hospital, according to Poser's criteria [46]. Inclusion criteria comprised patients aged from 18 to 60 years, with Expanded Disability Status Scale (EDSS) score between 3 and 6·5, and evidence of disease progression in the last 6 months. Exclusion criteria were co-morbidities (infectious diseases or haematological, kidney, pulmonary, hepatic and heart dysfunctions), cognitive or psychiatric disorders, pregnancy or acute relapses in the last 30 days. All MS patients must have failed first-line MS therapy and were off treatment before AHSCT. Specifically, IFN-β and glatiramer acetate were stopped for at least 30 days before the beginning of the transplant procedure.

For mobilization of haematopoietic stem cells, patients were treated with cyclophosphamide (CY, 2 g/m^2) followed by granulocyte colony-stimulating factor (G-CSF) injections until reaching 10^3 leucocytes/mm^3, and at least 10 CD34$^+$ cells/mm^3. Patients transplanted from 2001 to 2004 were conditioned subsequently with BEAM/hATG at a total dose of 60 mg/kg. Further patients were treated with CY/rATG (200mg/kg cyclophosphamide plus rabbit ATG at the total dose of 4·5 mg/kg). The reason that conditioning regimens were changed was excessive toxicity observed in the former, as reported previously [47]. All procedures were approved by the institutional ethics committee (process number 14105/06). Informed consent was obtained from MS patients and controls at enrolment.

Peripheral blood mononuclear cells (PBMCs) were collected from MS patients at the following time-points: baseline (pre-mobilization), 180 days (day + 180), 360 days (day + 360), 540 days (day + 540) and 720 days (day-+ 720) after AHSCT. The control group included 18 healthy subjects (10 females), with a median age of 41 years (ranging from 20 to 55). Control and transplant groups of patients were age- and sex-matched.

Table 1. Clinical data from multiple sclerosis patients before and after autologous haematopoietic stem cell transplantation

Controls gender/ age (years)	Patients gender/ age (years)	MS subtypes	Disease duration (years)	EDSS pre-AHSCT	EDSS day + 180/ day + 360/ day+ 720 post-AHSCT	MRI pre-AHSCT/ post-AHSCT	Previous therapy	Clinical response at day + 720 post-AHSCT
M/37	M/37	SP	14	6·5	6·5/6·5/6·0	Inactivity/inactivity	STER/IFN/GA	Non-progression
M/48	M/52	SP	09	6·5	6·0/6·0/7·5	Inactivity/n.d.	STER/IFN/GA	Progression
M/52	M/50	SP	09	6·5	8·0/7·5/7·5	Inactivity/inactivity	STER/IFN/GA	Progression
F/42	F/43	SP	12	6·5	8·0/7·5/7·5	Inactivity/inactivity	STER/IFN/GA	Progression
F/42	F/42	SP	11	7·0	7·0/9·0/9·0	Inactivity/n.d.	STER/IFN/GA	Progression
M/20	M/31	PP	04	6·5	6·5/6·5/6·5	Activity/inactivity	STER/IFN/GA	Non-progression
M/54	M/52	PP	11	6·5	6·5/6·5/7·5	Inactivity/n.d.	STER/IFN/GA	Progression
F/47	F/44	SP	20	6·5	6·5/6·5/6·5	Inactivity/inactivity	STER/IFN/GA	Non-progression
F/48	F/48	SP	10	4·5	2·5/3·0/3·0	Inactivity/inactivity	STER/IFN/GA	Non-progression
M/55	M/54	SP	09	6·5	6·5/6·0/6·0	Inactivity/inactivity	STER/IFN/GA	Non-progression
F/33	F/36	SP	14	6·5	6·5/7·0/8·0	Inactivity/inactivity	STER/IFN/GA	Progression
F/32	F/32	SP	06	6·0	6·0/6·0/6·0	Inactivity/inactivity	STER/IFN/GA	Non-progression
F/40	F/42	SP	10	6·0	6·0/6·0/6·0	Inactivity/inactivity	STER/IFN/GA	Non-progression
M/47	M/49	PP	10	6·5	6·5/6·5/6·5	Inactivity/inactivity	STER/IFN/GA	Non-progression
F/36	F/38	PP	09	6·5	6·5/6·5/6·5	Inactivity/inactivity	STER/IFN/GA	Non-progression
F/36	F/36	SP	11	5·0	2·5/2·5/2·5	Inactivity/inactivity	STER/IFN/GA	Non-progression
M/33	M/33	SP	11	6·5	6·5/6·5/7·0	Activity/inactivity	STER/IFN/GA	Progression
F/30	F/31	SP	06	6·5	5·5/5·0/4·5	Activity/inactivity	STER/IFN/GA	Non-progression

M = male; F = female; MS = multiple sclerosis; SP = secondary progressive; PP = primary progressive; EDSS = expanded disability status scale; MRI = magnetic resonance imaging; activity = contrast enhancement of any lesion; inactivity = no contrast enhancement; n.d. = not determined; STER = intravenous (i.v.) corticosteroids; IFN = β interferon; GA = glatiramer acetate. 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. Patients were treated with BCNU, Etoposide, AraC and Melphalan/horse anti-human thymocyte globulin (BEAM/hATG) (BEAM group; patients 1–7) or cyclophosphamide/rabbit ATG (CY/rATG) (CY group; patients 8–18) conditioning regimens. Based on EDSS changes after autologous haematopoietic stem cell transplantation (AHSCT), from baseline to last visit, patients were classified, retrospectively, as: progression = patients who presented an increase in the EDSS scores; non-progression = patients whose EDSS scores either decreased or remained stable.

Neurological disability was assessed through EDSS by a single certified neurologist who was not blinded to patient treatment. Disease progression and improvement were 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 alterations after AHSCT from baseline to last visit, patients were classified retrospectively as: progression – patients who presented an increase in the EDSS scores; and non-progression – patients whose EDSS scores either decreased or remained stable. Patients with progression of disability after AHSCT were not treated with any immunomodulatory or immunosuppressive drugs, except for two patients who received intravenous methylprednisolone pulses. In these cases, blood samples were collected at least 30 days after the last steroid infusion. Table 1 summarizes the demographic features of MS patients and controls.

Isolation of PBMCs

PBMCs from MS patients and healthy subjects were isolated from heparinized blood (20 ml) by centrifugation on a Ficoll-Hypaque density gradient (d = 1·077; Amersham-Pharmacia, Uppsala, Sweden). A total of $1·0 \times 10^7$ cells were resuspended in a guanidine isothyocyanate phenol solution (TRIzol; GIBCO BRL Life Technologies, Grand Island, NY, USA) and frozen at −80°C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated by TRIzol methodology according to the manufacturer's instructions (GIBCO BRL Life Technologies, Birmingham, AL, USA) and quantified by spectrophotometry. RNA concentration was adjusted to 1·0 μg/μl and cDNA synthesized using 2·0 μg of RNA by employing a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol. cDNA synthesis reaction was performed at 25°C for 10 min and then at 37°C for 2 h on a 9700 GeneAmp polymerase chain reaction (PCR) System (Applied Biosystems).

Relative quantification of gene expression by real-time PCR

Relative quantification of pro- and anti-apoptotic genes from the Bcl-2 family (intrinsic pathway), death receptor

family (extrinsic pathway) and the inhibitors of apoptosis proteins (IAP) family was performed with SYBR green PCR Master Mix Kit (Applied Biosystems) on a 7500 real-time PCR system (Applied Biosystems). The PCR mixture consisted of 7·5 µl of SYBR green PCR Master Mix, 10·0 µM of forward and reverse primers, 4·0 ŋg of cDNA and 4·5 µl of deionized water to a final volume of 15 µl. The PCR conditions comprised 1 cycle at 50°C for 2 min, 95°C during 10 min and 50 cycles at 95°C during 15 s, 54–62°C for 25 s (annealing temperatures were determined for each gene) and 72°C for 34 s.

The evaluated pro- and anti-apoptotic genes, sequence primers, annealing temperatures and amplicon size are described in Table 2. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *ACTB* (actin beta) were used as housekeeping genes and the relative expression of the studied target genes were obtained after normalizing using the geometric average of the housekeeping genes mRNA levels. All reactions were performed in duplicate and the gene expression was calculated by the relative expression units (REU) method [48].

Immunophenotypical analysis

PBMCs from MS patients and healthy individuals (5 ml) were collected in ethylenediamine tetraacetic acid (EDTA)

Table 2. Primer sequences, amplicon size and annealing temperature of apoptosis-related genes

mRNA targets	Oligonucleotides (5′–3′)	Amplicon (bp)	AT (°C)
BAD	F: CCG AGT GAG CAG GAA GAC TC	209 bp	60
	R: GGT AGG AGC TGT GGC GAC T		
BAK	F: TTT TCC GCA GCT ACG TTT TT	201 bp	60
	R: TGG TGG CAA TCT TGG TGA AGT		
BAX	F: CCC TTT TGC TTC AGG GTT TC	500 bp	56
	R: TCT TCT TCC AGA TGG TGA GTG		
BID	F: GCT TCC AGT GTA GAC GGA GC	203 bp	62
	R: GTG CAG ATT CAT GTG TGG ATG		
BIK	F: TCT GCA ATT GTC ACC GGT TA	193 bp	60
	R: TTG AGC ACA CCT GCT CCT C		
BIMEL	F: TCT GCA ATT GTC ACC GGT TA	196 bp	59
	R: AAG ATG AAA AGC GGG GCT CT		
BOK	F: TTT TCC GCA GCT ACG TTT TT	249 bp	59
	R: TGG TGG CAA TCT TGG TGA AGT		
NOXA	F: AGC TGG AAG TCG AGT GTG CT	167 bp	54
	R: ACG TGC ACC TCC TGA GAA AA		
A1	F: GGC TGG CTC AGG ACT ATC	227 bp	50
	R: CCA GTT AAT GAT GCC GTC		
BCL-2	F: ACG AGT GGG ATG CGG GAG ATG TG	241 bp	67
	R: GCG GTA GCG GCG GGA GAA GTC		
BCLW	F: AGT TCG AGA CCC GCT TCC	308 bp	60
	R: CCC GTC CCC GTA TAG AGC		
BCL-XL	F: CTG AAT CGG AGA TGG AGA CC	211 bp	60
	R: TGG GAT GTC AGG TCA CTG AA		
MCL1	F: AGA AAG CTG CAT CGA ACC AT	183 bp	56
	R: CC AGC TCC TAC TCC AGC AAC		
FAS	F: CAA GGG ATT GGA ATT GAG GA	203 bp	55
	R: TGG AAG AAA AAT GGG CTT TG		
FASL	F: AGG AAA GTG GCC CAT TTA AC	176 bp	55
	R: CAA GAT TGA CCC CGG AAG TA		
c-FLIPL	F: GCC GAG GCA AGA TAA GCA	464 bp	54
	R: GCC CAG GGA AGT GAA GGT		
c-IAP1	F: AGT CTT GCT CGT GCT GGT TT	550 bp	59
	R: ATG GAC AGT TGG GAA AAT GC		
c-IAP2	F: AGT CTT GCT CGT GCT GGT TT	432 bp	58
	R: TGC TTT TGC CAG ATC TGT TG		
ACTB	F: GCC CTG AGG CAC TCT TCC A	192 bp	58
	R: CCA GGG CAG TGA TCT CCT TCT		
GAPDH	F: GCC TCA AGA TCA TCA GCA ATG C	111 bp	60
	R: CAT GGA CTG TGG TCA TGA GTC CT		

F = forward primer; R = reverse primer; bp = base pairs; AT = annealing temperature.

(8·55 mg/tube) for blood counts and immunophenotyping. Briefly, 100 μl of peripheral mononuclear suspension $(1·0 \times 10^6)$ were labelled with 5 μl of monoclonal antibody and incubated for 15 min at 4°C in the dark. Frequencies of T lymphocyte subsets expressing Fas or FasL apoptosis-related molecules $(CD3^+CD4^+Fas^+$, $CD3^+CD8^+Fas^+$, $CD3^+CD4^+FasL^+$, $CD3^+CD8^+FasL^+)$ were analysed.

After incubation, cells were centrifuged for 5 min at 500 g, washed twice with fluorescence activated cell sorter (FACS) buffer [phosphate-buffered saline (PBS), 0·2% fetal bovine serum, 0·02% sodium azide], resuspended in 200 μl of FACS buffer and analysed by flow cytometry. Five thousand mononuclear cells were acquired using a FACSCanto Flow Cytometer (Becton-Dickinson, San Diego, CA, USA) and analysed by dot-plot using Diva version 6.0 software (Becton-Dickinson). Results are shown as absolute cell numbers (cells/μl), which were calculated by multiplying the percentage of positive cells by the lymphocytes numbers/μl, obtained from blood cell counts of peripheral blood samples.

Protein extraction and Western blotting analysis

Proteins were isolated by the TRIzol extraction method, according to the manufacturer's instructions (GIBCO BRL Life Technologies). Protein samples were diluted in sodium dodecyl sulphate 1% and quantified by using the bicinchoninic acid assay (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). A total of 30 μg of protein were separated using 10 or 15% polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride (PVDF) membranes using standard procedures. Following overnight incubation at 4°C with a blocking cocktail (5% light milk powder, Tris-HCl pH 7·5, 0·1% Tween 20 buffer), membranes were incubated with specific primary antibodies for 24 h at 4°C.

We used monoclonal antibodies anti-Bad, anti-Bax, anti-Bid and anti-Bcl-2 (BD Pharmingen, San Jose, CA, USA), polyclonal rabbit antibodies anti-Bak (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bim (BD Pharmingen), anti-Bcl-xL (BD Pharmingen) and anti-c-FlipL (Calbiochem, La Jolla, CA, USA). Primary antibodies were diluted at 1 : 1000 in blocking buffer plus 0·01% sodium azide. Following three washes in TBS-Tween buffer, membranes were labelled for 45 min with secondary antibody conjugated with horseradish peroxidase anti-mouse or anti-rabbit (GE Healthcare, Little Chalfont, UK). Bands were detected by the enhanced chemiluminescence (ECL)-plus system (GE Healthcare) and quantified densitometrically by Alphaease software (Alpha Innotech, San Leandro, CA, USA). Polyclonal rabbit antibody anti-γ-tubulin and monoclonal anti-β-actin were used as endogenous controls. Results are presented by ratio of the integrated density value (IDV) of target proteins by the endogenous control IDV.

Statistical analysis

The Mann–Whitney t-test was used to compare gene expression of MS patients with healthy subjects and MS patients at day + 720 post-AHSCT with controls. Longitudinal analyses of gene expression in MS patients treated with BEAM or CY/AHSCT were performed by a mixed-effects linear regression model. For multiple comparisons, we used orthogonal contrasts and the results were obtained using SAS® version 9 through PROC NPAR1WAY. P-values lower than 0·05 were considered statistically significant.

Results

Clinical aspects

Patients were evaluated thoroughly for clinical and laboratory aspects before, during and periodically after transplantation for the whole follow-up period. Seven of the 18 patients presented worsening of the EDSS scores at 2 years after transplantation when compared to baseline, and were thus labelled as the 'progression' group. The 11 remaining patients were responsive to the procedure, experiencing either improvement or stabilization of the neurological disability. There were no significant differences in the response rate between patients treated with either BEAM/hATG or CY/rATG conditioning regimens.

One patient experienced severe transplant-related toxicity, with bilateral bacterial pneumonia treated in the intensive care unit with broad-spectrum antibiotics under mechanical ventilation. This patient recovered fully from the complication after a few weeks of treatment. Three other patients presented mild allergic reactions to ATG infusions, which were treated successfully with steroids and histamine blockers. One patient presented severe hypotension during etoposide infusion, reversed with volume expansion. Three patients developed non-complicated, dermatome-limited herpes zoster rash several months after AHSCT. Two patients who had previous history of urinary dysfunction and recurrent infections developed lower urinary tract infections after transplant and required antibiotic treatment followed by continuous prophylaxis. There were no deaths due to transplant toxicity or disease progression in any of the studied patients. None of patients were treated with disease-modifying drugs during the length of this study.

Defective apoptosis-related gene expression in MS patients

To evaluate the apoptosis profile in MS patients, we analysed the expression of pro- and anti-apoptotic genes of the intrinsic and extrinsic pathways, and from IAP family members in PBMCs isolated from MS patients and healthy subjects. We observed a significant decrease in pro-apoptotic *BAD* (median: 0·032, $P < 0·01$), *BAX* (0·003, $P = 0·01$) and *FASL*

(6·966, $P = 0.03$) expression in the PBMCs of MS patients when compared with controls (*BAD*: 1·423; *BAX*: 9·625; *FASL*: 18·368) (Fig. 1a–c). In contrast, significant increase in *BID* (101·629, $P = 0.03$) and *BOK* (4·867, $P < 0.01$) expression was observed in MS patients compared with controls (*BID*: 13·499; *BOK*: 1·493) (Fig. 1d,e).

Furthermore, the anti-apoptotic gene *A1* (median 381·789, $P = 0.02$) was up-regulated significantly in MS patients compared with healthy individuals (*A1*: 79·972) (Fig. 1f), while *BCL-XL* (88·376, $P = 0.01$) and *c-IAP1* (11·145, $P = 0.04$) were down-regulated in patients compared with controls (*BCL-XL*: 232·31; *c-IAP1*: 54·135) (Fig. 1g-h). Supporting information, Table S1 shows gene expression values for all 18 genes evaluated in patients at the pretransplantation period (BEAM/hATG or CY/hATG groups) and controls. Figure 2a summarizes comparative gene expression data from MS patients and controls.

Apoptosis-related gene expression is modulated in MS patients after BEAM/hATG followed by AHSCT

Once we detected abnormal expression of several apoptosis-related genes in MS patients at baseline, we evaluated the effect of BEAM/AHSCT therapy on the expression of those genes after transplantation.

The pro-apoptotic *BAK* (median pre-AHSCT: 5·408; $P < 0.01$), *BIK* (0·747; $P = 0.02$), *BIMEL* (28·487; $P < 0.01$), *FAS* (53·659; $P = 0.03$) and *FASL* (7·676; $P = 0.01$) gene expressions were increased after AHSCT, mainly at day + 540 (*FAS*: 174·712; *FASL*: 29·565) and day + 720 (*BAK*: 603·477; *BIK*: 4·392; *BIMEL*: 172·688; *FASL*: 35·837) (Fig. 3a–e). Similarly, a significant increase in the anti-apoptotic *A1* (median pre-AHSCT: 173·596; $P = 0.02$), *BCL-2* (0·815; $P = 0.04$), *BCL-XL* (47·522; $P < 0.01$), *c-FLIPL* (21·004; $P = 0.03$) and *c-IAP2* (14·731; $P = 0.04$) gene expressions was detected at day + 360 (*c-FLIPL*: 95·651), day + 540 (*c-IAP2*: 31·553) and day + 720 (*A1*: 374·090; *BCL-2*: 12·353; *BCL-XL*: 295·262) (Fig. 3f–j).

Furthermore, we evaluated the outcomes of initially deregulated apoptosis-related gene expressions in MS patients after BEAM/hATG and AHSCT. At 2 years post-transplantation, expression levels of proapoptotic *BAK*, *BIK*, *BOK*, *FAS* and *FASL* genes in MS patients were similar to those from controls (median controls, *BAK*: 139·956; *BIK*: 1·989; *BOK*: 1·492; *FAS*: 76·422; *FASL*: 18·367). Expression of *BAD*, *BAX*, *BID*, *BIMEL* and *NOXA* was also modulated after transplantation, although not reaching control levels (median controls, *BAD*: 1·423; *BAX*: 9·625; *BID*: 13·498; *BIMEL*: 23·778; *NOXA*: 17·562). Additionally, at day + 720, expression of the anti-apoptotic genes *A1*, *BCL-2*, *BCLW*, *BCL-XL*, *MCL1*, *c-IAP1* and c-IAP2 was normalized in MS patients, similar to healthy subjects (median controls, *A1*: 79·971; *BCL-2*: 24·897; *BCLW*: 2·026; *BCL-XL*: 232·310; *MCL1*: 7537·153; *c-IAP1*: 54·135; *c-IAP2*: 125·443). Finally, the expression of *c-FLIPL* in MS patients

at day + 720 was different from that found in controls (median controls, *c-FLIPL*: 96·838). Supporting information, Tables S2–S19, show gene expression values for all 18 genes evaluated in patients (BEAM/hATG or CY/hATG groups) and controls. Figure 2b summarizes the results of comparative gene expression data from MS patients before and after BEAM/hATG followed by AHSCT.

Apoptosis-related gene expression is modulated in MS patients after CY/rATG followed by AHSCT

We observed increased expression of pro-apoptotic genes *BAX* (median pre-AHSCT: 0·011; $P < 0.01$) and *BID* (58·014; $P < 0.01$) at day + 180, day + 360, day + 540 and day + 720 post-transplantation. Conversely, decreased expression of *BIK* (median pre-AHSCT: 4·430; $P < 0.01$) was observed at day + 720 post-AHSCT (Fig. 4a–c).

In addition, a significant increase in anti-apoptotic *BCLW* (median pre-AHSCT: 1·277; $P = 0.02$), *c-FLIPL* (118·995; $P = 0.02$) and *c-IAP1* (6·086; $P < 0.01$) gene expressions at day + 180 (*c-IAP1*: 62·026), day + 360 (*BCLW*: 2·034; *c-IAP1*: 78·444), day + 540 (*c-IAP1*: 46·341) and day + 720 (*c-FLIPL*: 595·165; *c-IAP1*: 175·338) was detected (Fig. 4d–f).

Furthermore, expression of *BAD*, *BAK*, *BAX*, *BIK*, *BOK*, *NOXA*, *FAS* and *FASL* pro-apoptotic genes in MS patients were similar to control levels ($P > 0.05$) at day + 720 after transplantation (median controls, *BAD*: 1·423; *BAK*: 139·956; *BAX*: 9·625; *BIK*: 1·989; *BOK*: 1·492, *NOXA*: 17·562; *FAS*: 76·422; *FASL*: 18·367). Expression of *BID* and *BIMEL* genes was modulated during follow-up, although not reaching control levels at day + 720 (median controls, *BID*: 13·498; *BIMEL*: 23·778). In addition, we demonstrated re-establishment of normal expression of *BCL-2*, *BCLW*, *BCL-XL*, *MCL1* and *c-IAP2* anti-apoptotic genes in MS patients at day + 720 when compared to controls (median controls, *BCL-2*: 24·897; *BCLW*: 2·026; *BCL-XL*: 232·310; *MCL1*: 7537·153; *c-IAP2*: 125·443). In the CY group, expression of *A1*, *c-FLIPL* and *c-IAP1* genes did not reach control levels (median controls, *A1*: 79·971; *c-FLIPL*: 96,838; *c-IAP1*: 54·135) after AHSCT (Fig. 4e,f). Supporting information, Tables S2–S19, show gene expression values for all 18 genes evaluated in patients (BEAM/hATG or CY/hATG groups) and controls. Figure 2c summarizes the results of comparative gene expression data from MS patients before and after CY/rATG followed by AHSCT.

Apoptosis-related proteins expression in MS patients before and after AHSCT

To demonstrate further deregulated expression of the pro-apoptotic *FAS* and *FASL* genes in MS patients submitted to BEAM/AHSCT therapy, we analysed the expression of these molecules on CD4$^+$ and CD8$^+$ T cells by flow cytometry. We observed a significant decrease in the number of CD4$^+$Fas$^+$ T cells at day + 360 post-AHSCT (median:
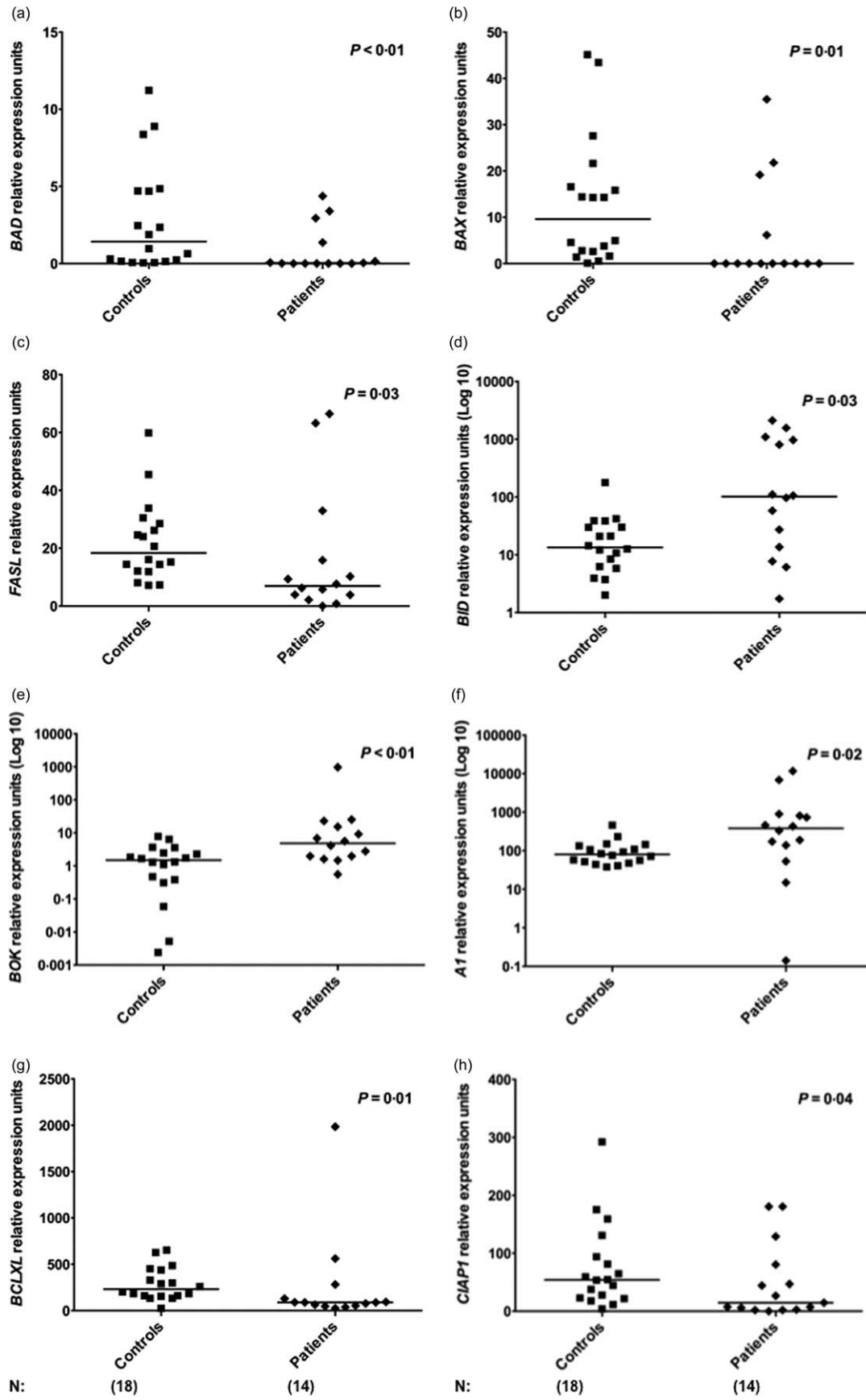
182·5 cells/μl, $P = 0.04$) compared with pretransplantation (660·5) (Fig. 5a). Conversely, we found increased numbers of CD8$^+$Fas$^+$ T cells at day + 180 (1110, $P = 0.04$) and day + 360 (795·7, $P = 0.02$) post-transplantation compared with baseline (333·4) (Fig. 5b). We did not find differences in the frequencies of CD4$^+$FasL and CD8$^+$FasL$^+$ subsets in MS patients after transplantation (Fig. 5c,d).

To evaluate further whether pro- and anti-apoptotic gene expression results correlated with their respective protein expressions in patients who underwent AHSCT with the CY/rATG regimen, we performed Western blotting analysis for the Bad, Bak, Bax, Bid, Bim, Bcl-2, Bcl-xL and c-FlipL proteins. PBMCs from MS patients, at baseline and day + 360 post-AHSCT, and controls were analysed.

At day + 360 post-AHSCT, Bak (IDV median: 0·046), Bim (0·231), Bcl-xL (0·251) and c-FlipL (0·158) protein expressions were decreased significantly ($P = 0.04$ for all comparisons) compared to baseline (median Bak: 0·152, Bim: 1·564, Bcl-xL: 0·624, cFlip-L: 0·415). At baseline, there were no differences in Bak, Bim, Bcl-xL and c-FlipL protein expressions when patients were compared to controls (Fig. 6a–e). At 1 year post-transplantation, the expression of Bak, Bim, Bcl-xL and c-FlipL protein was modulated during follow-up, although not reaching control levels.

Expression of the anti-apoptotic protein Bcl-2 was increased in patients before transplantation (median 4·210) when compared with controls (1·389, $P = 0.02$). At day-+ 360 post-AHSCT, Bcl-2 protein expression was decreased (0·754, $P = 0.007$) when compared with pretransplant levels (Fig. 6c). At 1 year post-transplantation, we found that expression levels of Bcl-2 protein in MS patients were similar to those found in healthy subjects. We found no differences in Bad, Bax and Bid protein expression at baseline and day + 360, compared with controls (data not shown).

Discussion

Mechanistic studies in autoimmune diseases indicate that AHSCT is able to eliminate the immune cell repertoire, promote reconstitution of a new immune system from haematopoietic stem cell precursors (immune resetting) and restore immune tolerance, thereby interrupting inflammatory activity and preventing disease progression [42,49,50]. To date, more than 800 MS patients have been treated worldwide with AHSCT [44]. Currently, BEAM/ATG is the most frequently used AHSCT regimen for MS in Europe, and has also been used by some North American centres [51]. Nevertheless, CY/ATG has been
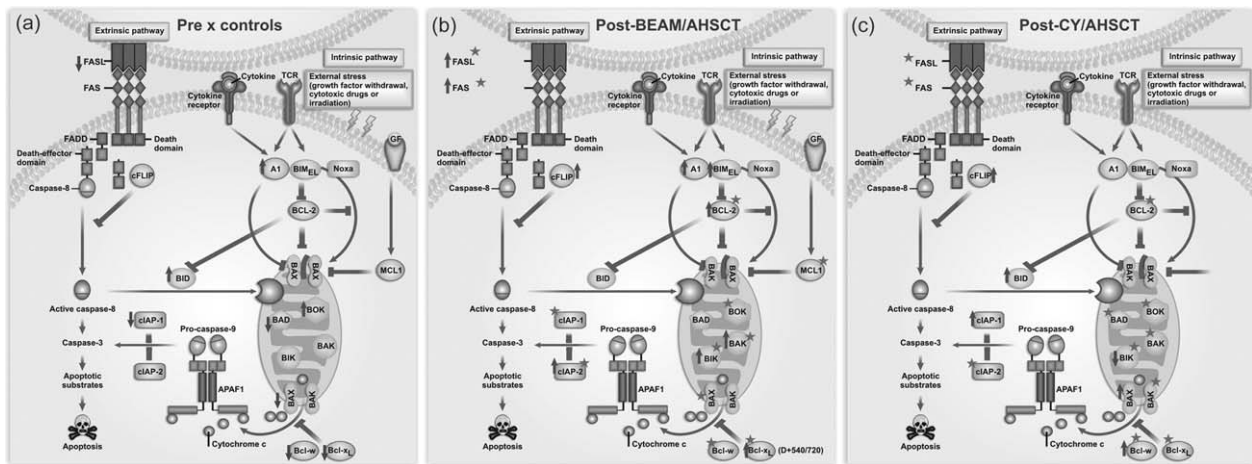
Fig. 2. Schematic diagram of the extrinsic and intrinsic apoptotic pathways highlighting results of the study. (a) Alterations of gene expression in peripheral blood mononuclear cells (PBMCs) from multiple sclerosis (MS) patients compared with those from healthy subjects. Red arrows indicate up-regulated genes and green arrows indicate down-regulated genes. (b) Abnormalities of gene expression in PBMCs from MS patients after BCNU, Etoposide, AraC and Melphalan/horse anti-human thymocyte globulin autologous haematopoietic stem cell transplantation (BEAM/hATG AHSCT) (day + 720) compared with pre-AHSCT values. (c) Abnormalities of gene expression in PBMCs from MS patients after cyclophosphamide (CY)/rabbit ATG (rATG) AHSCT (day + 720) compared with pre-AHSCT values. In (b) and (c), red arrows indicate up-regulated genes and green arrows indicate down-regulated genes in MS patients following transplantation. Stars indicate modulated genes that reached healthy control levels at day + 720. [Colour figure can be viewed at wileyonlinelibrary.com]
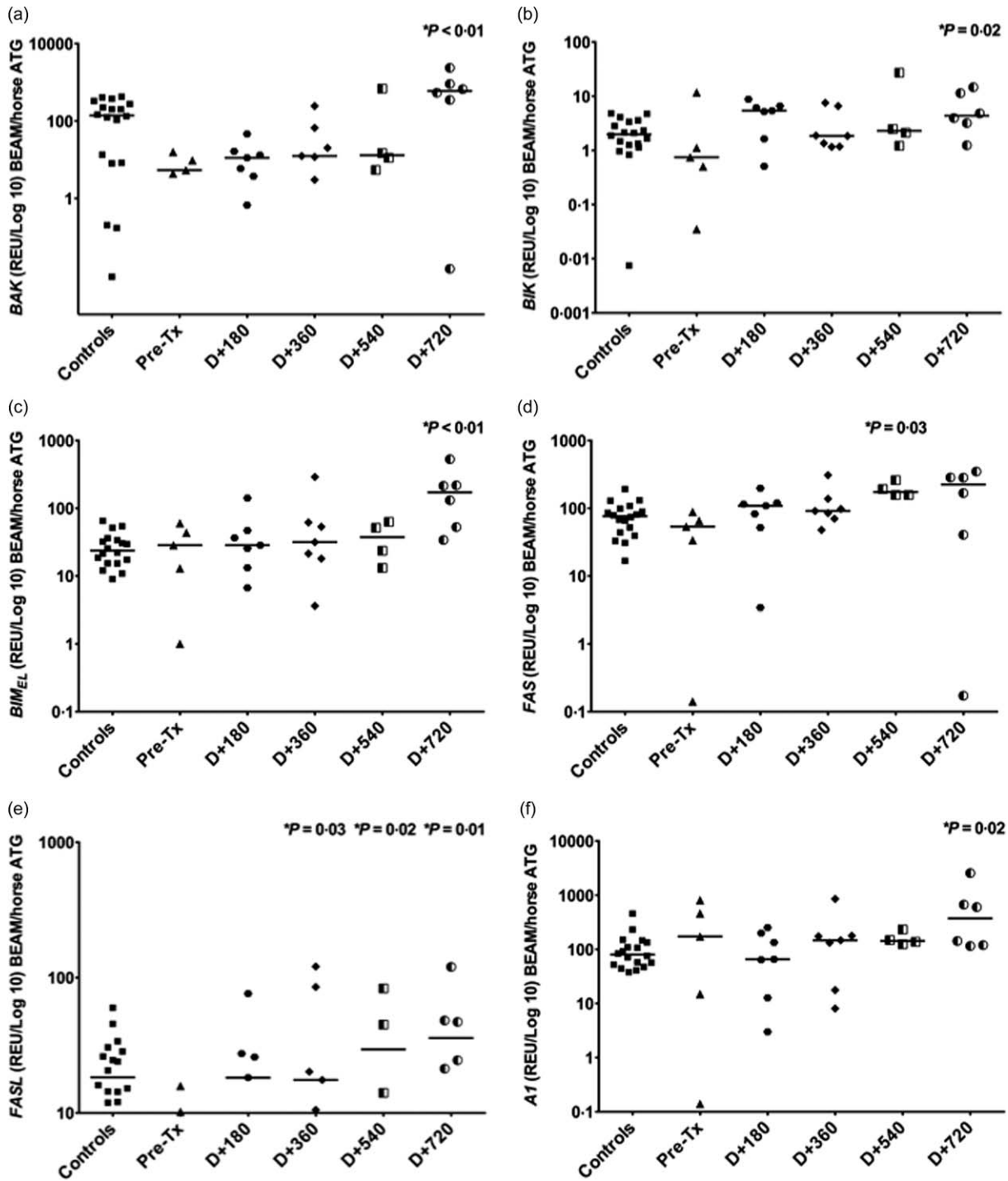
Fig. 3. Continued.

adopted by other transplant centres to treat MS and several other autoimmune diseases, with equally positive results [52,53]. Recently, one single North American centre has shown safety and long-term activity-free survival in a cohort of relapsing–remitting MS patients treated with CY/ATG conditioning regimen [54]. The issue is a matter of debate, and the Italian group still believes that BEAM/ATG may be a more effective scheme for MS patients, leading to longer disease activity-free survival [55]. While a consensus has not yet been achieved, we believe that mechanistic investigations may aid to consolidate one or other regimen in the context of AHSCT for MS patients and perhaps for other autoimmune and inflammatory disorders.
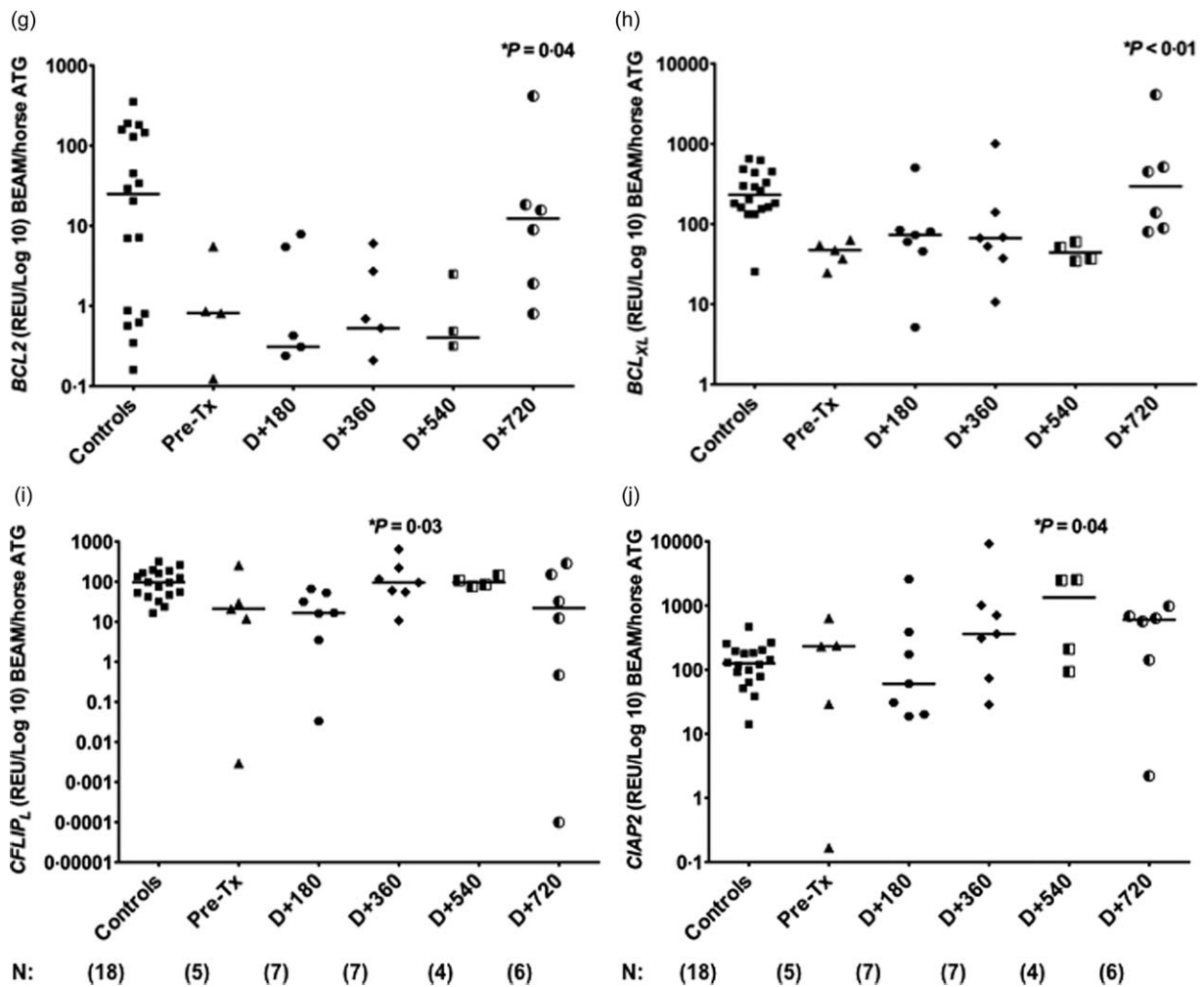
Fig. 3. Modulation of apoptosis-related genes in multiple sclerosis (MS) patients after AHSCT with BCNU, Etoposide, AraC and Melphalan/horse anti-human thymocyte globulin autologous haematopoietic stem cell transplantation (BEAM/hATG AHSCT) conditioning regimen. (a–e) Expression of *BAK, BIK, BIM_EL, FAS* and *FASL* pro-apoptotic genes in peripheral blood mononuclear cells (PBMCs) from healthy controls ($n = 18$) and MS patients ($n = 5$) before and at day + 180 ($n = 7$), day + 360 ($n = 7$), day + 540 ($n = 4$) and day + 720 ($n = 6$) post-AHSCT with BEAM/hATG conditioning regimen. (f–j) Expression of *A1, BCL2, BCLX_L, CFLIP_L* and *CIAP2* anti-apoptotic genes in PBMCs from healthy controls and MS patients before and at day + 180, day + 360, day + 540 and day + 720 post-AHSCT with BEAM/hATG conditioning regimen. Apoptosis-related gene expression was evaluated by real-time polymerase chain reaction (PCR). Statistical analyses were performed by linear regression model with mixed effects. For multiple comparisons, we used orthogonal contrasts post-test. Significance was set at $P < 0.05$. Horizontal bars represent medians. Symbols correspond to individual subjects.

Defective apoptosis affects tolerance processes and deletion of autoreactive cells and may be involved in the development of EAE in mice and MS in humans [10,23–25,27–29]. Therefore, we investigated further the expression profile of apoptosis-related molecules in MS patients and their modulation for the first 2 years following treatment with AHSCT using BEAM or CY-based conditioning regimen.

Before transplantation, we detected abnormal expression of genes from the intrinsic apoptosis pathway in MS patients (decreased *BAD* and *BAK*, increased *A1* and Bcl-2 protein), suggesting a role of defective apoptosis in disease pathogenesis. Similarly, previous studies have shown alterations in

mitochondria-based apoptosis, such as decreased BAX/BCL-2 protein ratio in peripheral lymphocytes from MS patients compared to controls [23]. Up-regulated *BCL-2* gene expression has been described in PBMCs from MS patients [56]. Moreover, increased Bcl-xL expression has been correlated with higher resistance to AICD [24].

After AHSCT, we showed that several genes from the intrinsic pathway were modulated in PBMCs, mainly in the BEAM-conditioned (increased *BAK, BIK, BIMEL, A1, BCL-2, BCL-XL*) compared to the CY-conditioned group (increased *BAX, BID, BCLW* and decreased *BIK*). At 2 years after transplantation, most genes from mitochondria-based apoptosis reached levels similar to healthy controls, both in
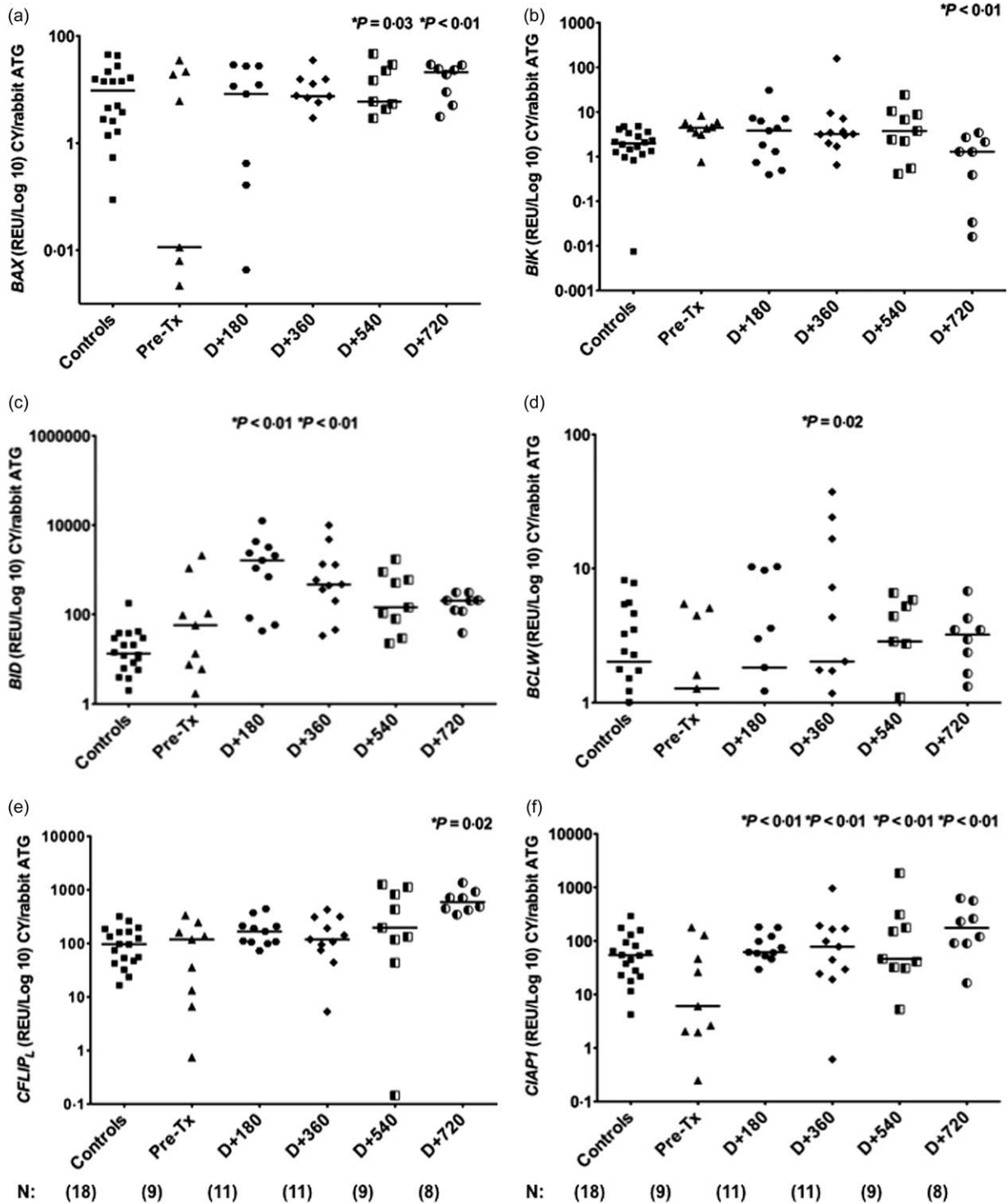
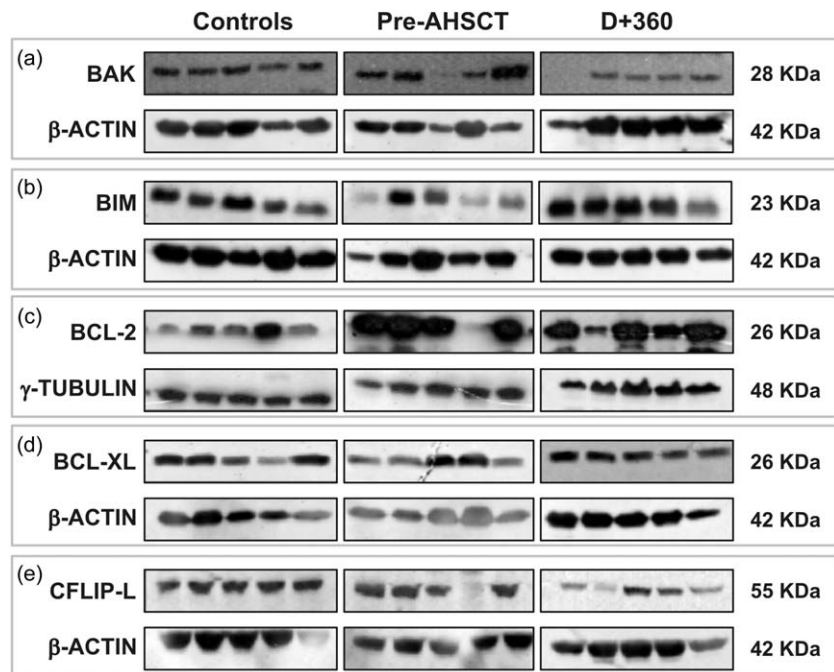**Fig. 4.** Modulation of apoptosis-related genes in multiple sclerosis (MS) patients after autologous haematopoietic stem cell transplantation (AHSCT) with cyclophosphamide/rabbit anti-human thymocyte globulin (CY/rATG) conditioning regimen. (a–c) Expression of *BAX*, *BIK* and *BID* pro-apoptotic genes in peripheral blood mononuclear cells (PBMCs) from healthy controls ($n = 18$) and MS patients ($n = 9$) before and at day + 180 ($n = 11$), day + 360 ($n = 11$), day + 540 ($n = 9$) and day + 720 ($n = 8$) post-AHSCT with CY/rATG conditioning regimen. (d–f) Expression of *BCLW*, *CFLIP*$_L$ and *CIAP1* anti-apoptotic genes in PBMCs from healthy controls and MS patients before and at day + 180, day + 360, day + 540 and day = 720 post-CY/rATG conditioning regimen. Apoptosis-related gene expression was evaluated by real-time polymerase chain reaction (PCR). Statistical analyses were performed by linear regression model with mixed effects. For multiple comparisons we used the post-test by orthogonal contrasts. Significance was set at $P < 0.05$. Horizontal bars represent medians and symbols correspond to individual subjects.
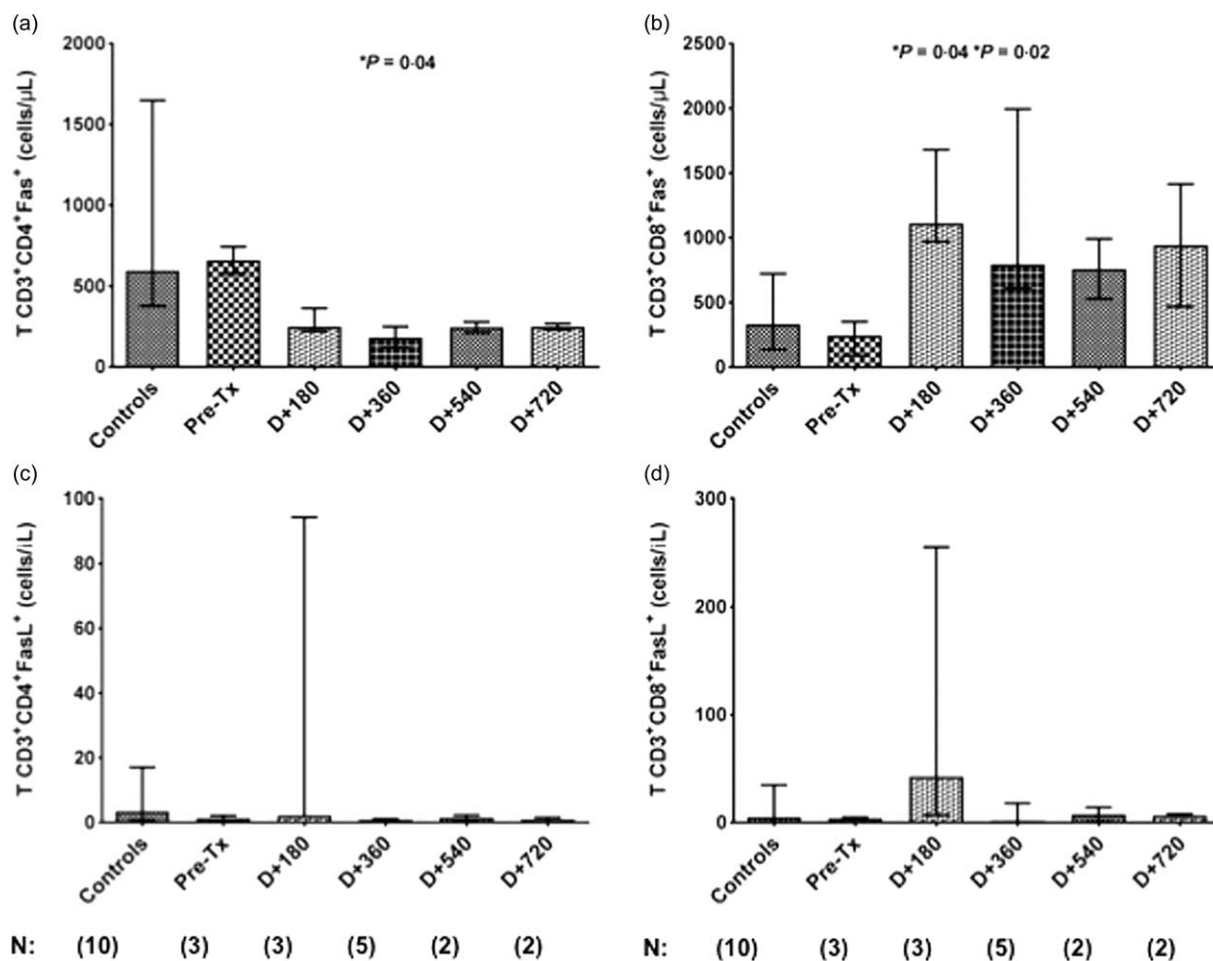
Fig. 5. Reconstitution of CD4⁺Fas⁺ and CD8⁺Fas⁺ T cells in multiple sclerosis (MS) patients after AHSCT. (a) CD4⁺Fas⁺ T cell counts (cells/μl) in healthy controls ($n = 10$) and in MS patients before ($n = 3$) and at day (D) + 180 ($n = 3$), day + 360 ($n = 5$), day + 540 ($n = 2$) and day + 720 ($n = 2$) post-BCNU, Etoposide, AraC and Melphalan/horse anti-human thymocyte globulin autologous haematopoietic stem cell transplantation (BEAM/hATG AHSCT) conditioning regimen. (b) CD8⁺Fas⁺ T cell counts (cells/μl) in healthy controls and in MS patients before and at day + 180, day + 360, day + 540 and day + 720. Statistical analyses were performed by linear regression model with mixed effects. For multiple comparisons, we used the post-test by orthogonal contrasts. Significance was set at $P < 0.05$. Bars represent the medians with the range.

the BEAM and CY-conditioned groups, suggesting that AHSCT plays an essential role in re-establishing the apoptosis machinery in MS patients early after therapy. To our knowledge, there are no reports in the literature about modulation of apoptosis-related molecules from the intrinsic pathway after AHSCT.

In the extrinsic apoptosis pathway, we found decreased expression of *FASL* in PBMCs from our MS patients when compared to baseline. Indeed, studies in mice and humans with autoimmune diseases have demonstrated decreased expression of *FAS* and *FASL* in peripheral lymphocytes [57–59]. They also suggest that the breakdown of immune tolerance to self-antigens could be associated with defective AICD, which is the major mechanism of peripheral tolerance induction, playing an important role in the deletion of autoreactive lymphocytes that have escaped from central tolerance process [58,60].

After transplantation, we showed that genes from the extrinsic pathway were modulated during follow-up in both groups, conditioned with BEAM (increased *FAS*, *FASL* and *c-FLIPL*) and CY (increased *c-FLIPL*), suggesting the role of these molecules in death of residual autoreactive lymphocytes. In fact, we have demonstrated *FAS* and *FASL* up-regulation previously in type 1 diabetes patients after AHSCT with CY conditioning regimen [22]. In the present study, we detected normalization of *FAS* and *FASL* expression towards levels similar to controls, both in BEAM and CY groups, at day + 720, suggesting the re-establishment of peripheral tolerance and AICD. In addition, cytometric analysis showed an increase of CD8⁺Fas⁺ T cells at day-+ 180 and day + 360 when compared to baseline. Previous observations have shown Fas up-regulation on immune cells after immunosuppression with rabbit ATG [61–63]. Furthermore, Muraro and co-workers showed increased

Fig. 6. Expression of apoptosis-related proteins in multiple sclerosis (MS) patients after cyclophosphamide/autologous haematopoietic stem cell transplantation (CY/ AHSCT). (a,b) Expression of Bak and Bim pro-apoptotic proteins in healthy controls and MS patients before and at day + 360 post-AHSCT with CY/rATG conditioning regimen ($n = 5$ for each group). Protein expression was determined by Western blotting. (c–e) Bcl-2, Bcl-xL and cFlip-L anti-apoptotic protein expression in healthy controls and MS patients before and at day + 360 post-AHSCT with CY/rATG conditioning regimen. Statistical analyses were performed by Kruskal–Wallis test followed by Dunn's post-test. Significance was set at $P < 0.05$.

frequency of CD4$^+$Fas$^+$ and CD8$^+$Fas$^+$ T cells in MS patients treated with AHSCT during the first months of immune reconstitution [64]. Based on the immune reconstitution profile of these patients, we suggest that the increase of the CD4$^+$ T cell numbers at day + 720 may account for the observed normalization of the apoptotic gene expression [65]. However, this correlation must be confirmed by future experiments. Our results and these reports highlight the possible role of apoptosis modulation in the re-establishment of immunological tolerance after AHSCT.

Regarding IAP family molecules, we detected up-regulation of *c-IAP1* in PBMCs from MS patients when compared with healthy counterparts, suggesting deregulated apoptosis in MS. Similarly, Sharief and Semra showed higher expression of IAP proteins (IAP-1, IAP-2 and XIAP) in peripheral T lymphocytes from MS patients [66]. Hebb and colleagues reported increased mRNA levels of *IAP1*, *IAP2* and *XIAP* in the peripheral blood of active MS patients [67]. In our study, *c-IAP2* and *c-IAP1* were modulated after AHSCT in both BEAM and CY-conditioned groups, reaching levels similar to controls at 2-year follow-up, indicating normalization of apoptosis processes. Such findings have not yet been described in the literature.

In a recent study concerning immune mechanisms after AHSCT in MS patients, we have demonstrated that CD4$^+$ and CD8$^+$ T cells presented decreased *FOXP3*, *FOXO1*, *PDCD1* and *IRF2BP2* genes and increased miR-16, miR-155 and miR-142-3p before transplantation. At 2 years post-transplantation the expression of these genes increased, reaching control levels. This study suggests that AHSCT normalizes gene and microRNA expression,

thereby improving the immunoregulatory network. These immune mechanisms may be important for disease control in the early periods following AHSCT in MS patients [65].

We have compared previously the clinical aspects of MS patients who underwent AHSCT with two different conditioning regimens, BEAM/hATG and CY/rATG, showing less toxicity in the CY group [47]. However, as evidenced by the different baseline EDSS scores in each group, patient characteristics were very dissimilar, having patients with more advanced disease, mainly in the secondary progressive phase, included in the BEAM group [47]. Indeed, in the present analysis, we observed increasing EDSS scores after transplantation in the BEAM group, while they stabilized in the CY group. Therefore, due to the clinical heterogeneity between BEAM and CY-conditioned patients, our study does not allow groups to be compared. We were, however, able to evaluate the effects of AHSCT on the apoptosis-related gene expression within each of the conditioning regimen groups. Both BEAM and CY regimens were able to modulate expression of apoptosis-related genes in MS patients throughout follow-up. In the BEAM-conditioned patients, we observed that *BAD*, *BAX*, *BID*, *BIMEL*, *NOXA* and *c-FLIPL*, which are involved in inhibition of the extrinsic apoptosis pathway, did not reach control levels at 2 years post-transplantation. Deregulation of apoptosis-related molecules may be involved with the perpetuation of residual memory autoreactive lymphocytes and possibly with disease progression of MS patients.

Normalization of apoptosis-related molecules during the first 2 years after AHSCT, mainly modulation of *FAS* and *FASL*, suggests re-establishment of peripheral tolerance mechanisms mediated by AICD, possibly increasing

apoptosis susceptibility of residual autoreactive lymphocytes. Therefore, we believe that *FAS* and *FASL* may be identified as biomarkers for clinical neurological outcomes in MS patients treated with AHSCT. Furthermore, these molecules may become targets for future therapeutic interventions.

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Disclosure

The authors report no disclosures.

Author contributions

G. L. V. de O. participated in all aspects of the study, experimental conception, sample collection, PCR and protein extraction standardization, experimental procedures, data acquisition, data analysis, interpretation and manuscript writing. A. F. F. and E. P. L. G. helped with PCR and WB standardizations; C. R. T., A. A. B. and D. G. B. were responsible for all clinical aspects involving MS patients. S. K. and D. T. C. provided administrative and financial support. J. C. V. contributed to conception and study design. M. C. O., F. A. de C. and K. C. R. M. participated in experimental conception, interpretation and drafting/revising the manuscript.

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