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Original Article

Lymphocyte reconstitution following autologous stem cell transplantation for progressive MS

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Abstract

Background: Autologous stem cell transplantation (ASCT) for progressive multiple sclerosis (MS) may reset the immune repertoire.

Objective: The objective of this paper is to analyse lymphocyte recovery in patients with progressive MS treated with ASCT.

Methods: Patients with progressive MS not responding to conventional treatment underwent ASCT following conditioning with high-dose cyclophosphamide and antithymocyte globulin. Lymphocyte subset analysis was performed before ASCT and for two years following ASCT. Neurological function was assessed by the EDSS before ASCT and for three years post-ASCT.

Results: CD4+ T-cells fell significantly post-transplant and did not return to baseline levels. Recent thymic emigrants and naïve T-cells fell sharply post-transplant but returned to baseline by nine months and twelve months, respectively. T-regulatory cells declined post-transplant and did not return to baseline levels. Th1 and Th2 cells did not change significantly while Th17 cells fell post-transplant but recovered to baseline by six months. Neurological function remained stable in the majority of patients. Progression-free survival was 69% at three years.

Conclusion: This study demonstrates major changes in the composition of lymphocyte subsets following ASCT for progressive MS. In particular, ablation and subsequent recovery of thymic output is consistent with the concept that ASCT can reset the immune repertoire in MS patients.

***Keywords*:** Multiple sclerosis, autologous stem cell transplantation, lymphocyte reconstitution, immune reconstitution

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Introduction

Autologous stem cell transplantation (ASCT) following intensive immunosuppression has been widely reported as an effective therapeutic strategy for patients with multiple sclerosis (MS).[1–8] The cumulative experience is now substantial and clinical parameters which are associated with a more favourable outcome from ASCT have been identified.[9,10] However, the precise mechanism by which ASCT confers clinical benefit in MS is not well established. ASCT is intensely immunosuppressive and has the potential to eradicate self-reactive immune cells. There is, in addition, evidence to indicate some form of ''resetting'' of the immune repertoire in the post-transplant phase. The contributions of marked immunosuppression versus resetting of the immune repertoire are a matter of debate and ongoing investigation.[11] Qualitative changes in the T-cell compartment following ASCT for MS[12] and systemic lupus erythematosus (SLE)[13] have been reported, including an increased frequency of naïve CD4+ T-cells with features of recent thymic origin and expansion of clonal diversity. However, others have found no difference in the phenotype or clonal diversity of reconstituting T-cells.[14]

Specific T-cell subsets have been implicated in the pathogenesis of MS, with Th1-polarised CD4+ T-cells initially suggested as an effector cell.[15] However, more recently defined immune effector cells, particularly Th17 and T-regulatory cells, may also be important in disease pathogenesis. The

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pro-inflammatory Th17 cell has been implicated in demyelinating disease,[16] and diminished Th17 responses following ASCT for MS have been shown.[17] Restoration of immunosuppressive CD4+CD25+ regulatory T-cells following ASCT for juvenile idiopathic arthritis (JIA) has been demonstrated.[18]

Therefore, we undertook a comprehensive analysis of lymphocyte reconstitution for two years following ASCT with cyclophosphamide/antithymocyte globulin (ATG) in a cohort of patients with progressive MS.

Participants and methods

Patients

From March 2010 to May 2012, 13 patients with progressive MS underwent ASCT. Initial patient selection was made by a neurologist with subsequent review at a multidisciplinary clinic including a haematologist, neurologist, stem cell transplant nurse practitioner and neurology clinical nurse specialist. Selection criteria included progressive disease not responsive to other therapies which met one of the following: (1) progression of sustained disability of at least one point out of 10 of Expanded Disability Status Scale (EDSS) score over 12 months, (2) advanced MS (EDSS ≥ 6) with threatened loss of ambulation, (3) rapidly progressive disease not adequately assessed by EDSS, including (i) progressive cognitive deterioration, (ii) loss of upper limb or bulbar function, (iii) radiologically aggressive MS and (iv) progressive disease in limbs or brain, early paraplegia.

Clinical review with neurological assessment including EDSS was performed at baseline, three-monthly for the first year, six-monthly until two years and then at three years. Magnetic resonance imaging (MRI) scan with and without contrast was performed at baseline, 12 months and 24 months post-transplant. Progression-free survival was defined as no increase in the EDSS of at least 0.5 points between the pre-ASCT assessment and the final assessment.

Ten of the patients underwent an analysis of post-transplant lymphocyte reconstitution with assessment of total lymphocyte count, T-cell, B-cell, natural killer (NK)-cell, CD4, CD8, naïve T-cell, recent thymic emigrant (RTE), T-regulatory (T-reg), Th1, Th2 and Th17 numbers. Time-points for assessment included baseline (pre-treatment), after stem cell mobilisation and 1, 3, 6, 9, 12, 18 and 24 months post-transplant. Results were compared to a cohort of normal healthy volunteers ($n = 10$).

Standard protocol approvals, registrations and patient consents

Approval for the lymphocyte reconstitution study was obtained from the local human research ethics committee (HREC No: 2011-012). Written informed consent was obtained from all participants in the study.

ASCT

Stem cell mobilisation was with cyclophosphamide 2.0 g/m^2 and granulocyte-colony stimulating factor (G-CSF) 5 μg/kg twice a day (bd) from day 4 (prednisolone 50 mg daily concurrent with G-CSF). Transplant conditioning consisted of rabbit ATG (0.5 mg/kg day 6; 1.0 mg/kg days 5, 4, 3, 2; concurrent methylprednisolone 125 mg) and cyclophosphamide (50 mg/kg days 4, 3, 2, 1). Stem cell reinfusion occurred on day 0 and G-CSF 5 μg/kg was administered from day +4 (with concurrent prednisolone 50 mg) until neutrophil recovery.

Flow cytometry

Flow cytometric analysis for all cell types was performed on a BD FACSCantoII Flow Cytometer and data were analysed using BD FACS Diva software (BD Biosciences, San Jose, CA, USA). The percentage and absolute counts for T, B, NK, CD4 and CD8 cells was determined using the BD Multitest 6-color TBNK Reagent with BD Trucount tubes. The naïve CD4+ T-cell population was identified by expression of CD45RA and CD27. RTEs were identified by expression of CD45RA and CD31. The following antibodies were used: CD3 fluorescein isothiocyanate (FITC) (IOT), CD31 PE (BD), CD45 PercpCy5.5 (BD), CD27 PE-Cy7 (BD), CD4 APC (BD), and CD45RA APC-H7 (BD). CD4+ T-regulatory cells were identified by expression of CD4/CD25/FoxP3. Fixation and permeabilisation was performed using the FoxP3 Buffer Set (BD). Cells were stained with the following antibodies for expression of surface antigens: CD3 FITC (IOT), CD45 PerCP Cy5.5 (BD), CD25 PE-CY7 (BD), CD4 APC (BD). Cytoplasmic staining was performed with the antibody FoxP3 PE (BD).

The percentage and absolute counts for the Th1, Th2 and Th17 compartments were analysed using the Human Th1/Th2/Th17 Phenotyping Kit from BD Biosciences. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by Ficoll-Paque centrifugation methodology. The PBMCs were stimulated for five hours in

Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco, Grand Island, NY, USA) with 10% foetal bovine serum (Bovogen Biologicals, VIC, Australia) and 50 ng/ml phorbol myrisate acetate (PMA) (Sigma Aldrich, St Louis, MO, USA) and 1 μg/ml (Sigma Aldrich) ionomycin in the presence of GolgiStop™ (BD Biosciences) at 37°C in air with 5% CO_2. The cells were fixed with Cytofix™ Buffer (BD Biosciences) and stored overnight at 4°C. They were then permeabilised and stained with CD3 PECY7 (BD), CD45 APC-H7 (BD) and the Human Th1/Th2/Th17 Phenotyping Cocktail (BD) containing CD4 PerCP-Cy5.5, interleukin (IL)-17A PE (for Th17), interferon-gamma (IFN-γ) FITC (for Th-1) and IL-4 APC (for Th2).

During data analysis fluorescence minus one (FMO) controls were used to set thresholds to distinguish between positive and negative fluorescence signals on naïve T-cells, RTEs and T-reg cells. FMO controls were also used to distinguish the proportion of cytokine-positive cells.

Statistical analysis

Measurements of lymphocytes were grouped as Initial (before treatment), Pre-SCT (after stem cell collection), 1, 3, 6, 9, 12, 18 and 24 months post-treatment, and Controls. Analyses were carried out on the log10 scale to better homogenise variability across the different groups. One zero value for naïve T-cells and two consistently outlying values for one individual at 18 and 24 months were excluded from the analyses. Comparisons across the different times and with controls were carried out via linear mixed models to account for correlations between repeated measurements taken on the same individuals. Time order amongst the groups was not taken into account in the analyses. All analyses were carried out using TIBCO Spotfire S+ 8.2 (Somerville, MA, USA).

Results

Patients

Patient characteristics are reported in Table 1. All patients had experienced disease progression in the 24 months leading up to ASCT. Four patients had developed gadolinium-enhancing lesions or new T2-weighted lesions in the 12 months before ASCT. All patients had completed their most recent treatment at least three months prior to stem cell transplantation.

ASCT

Stem cell mobilisation was successful in all cases. No major clinical adverse events were observed during this phase of treatment. Un-manipulated stem cells were reinfused without incident with a median viable CD34+ cell dose of 6×10^6/kg. Median time to neutrophil count of 1.0×10^9/l was 10 days. Median time to unsupported platelet count of 20×10^9/l was nine days. Patients were admitted to hospital for the entire procedure and the median length of stay was 17 days. Nine patients were discharged directly home and four patients were referred for a period of rehabilitation before going home.

Febrile neutropenia requiring antibiotic therapy occurred in 12 patients (92%). Six patients developed fluid retention. One patient developed a pericardial effusion without tamponade which resolved without intervention. One patient developed atrial fibrillation and pulmonary oedema requiring medical treatment. Eight patients (62%) developed haematuria (seven microscopic, one macroscopic). Two patients experienced neurological deterioration during conditioning chemotherapy which resolved spontaneously within five days. Unexpected complications included one case each of John Cunningham (JC) virus cystitis, cytomegalovirus (CMV) reactivation and immune-mediated thrombocytopenia (ITP; 12 months post-transplant). Most patients experienced some functional decline in the immediate post-transplant period which returned to baseline by approximately three months.

Neurological status

Neurological status assessed by EDSS was stable in the majority of patients through the three-year follow-up period (Table 2). In the first 12 months post-transplant, one patient improved her neurological function but returned to baseline status by 24 months. Progression-free survival at three years was 69%. EDSS score deteriorated by 0.5 points at three years in four patients while for the remaining nine patients the score was unchanged from baseline. None of the patients had a three-year EDSS score lower than baseline.

MRI scans were performed 12 months post-ASCT in 12 patients. No gadolinium-enhancing lesions were detected. Two patients had new T2-weighted lesions. MRI scans were performed 24 months post-ASCT in 10 patients. No gadolinium-enhancing lesions or new T2-weighted lesions were detected.

Lymphocyte reconstitution

Results for lymphocyte subset analyses are shown in Supplementary Table 1. There was no significant difference between patient baseline counts and healthy controls. Total lymphocyte counts fell

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Table 1. Patient characteristics.

Patient	Sex	Age at ASCT	Duration of MS (years)	Baseline EDSS score	Baseline MRI	Prior treatment	Disease course
MS1	F	38	5	7.0	–	Steroids, interferon-β, natalizumab	SPMS
MS2	F	22	10	8.0	1 Gd+	Steroids, interferon-β, natalizumab	SPMS
MS3	F	53	6	6.5	–	Interferon-β, minocycline, steroids, cyclophosphamide	SPMS
MS4	F	34	5	8.0	2 Gd+	Interferon-β, steroids, mitoxantrone	PPMS
MS5	F	37	3	7.5	–	Glatiramer acetate, natalizumab, steroids	PPMS
MS6	F	47	13	6.5	Three new T2 lesions	Interferon-β, methotrexate, steroids	SPMS
MS7	F	57	30	8.5	–	Interferon-β, methotrexate, minocycline, steroids	SPMS
MS8	F	41	11	8.0	One new T2 lesion	Interferon-β, mitoxantrone, minocycline, methotrexate	SPMS
MS9	M	60	15	6.0	–	Interferon-β, steroids, mitoxantrone, minocycline, methotrexate	PPMS
MS10	M	49	12	6.5	–	Interferon-β, steroids, methotrexate, mitoxantrone	SPMS
MS11	F	47	16	6.5	–	Steroids, interferon-β, mitoxantrone, minocycline	SPMS
MS12	F	50	17	6.5	–	Interferon-β	SPMS
MS13	F	48	19	7.5	–	Steroids, interferon-β, mitoxantrone, glatiramer acetate	SPMS

SPMS: secondary progressive multiple sclerosis; PPMS: primary progressive multiple sclerosis; Gd+: gadolinium-enhancing lesion; F: female; M: male; ASCT: autologous stem cell transplantation; MS: multiple sclerosis; EDSS: Expanded Disability Status Scale; MRI: magnetic resonance imaging.

following stem cell mobilisation and returned to baseline by nine months post-transplant (Figure 1). This was primarily due to a drop in the CD4 count which reached a nadir at three months post-transplant and then slowly recovered but never reached pre-treatment levels. CD8 cells increased one month post-transplant and then returned to baseline. B-cells fell sharply to a nadir one month post-transplant and recovered by three months. Subsequent levels were not different to baseline apart from the 18-month time-point.

Naïve T-cells and RTEs fell significantly to a nadir one month post-transplant and steadily recovered to reach baseline levels by 12 months and nine months, respectively (Figure 2). The pattern was similar for naïve T-cells and RTEs calculated as a percentage of total CD4 cells, with recovery at nine months and six months, respectively (Supplementary Figure 1).

Regulatory T-cells fell post-transplant and did not recover to baseline over the two-year follow-up (Figure 3). T-regs as a percentage of total CD4 cells did not change significantly throughout the study (supplementary Figure 2).

Th1 and Th2 cells did not change significantly throughout the two-year follow-up (Figure 3). Th17 cells fell significantly post-transplant and recovered by six months (Figure 3). As a percentage of the CD4 count, both Th1 and Th2 cells increased significantly in the first three months post-transplant and then

Table 2. Changes in Expanded Disability Status Scale (EDSS) score.

Patient	Baseline EDSS	3-month EDSS	6-month EDSS	12-month EDSS	24-month EDSS	36-month EDSS
MS1	7.0	7.0	7.0	7.0	7.0	7.0
MS2	8.0	8.0	8.0	7.5	8.0	8.0
MS3	6.5	6.5	6.5	6.5	6.5	6.5
MS4	8.0	8.0	8.0	8.0	8.0	8.0
MS5	7.5	7.5	7.5	7.5	7.5	7.5
MS6	6.5	6.5	6.5	6.5	6.5	6.5
MS7	8.5	8.5	8.5	8.5	8.5	8.5
MS8	8.0	8.0	8.0	8.0	8.0	8.0
MS9	6.0	6.0	6.0	6.0	6.0	6.5
MS10	6.5	6.5	6.5	7.0	7.0	7.0
MS11	6.5	6.5	6.5	6.5	6.5	6.5
MS12	6.5	6.5	6.5	6.5	7.0	7.0
MS13	7.5	7.5	7.5	8.0	8.0	8.0

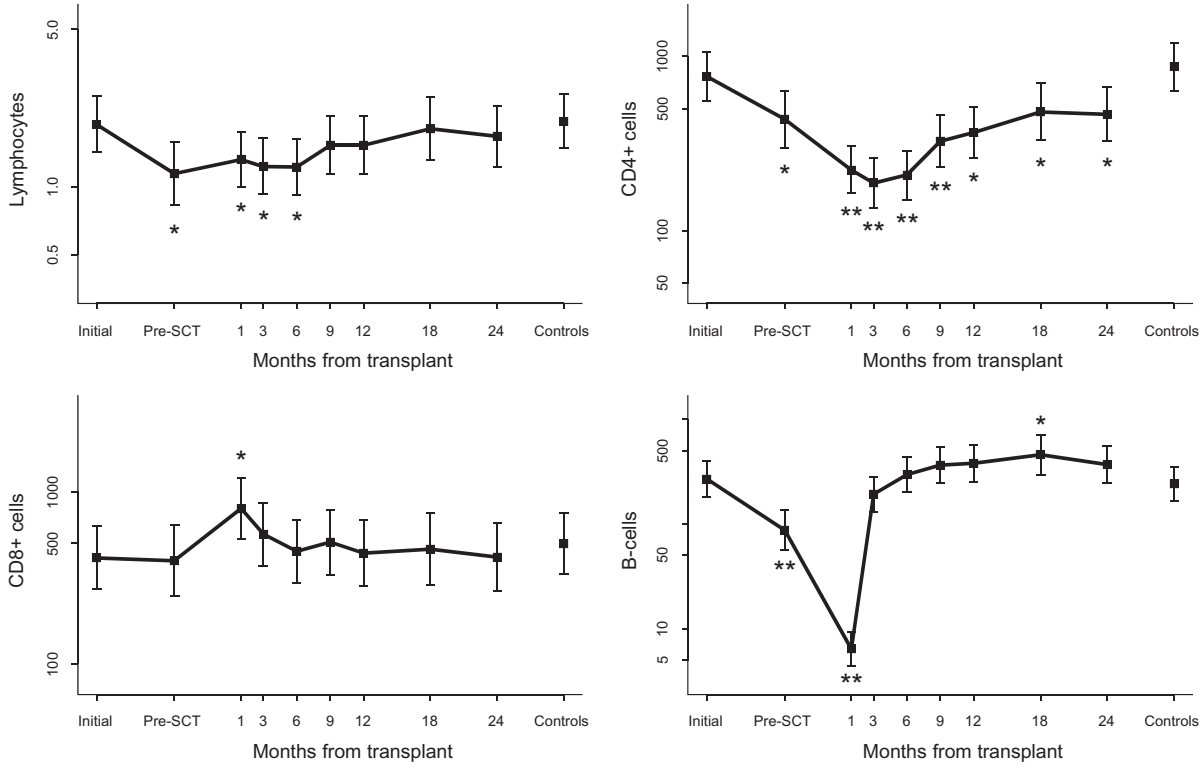

Figure 1. Total lymphocytes, CD4 T-cells, CD8 T-cells and B-cells.
Mean absolute values ± 2SE (log scale) for total lymphocytes, CD4+ T-cells, CD8+ T-cells and B-cells. Total lymphocyte count is $\times 10^9$/l and other values are $\times 10^6$/l. *$p < 0.05$ vs Initial, **$p < 0.0005$ vs Initial. Pre-stem cell transplantation (SCT) is after stem cell mobilisation and before autologous stem cell transplantation (ASCT).

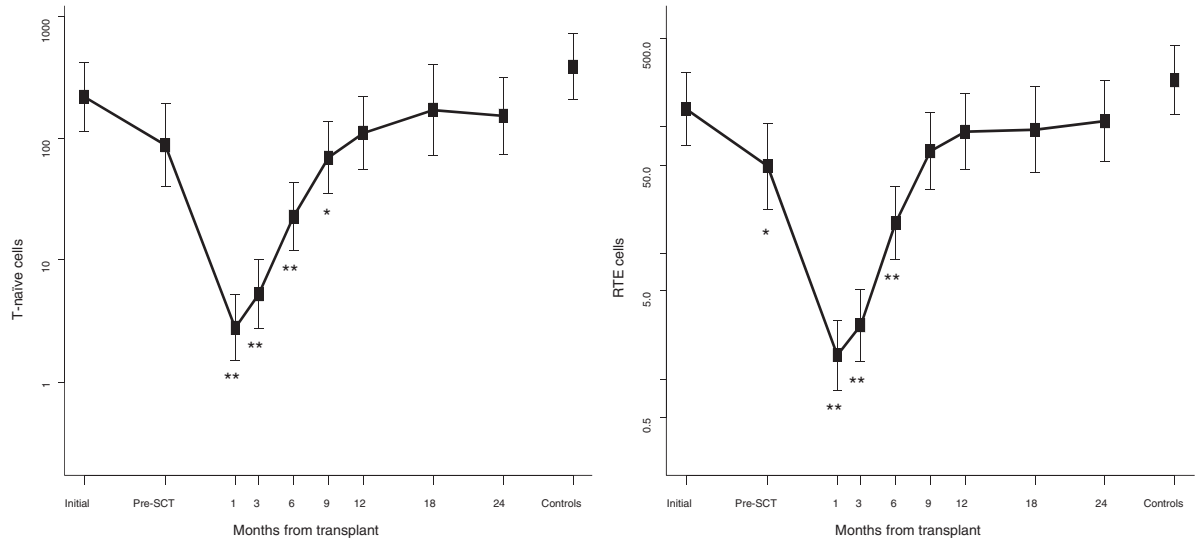

Figure 2. T-naïve cells and recent thymic emigrants.
Mean absolute values ± 2SE (log scale) for T-naïve cells and recent thymic emigrants (RTE cells). Values are $\times 10^6$/l. *$p < 0.05$ vs Initial, **$p < 0.0005$ vs Initial. Pre-stem cell transplantation (SCT) is after stem cell mobilisation and before autologous stem cell transplantation (ASCT).

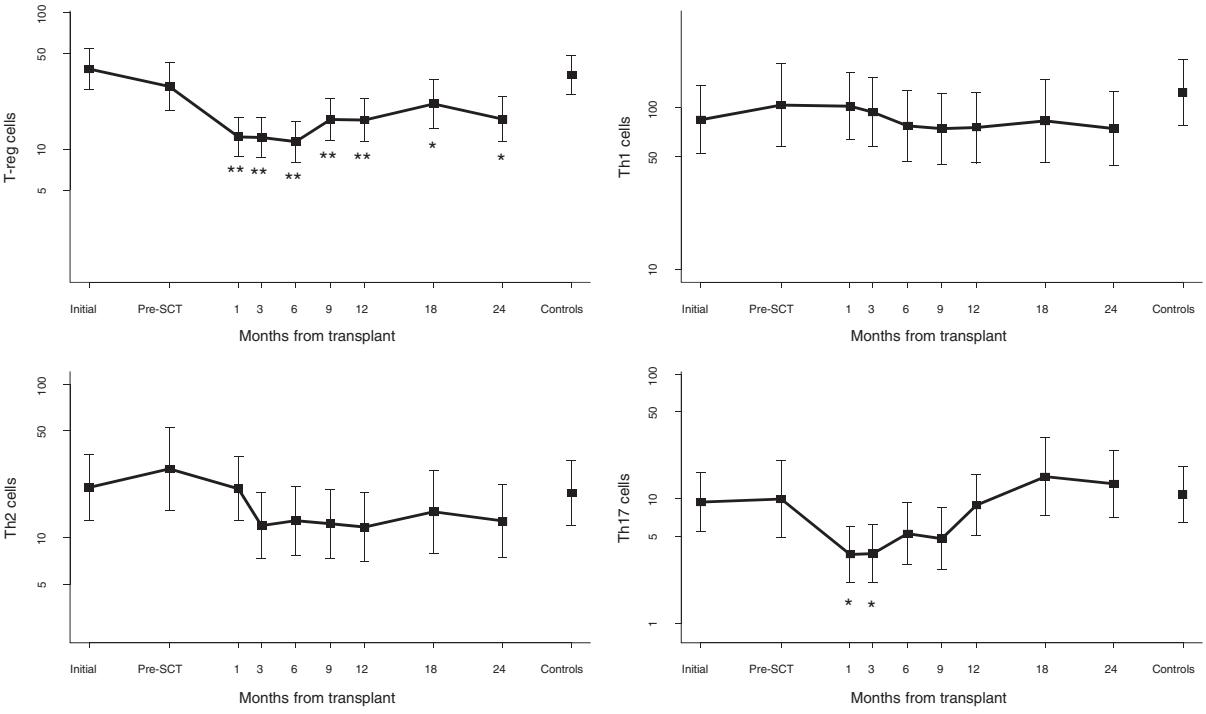

Figure 3. T-reg, Th1, Th2 and Th17 cells.
Mean absolute values ± 2SE (log scale) for T-reg cells, Th1 cells, Th2 cells and Th17 cells. Values are $\times 10^6$/l. *$p < 0.05$ vs Initial, **$p < 0.0005$ vs Initial. Pre-stem cell transplantation (SCT) is after stem cell mobilisation and before autologous stem cell transplantation (ASCT).

gradually returned to baseline (Supplementary Figure 2). Th17 cells increased as a percentage of CD4 cells following transplant, reaching statistical significance by six months and this was maintained through to 24 months (Supplementary Figure 2). These changes were primarily due to the significant reduction in total CD4 count post-transplant.

Discussion

For patients with aggressive relapsing–remitting MS who have not adequately responded to conventional therapies, ASCT is a promising treatment.[2,4,5,7] In this study, ASCT for advanced and progressive MS did not improve neurological disability. However, in most cases there was stabilisation of the neurological status. The progression-free survival of 69% at three years is consistent with previous reports of ASCT in patients with progressive MS.[1,3,6] Of note, the one patient who had a temporary improvement in neurological function had gadolinium-enhancing activity on the pre-ASCT MRI, consistent with previous reports that patients with active inflammation have a better outcome from ASCT. Indeed, a randomised study has shown superiority of ASCT over conventional therapy with mitoxantrone in MS patients with gadolinium-enhancing lesions.[4] A recent study also demonstrated long-term suppression of inflammatory activity in patients with active and progressing MS treated with intensive conditioning (busulfan, cyclophosphamide and rabbit ATG) and ASCT.[19] While the ideal candidate for ASCT in MS has not been clearly established, international working groups have suggested that best outcomes might be achieved in younger patients with relapsing–remitting disease and active inflammation who have failed conventional treatments.[9,10,20]

The initial clinical data from ASCT in patients with progressive MS prompted us to use the less intensive conditioning regimen of cyclophosphamide and ATG. There were no transplant-related deaths. Transplant-related morbidity with this cyclophosphamide/ATG conditioning regimen was generally as expected but nonetheless considerable, with febrile neutropenia the most common side-effect. Despite the use of steroids, two patients had acute neurological deterioration during conditioning therapy which resolved spontaneously. Most patients experienced some functional decline and, while they usually returned to baseline by three months post-transplant, a period of rehabilitation was required in four of the 13 patients. This highlights the importance of patient selection and underscores the need to assess patient-related factors (age, comorbidity) as well as disease-related parameters (disease stage, disease duration and presence of inflammatory features on imaging).

ASCT following intensive immunosuppression with cyclophosphamide/ATG induced major changes in lymphocyte subsets upon immune reconstitution. The total lymphocyte count returned to baseline by nine months; however, the CD4 count fell significantly and did not return to baseline by two years. Due to reduced numbers of CD4+ T-cells in the post-transplant phase, the CD4/CD8 ratios were significantly decreased compared to baseline. This contrasts with another report in which there was gradual normalisation of CD4+ and CD8+ T-cells, with CD4/CD8 ratios reverting toward baseline at one year and not significantly different at two years' follow-up.[12] This study also demonstrated that naïve T-cells fell post-transplant and then steadily increased to reach higher levels than baseline at two years. We also found that RTEs and naïve T-cells fell sharply post-transplant but, while returning to baseline by one year, did not increase at two years. One explanation for these differences is the different conditioning regimens used, with the less intensive cyclophosphamide/ATG combination in our study and the more intensive cyclophosphamide/total body irradiation (TBI) combination in the study by Muraro et al.[12] It is certainly conceivable that different conditioning regimens will modify lymphocyte reconstitution post-transplant in different ways and this has the potential to influence the clinical outcome.

The sharp drop in cells of thymic origin followed by a relatively rapid recovery of thymic output, as assessed by naïve T-cells and RTEs, is consistent with a resetting of the immune repertoire but definitive confirmation requires a functional assessment. This has been demonstrated previously with normalisation of the T-cell receptor (TCR) repertoire usage following ASCT in SLE.[13] Also, in patients undergoing ASCT for MS, ablation of existing TCR clones and development of a new repertoire has been reported.[12,21] Similarly, in four patients receiving ASCT for JIA and juvenile dermatomyositis, strong diversification of the TCR was shown, including eradication of pre-existing clones.[22]

T-reg number and frequency was not different from healthy controls at baseline. The absolute number of T-regs fell post-transplant but did not change significantly as a percentage of CD4 cells. This contrasts with reports in JIA, SLE and systemic sclerosis showing restoration of T-regs following ASCT.[13,18,23] However, as demonstrated recently, it may well be that diversification of the T-reg repertoire is more important than absolute numbers or frequency.[22]

The clinical relevance of the changes in peripheral blood Th1, Th2 and Th17 cells is uncertain. A Th1 response has been suggested as important in the pathology of MS, though recent evidence indicates

a more complex process than Th1/Th2 polarisation and the involvement of other immune cells, particularly Th17 cells.[16] While an increase in Th1 and Th17 cells has been reported,[24,25] our cohort of MS patients with progressive disease had baseline levels similar to healthy volunteers. Following ASCT, absolute numbers of Th1 and Th2 cells did not change significantly while Th17 cells fell and then returned to normal by six months. The frequency of Th1 and Th2 cells increased post-ASCT and returned to normal at 18 and nine months, respectively. While the absolute numbers of Th17 cells fell briefly post-ASCT, their frequency increased. Recent reports have shown a suppressed Th17 response to myelin antigens,[17,25] and it is likely that assessment of target-specific T-cell responses by each T-cell subset will provide more information than their absolute numbers or frequency.

B-cells recovered by three months and remained at pre-ASCT levels for all but the 18-month time-point. The limited comparative data available suggest naïve B-cells return to normal levels by 12 months whereas memory B-cells may remain suppressed for up to three years.[26]

In summary, in a cohort of patients with progressive MS, ASCT following cyclophosphamide/ATG conditioning results in significant changes in lymphocyte subsets upon immune reconstitution. These changes, particularly ablation and subsequent recovery of thymic output, have the potential to reset the immune repertoire from a self-reactive to a more tolerant response. This is more likely to be effective in the early stages of disease where there is active inflammation than in the more advanced stages where central nervous system damage is more severe and often irreversible. Ongoing study to assess the nature of the reconstituting immune system following ASCT for MS is required, and the recommendations from the European Society for Blood and Marrow Transplantation provide a useful reference in this regard.[27] In particular, long-term analysis of lymphocyte reconstitution after different intensity conditioning regimens, including assessment of relevant antigen-specific responses, is needed to more fully elucidate which preparative approach induces a tolerant immune system with the capacity for the greatest clinical benefit.

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Conflicts of interest

Dr Gavin Cull has received travel sponsorship from Amgen Australia, served on an advisory board for Gilead and Abbvie Ltd (no honoraria) and presented at an educational forum organised by Roche (no honoraria).
Dr Dustin Hall has no conflicting interests.
Dr Fabis-Pedrini has received travel sponsorship from Biogen Australia and New Zealand.
Prof William Carroll has been the recipient of travel sponsorship, and provided advice to, Bayer Schering Pharma, Biogen-Idec, Novartis, Genzyme, Sanofi-Aventis, BioCSL and Merck-Serono.
Dr Luke Forster has no conflicting interests.
Fiona Robins has no conflicting interests.
Dr Reza Ghassemifar has no conflicting interests.
Christina Crosbie has no conflicting interests.
Susan Walters has no conflicting interests.
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