

Characteristics of T-cell receptor repertoire and myelin-reactive T cells reconstituted from autologous haematopoietic stem-cell grafts in multiple sclerosis

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Summary

Multiple sclerosis is thought to involve aberrant immune responses to myelin autoantigens. Haematopoietic stem-cell transplantation (HSCT) is in clinical trials for progressive multiple sclerosis based on the rationale that it destroys aberrant immune system, while recapitulation of lymphocyte ontogeny might alter the immune system and slow down disease progression. This study was undertaken to analyse characteristics of the T-cell receptor (TCR) repertoire, serum cytokine profile and the T-cell responses to myelin basic protein (MBP) in the reconstituted immune system in progressive multiple sclerosis. The study revealed that, following autologous HSCT, the T-cell immunity recovered in two distinctive phases. The first phase was characterized by limited T-cell immunity as a result of selective expansion of pre-existing T cells commonly expressing

the TCR β chain variable region (TCR BV) 20 and increased serum cytokine production during the first several months. The second phase of T-cell reconstitution coincided with increased thymic T-cell output 9–12 months after HSCT. T cells reconstituted from stem-cell grafts had the distinctive properties of comprehensive T-cell immunity and a broad TCR repertoire. T cells recognizing MBP were initially depleted by immunablation and rapidly expanded from the reconstituted T-cell repertoire in 12 months. The reconstituted MBP-reactive T cells exhibited a broader epitope recognition repertoire while maintaining the same skewed reactivity pattern compared with that seen at baseline. The findings have important implications in the understanding of the role of HSCT as a potential treatment for multiple sclerosis.

Keywords: haematopoietic stem-cell transplantation; multiple sclerosis; myelin basic protein; T-cell receptor

Abbreviations: CDR3 = complementarity-determining region 3; EDSS = Expanded Disability Status Scale; ELISA = enzyme-linked immunosorbent assay; ELISPOT = enzyme-linked immunospot assay; HSCT = haematopoietic stem-cell transplantation; Ig = immunoglobulin; IL = interleukin; MBP = myelin basic protein; PBS = phosphate-buffered saline; TCR = T-cell receptor; TCR BC = T-cell receptor β chain constant region; TCR BV = T-cell receptor β chain variable region; TREC = T-cell receptor rearrangement excision circles

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Introduction

Although the aetiology and pathogenesis of multiple sclerosis remain elusive, there is increasing evidence suggesting that the disease is associated with aberrant immune responses

(Martin *et al.*, 1992; Noseworthy *et al.*, 2000). Among multiple abnormalities that have been reported in multiple sclerosis, aberrant T-cell and antibody reactivity to myelin

antigens, such as myelin basic protein (MBP), is thought to play an important role in the disease process (Genain *et al.*, 1990; Ota *et al.*, 1990; Martin *et al.*, 1991; Zhang *et al.*, 1994; Bieganowska *et al.*, 1997; Madsen *et al.*, 1999). These autoreactive T cells have been found to undergo *in vivo* activation and expansion in the blood and cerebrospinal fluid of patients with multiple sclerosis (Allegretta *et al.*, 1990; Chou *et al.*, 1992; Zhang *et al.*, 1994). There is further evidence suggesting the presence of MBP-reactive T cells in brain lesions of multiple sclerosis patients (Oksenberg *et al.*, 1993; Krogsgaard *et al.*, 2000). In addition to the role of autoreactive T-cell and antibody reactivity, multiple sclerosis is also associated with an abnormal cytokine milieu as part of the inflammatory processes, which is characterized by the increased production of proinflammatory cytokines and chemokines/receptors (Balashov *et al.*, 1999; Zang *et al.*, 2000; Ozenci *et al.*, 2002). At a more general level, aberrant immune responses in multiple sclerosis may be attributable to a dysfunctional immune regulatory network that normally keeps autoreactive T cells and B cells in check (Weber *et al.*, 1998; Mycko *et al.*, 2003). The underlying mechanism is poorly understood at the present time. Currently, the standard treatment options for multiple sclerosis include β -interferons and glatiramer acetate, whose main mechanism of action is to modulate the immune system (Zhang *et al.*, 2002). Both immunomodulatory agents have been shown to reduce disease activity in relapsing–remitting multiple sclerosis (Noseworthy, 2003). However, these drugs, including mitoxantrone, have a limited clinical effect on the clinical course of secondary progressive multiple sclerosis (European Study Group, 1998; Miller *et al.*, 1999; Hartung *et al.*, 2002). At the present time, control of the disease activity is not satisfactory in a significant proportion of multiple sclerosis patients treated with the drugs, while others do not respond at all. A relatively high percentage of breakthrough disease while on the treatment is another challenge in our effort to treat multiple sclerosis. It is clear that alternative treatment strategies need to be investigated.

Recently, haematopoietic stem-cell transplantation (HSCT) has been tested in clinical trials to treat clinically advanced secondary progressive multiple sclerosis patients (Fassas *et al.*, 1997, 2000, 2002; Burt *et al.*, 1998; Kozak *et al.*, 2000; Openshaw *et al.*, 2000) based on its effectiveness in the treatment of various forms of autoimmune diseases in experimental animals, including experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis (van Bakkum *et al.*, 2002). The commonly used protocol involves high-dose immunosuppression (conditioning regimen) followed by autologous HSCT (Comi *et al.*, 2000). The rationale is that ablation of aberrant immune system followed by reconstitution of the new immune system from haematopoietic stem cells may significantly alter the characteristics of the T-cell responses and other immunological properties, which may improve the clinical course of multiple sclerosis. In more than 100 multiple sclerosis patients treated so far, an autologous HSCT procedure seemed to have some variably

positive impact on disease progression of multiple sclerosis (Fassas *et al.*, 1997, 2000, 2002; Burt *et al.*, 1998; Kozak *et al.*, 2000; Openshaw *et al.*, 2000). However, it is unknown whether the observed clinical benefit of autologous HSCT seen in early trials is due to the conditioning regimen and whether the clinical effect of HSCT can be sustained over time. It is speculated that sustained clinical benefit may be achieved if the functional characteristics and the T-cell repertoire of the immune system are significantly altered during reconstitution and such an effect persists over time. This possibility is less likely to occur for autologous HSCT as the new immune system is reconstituted from haematopoietic stem cells of autologous origin. Thus, detailed analysis of the characteristics of the reconstituted immune system, in particular the T-cell receptor (TCR) repertoire and T-cell responses to myelin autoantigens, is important for the understanding of the mechanism of action and for the clinical justification of using autologous HSCT for the treatment of multiple sclerosis, as the procedure is associated with significant mortality (Fassas *et al.*, 1997). The present study was not intended to evaluate the clinical effect of autologous HSCT in a small number of multiple sclerosis patients. It was undertaken to address the above-mentioned specific issues through detailed characterization of various components of the original and reconstituted immune systems. The study, though in a small number of multiple sclerosis patients, has provided important findings that have important implications in the understanding of the role of HSCT in the treatment of multiple sclerosis.

Material and methods

Patients and specimens

Four patients with progressive multiple sclerosis were included in the study. The patients had a mean Expanded Disability Status Scale (EDSS) score of 6.25 and had failed other conventional treatment options. Treatments before the study included β -interferon, glatiramer acetate, cladribine and mitoxantrone. Blood specimens were collected before the procedures at baseline and at various time-points within the first month then every 3 months after HSCT. CSF was obtained through lumbar puncture at baseline and after HSCT for oligoclonal banding. Neurological examinations and EDSS (Poser, 1983) were carried out at baseline and every 3 months through the first year after HSCT. MRI examinations of the brain and spine were performed with and without contrast at baseline and 6 and 18 months after HSCT. The study protocol was approved by the institutional human subjects review board.

HSCT treatment protocol

Peripheral blood stem cells were mobilized with granulocyte colony-stimulating factor (10 $\mu\text{g}/\text{kg}/\text{day}$) and collected after day 4. Patients received prednisone (1 $\text{mg}/\text{kg}/\text{day}$) during administration of granulocyte colony-stimulating factor to prevent exacerbation of multiple sclerosis. Cells were selected for CD34⁺ cells using the Isolex 300i device. Daily leucopheresis was done until 2 million CD34⁺ cells/kg had been collected. Patients received myeloablative chemotherapy, consisting of cyclophosphamide (90 mg/kg), total

Table 1 Primers for 25 TCR BV genes and the TCR BC gene

Gene	Sequence 5' à 3' (forward)	Sequence 5' à 3' (reverse)	Amplicon (bp)
BV1	AAGCACCTGATCACAGCAACT	TAGTTCAGAGTGCAAGTCAGG	209
BV2	GGTTATCTGTAAAGAGTGGAACT	AGGATGGGCACTGGTCACTGT	229
BV3	TCGAGATATCTAGTCAAAGGACG	GGTGCTGGCGGACTCCAGAAT	228
BV4	AAGCAGGGATATCTGTCAACGT	TTCAGGGCTCATGTTGCTCAC	235
BV5	GATCAAAACGAGAGGACAGCA	AGCACCAAGGCGCTCACATTCA	217
BV6	CTCAGGTGTGATCCAATTTCA	CCCCGCTCTGTGCGCTGGAT	195
BV7	CATGGGAATGACAAATAAGAAGTCT	TGGCTGCAGGGCGTGTAGGTG	214
BV8	CCCCGCCATGAGGTGACAGAG	GAGTCCCTGGGTTCTGAGGGC	239
BV9	CCAAAATACCTGGTCACACAG	CCAGGGAATTGATGTGAAGATT	207
BV10	ACCTAGACTTCTGGTCAAAGCA	GGACTGGATCTCCAAGGTACA	223
BV11	TTATAGGGACAGGAAAGAAGATC	ATGTGAGGGCCTGGCAGACTC	224
BV12	CAAGACACAAGATCACAGAGACA	GGCAGCAGACTCCAGAGTGAG	224
BV13	TGAAGACAGGACAGAGCATGACA	CACAGATGTCTGGGAGGGAGC	227
BV14	ACCCAAGATACCTCATCACAGTG	AGAGGTCTGGTTGGGGCTGGG	242
BV15	TCACAAAGACAGGAAAGAGGATT	GGGGATGGCAGACTTAGGGA	215
BV16	GTTCCCCAGCCACAGCGTAATA	CAGTTCGTCAGGCTGCACCTT	235
BV17	GTCCCCAAAGTACCTGTTCAGA	AGCTGTCGGGTTCTTTTGGGC	244
BV18	AGACACCTGGTCAGGAGGAGG	TGCCGAATCTCCTCGCACTAC	240
BV19	CCAGGACATTTGGTCAAAGGAAAA	CAGTGCCGTGTCTCCCGGTTT	246
BV20	GACCCTGGTGCAGCCTGTG	GAGGAGGAGCTTCTTAGAACT	223
BV21	CCCAGATATAAGATTACAGAGAAA	CTGGATCTTGAGAGTGGAGTC	219
BV22	CACAGATGGGACAGGAAGTGATC	GTCCTCCAGCTTTGTGGACCG	221
BV23	AAGAGGGAAACAGCCACTCTG	CAGCTCCAAGGAGCTCATGTT	207
BV24	CCAAGATACCAGGTTACCCAGTTT	CAGGCCTGGTGAGCGGATGTC	228
BV25	AAAACATCTTGTGTCAGAGGGGAA	TGAATCCTCAAGCTTCGTAGC	238
TCR BC	CAGCGCCCTTGTGTTGATG	AAGCGCTGGCAAAAAGAAGAA	121

body radiation (1000 cGy) and anti-thymocyte globulin (30 mg/kg). A median dose of 3.9 million CD34⁺ cells/kg (range 2.6–7.4 million) was thawed and infused through a central venous catheter. A median of 1.1×10^4 CD3⁺ T cells/kg were infused. Granulocyte colony-stimulating factor (5 µg/kg/day) was started on day 1 after transplantation and was given until the neutrophil count reached at least 1000/µl.

Determination of TCR β chain variable region (TCR BV) gene usage by real-time PCR analysis

Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and was treated with DNase (RNase-free DNase Set; Qiagen, Hilden Germany) to remove contaminating genomic DNA.

Twenty-five TCR BV and TCR β chain constant region TCR BC gene segments were cloned by using the TA Cloning kit (Invitrogen, San Diego, CA, USA) and One Shot TOP10 *E. coli* competent cells (Invitrogen) according to the manufacturer's protocol. The oligonucleotide sequences of the BV-specific primers are shown in Table 1. cDNA was synthesized from RNA using random primers and Superscript II (Invitrogen) in a 20-µl reaction. TCR BV gene expression was analysed by real-time quantitative PCR. An internal reference control for BV-BC amplification and a non-template control containing no cDNA were added to each reaction. Real-time PCR was performed in 96-well optical PCR plates on an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Briefly, an aliquot of cDNA sample (0.7 µl) was mixed with 25 pairs of BV-specific primers and one pair of BC primers (0.1 mM in the final solution), respectively, together with SYBR Green PCR Master Mix (Applied Biosystems) to a final reaction volume of 50

µl. All reactions were done in duplicate. The reaction was performed at 50°C for 2 min and at 95°C for 10 min as a hot-start activation, which was followed by 40 cycles of reaction at 95°C for 15 s and at 60°C for 1 min. The expression of individual BV genes was calculated based on signal intensity of the PCR reactions according to the following formula:

$$\text{TCR BV}_n (\%) = [2 - (\text{BV}_n C_T - \text{BC } C_T) \times 100] / \sum (2 - (\text{BV}_{1-25} C_T - \text{BC } C_T) \times 100) \times 100$$

(C_T refers to the threshold cycle).

The real-time PCR method used in this study was sensitive and specific for the detection of selective expansion of T cells based on the BV expression pattern. As shown in Fig. 1, selective expansion of BV2 was readily detected by real-time PCR analysis in four peripheral blood mononuclear cell preparations after stimulation with toxic shock syndrome toxin, a superantigen known to activate Vβ2⁺ T cells.

Quantitative analysis of TCR rearrangement excision circles by real-time PCR

TCR rearrangement excision circles (TREC) in DNA of PBMCs were performed by quantitative real-time PCR. DNA was extracted from PBMCs using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA) and 100 ng of DNA was added to each reaction with the primers CAC ATC CCT TTC AAC CAT GCT and GCC AGC TGC AGG GTT TAG G (Applied Biosystems). SYBR Green PCR Master Mix (Applied Biosystems) and PCR water were added to a final reaction volume of 50 µl. An additional set of primers to albumin was used to normalize for the relative quantity of genomic TREC

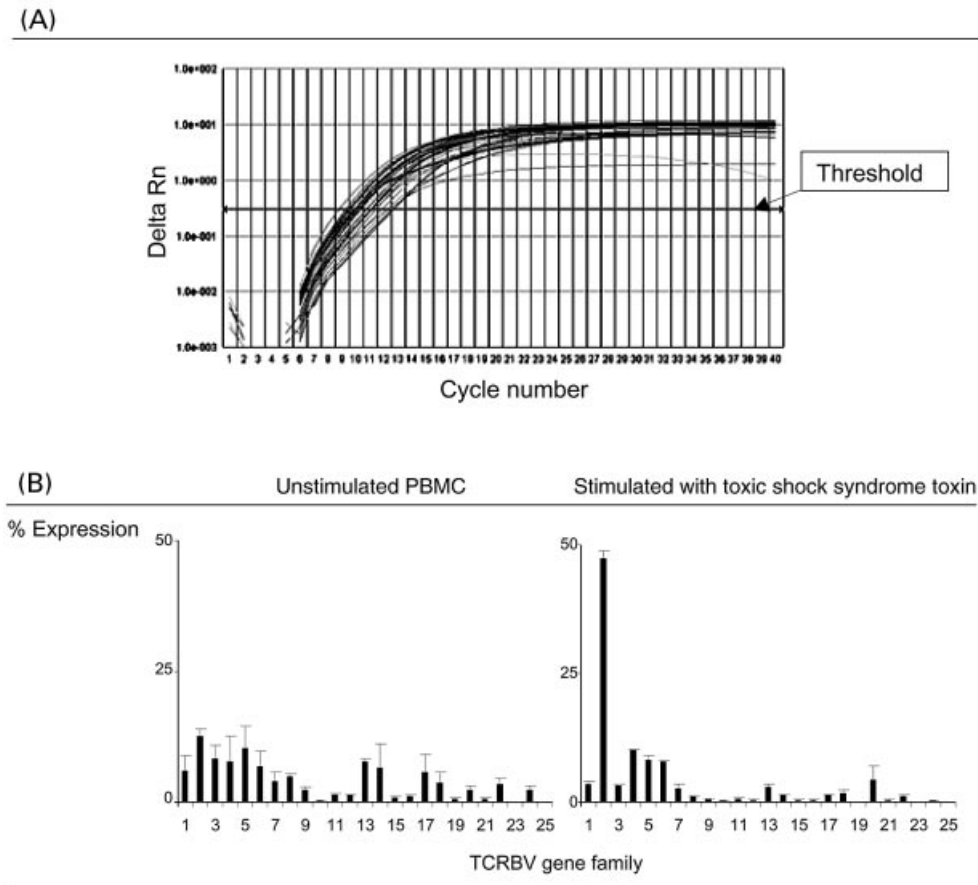


Fig. 1 Optimization of BV-specific primers for real-time PCR analysis and BV gene analysis of peripheral T cells after stimulation with a superantigen. **(A)** A set of oligonucleotide primers specific for 25 BV genes and the TCR BC gene were tested for PCR amplification efficiency using the ABI 7000 Sequence Detection System. Individual curves/profiles represent cycle-dependent fluorescence intensities (Delta Rn) of PCR amplifications by all 25 BV-specific primers. The C_T (cycle threshold) value for each specific BV gene is expressed as the number of cycles at the indicated threshold. Similar slopes of the curves as a function of cycle numbers indicate similar amplification efficiency of 25 BV genes and the TCR BC gene as a reference under the PCR conditions described in Material and methods. **(B)** PBMCs were prepared separately from four healthy individuals and cultured in the presence and absence of toxic shock syndrome toxin at the predetermined concentration of 1 $\mu\text{g}/\text{ml}$ for 7 days. Cells were collected and washed by centrifugation, and RNA was extracted for real-time PCR analysis using primers specific for 25 BV genes. The PCR conditions are described in Material and methods. Results are presented as mean percentage expression of BV genes relative to BC expression in four cell preparations.

(Douek *et al.*, 2000). The reaction was performed at 50°C for 2 min and at 95°C for 10 min as a hot-start activation, which was followed by 40 cycles of reaction at 95°C for 15 s and at 60°C for 1 min. The TREC values were expressed as $2^{-\Delta C_T}$ ($\Delta C_T = C_T$ of TREC – C_T of albumin).

Immunoscope analysis

PCR reactions were performed with 1 μl of cDNA sample derived from PBMCs in the following amplification mixture: 5 μl 10 \times PCR buffer (100 mM Tris-HCl, pH 8.3 and 500 mM KCl), 3 μl 25 mM magnesium chloride, 1 μl of 10 mM dNTP mix, 0.5 μl of *Taq* polymerase (5 U/ μl) (Invitrogen, Carlsbad, CA, USA), 20 pmol of primers (specific BV forward primer and BC primer 5'-CGA CCT CGG GTG GGA ACA). The PCR amplification profile used was 30 s at 94°C for denaturation, 30 s at 57°C for annealing and 30 s at 72°C for extension in a total of 40 cycles. Immunoscope analysis was performed with a modified protocol. Two microlitres of BV-specific PCR products were used as templates and run-off reactions were

performed with a single internal fluorescent label for each of the 6-FAM (6-carboxy fluorescein)-labelled BC (5',6-FAM-CAC AGC GAC CTC GGG TGG G). The reaction profile consisted of 30 s at 94°C denaturation temperature and 15 cycles at 94°C for 45 s; at 55°C for 45 s, at 72°C for 1 min followed by 72°C for 5 min as an extension step. The resulting PCR products were then denatured in formamide and analysed on an Applied Biosystems 3100 Prism using GeneScan 3.7 software (Perkin-Elmer, Boston, MA, USA). Labelled products were analysed separately as one-colour electro-fluorographs. The relative intensity of signal corresponding to complementarity-determining region 3 (CDR3) length was expressed as the area under the experimental peak divided by the area under the control peak found within a Gaussian distribution.

Serum cytokine measurement by enzyme-linked immunosorbent assay (ELISA)

Serum specimens were measured for the concentrations of cytokines using ELISA kits according to the manufacturer's instructions (Pharmingen, San Diego, CA, USA). Microtitre plates were coated

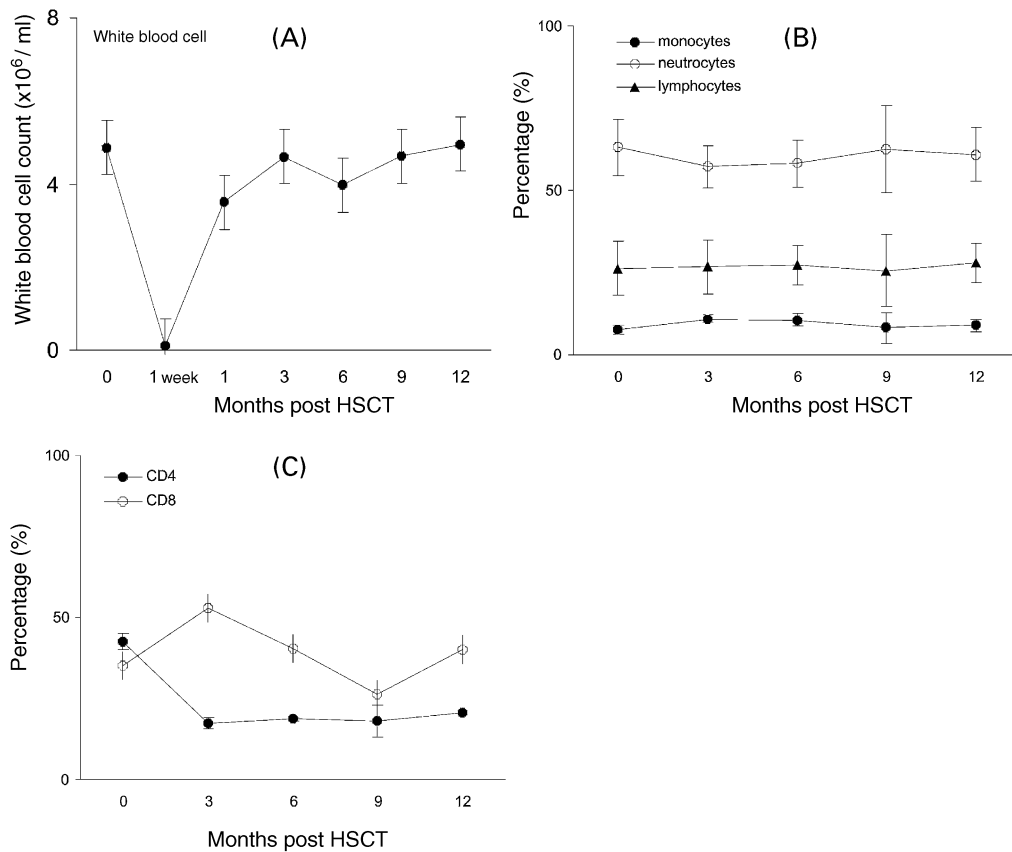


Fig. 2 Changes in white blood cells and ratio of CD4 and CD8 T cells after HSCT. White blood cells and differentiation were analysed at baseline and the indicated time points following HSCT. T cells of the CD4 and CD8 phenotypes in peripheral blood specimens were examined by flow cytometry. The results are expressed as the percentage of total lymphocytes.

Table 2 Demographic data and clinical characteristics of patients

Subject	Diagnosis	Age (years)	Sex	Disease duration (years)	EDSS	HLA haplotypes	
						Class I	Class II
MS-1	SPMS	44	M	19	6.0	A2, A3, B44, B40	DR4, DR11, DRB3, DRB4
MS-2	SPMS	38	F	10	7.0	A2, A24, B18, B57	DR7, DR13, DRB3, DRB4
MS-3	PPMS	50	F	10	6.0	A11, A68, B14, B55	DR13, DR15, DRB3, DRB5
MS-4	SPMS	50	M	7	6.0	A11, A68, B27, B35	DR8, DR15, DRB5
Mean		45.5		11.5	6.25		

SPMS = secondary progressive multiple sclerosis; PPMS = primary progressive multiple sclerosis; HLA = histocompatibility leucocyte antigen.

overnight at 4°C with mouse monoclonal antibodies (capturing antibody). Wells were then blocked at 37°C for 2 h with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (Sigma, St Louis, MO, USA) and were subsequently washed four times with 0.02% Tween 20 in a 0.9% NaCl solution. Samples were added in duplicate and incubated for 2 h with a matched biotinylated detecting antibody (0.25 µg/ml of each monoclonal antibody, respectively) in 2% bovine serum albumin/PBS/Tween 20. After washing, avidin-conjugated horseradish peroxidase was added and plates were incubated for 1 h. The substrate used for colour development was 3,3',5,5'-tetramethylbenzidine (Sigma). Optical density was

measured at 450 nm using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA) and cytokine concentrations were quantitated by Microplate computer software (Bio-Rad) using a double eight-point standard curve. The detection limits for all four cytokines were <15 pg/ml in all assays.

The frequency analysis and epitope recognition of MBP-reactive T cells

Enzyme-linked immunospot (ELISPOT) assays were performed using ELISPOT γ -interferon sets obtained from BD Pharmingen

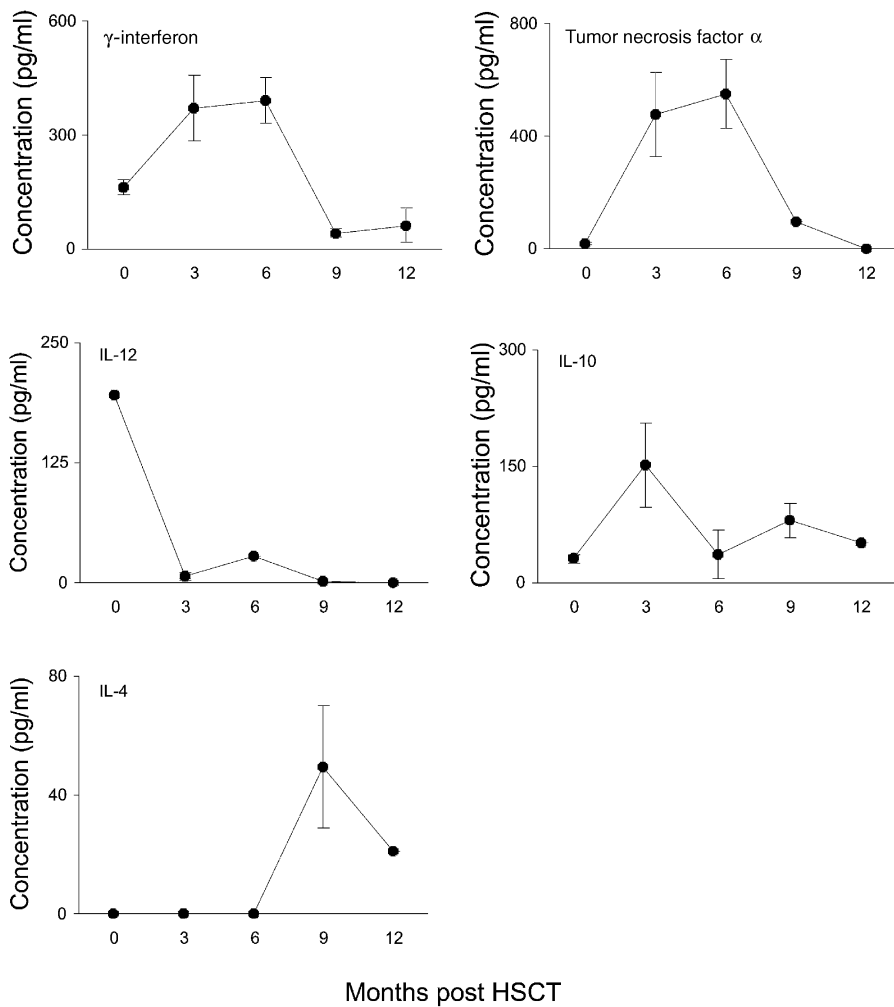


Fig. 3 Serum cytokine production profile before and after HSCT. Serum levels of selected cytokines, including γ -interferon, tumour necrosis factor α , IL-10 and IL-4, were measured quantitatively using ELISA at baseline (time 0) and the indicated time-points after HSCT.

(San Diego, CA, USA) (Pelfrey *et al.*, 2000; Targoni *et al.*, 2001). The ImmunoSpot plates were coated overnight at 4°C with a capture antibody dissolved in PBS. Non-specific binding sites were blocked using 1% bovine serum albumin in PBS for 1 h and washed three times with PBS. PBMC preparations were seeded out at 200 000 per well in triplicate wells in the presence and absence of MBP (40 μ g/ml) or the overlapping peptides of MBP (20 μ g/ml). RPMI1640 medium containing 5% fetal calf serum was used. Phytohaemagglutinin-protein was used as a positive control. After 48 h, the plates were washed and a biotinylated detection antibody was added and incubated for 12 h at 4°C. Subsequently, streptavidin–horseradish peroxidase in PBS/bovine serum albumin/Tween was added and incubation was performed for 2 h at room temperature. Spots were developed using AEC (Pierce Pharmaceuticals, Rockford, IL, USA) solution for 10–20 min. The reaction was stopped by rinsing plates with water until clear spots were visualized microscopically. The plates/membranes were then air-dried overnight before the plates were subjected to image analysis. The analysis was performed using a Series 1 ImmunoSpot

Image Analyzer (Cellular Technology, Cleveland, OH, USA) specifically designed for ELISPOT assay. Digitized images were analysed and results were expressed as number of positive spots per total number of cells per well.

Results

Clinical findings and the general characteristics of the reconstituted immune system after autologous HSCT

Four patients with clinically definite multiple sclerosis were included in the study. Three patients had secondary progressive multiple sclerosis and one had primary progressive multiple sclerosis. Table 2 shows the baseline demographics and histocompatibility leucocyte antigen (HLA) haplotypes of the patients. Mean age at the time of HSCT was 45.5 years, with a mean duration of the progressive phase of multiple

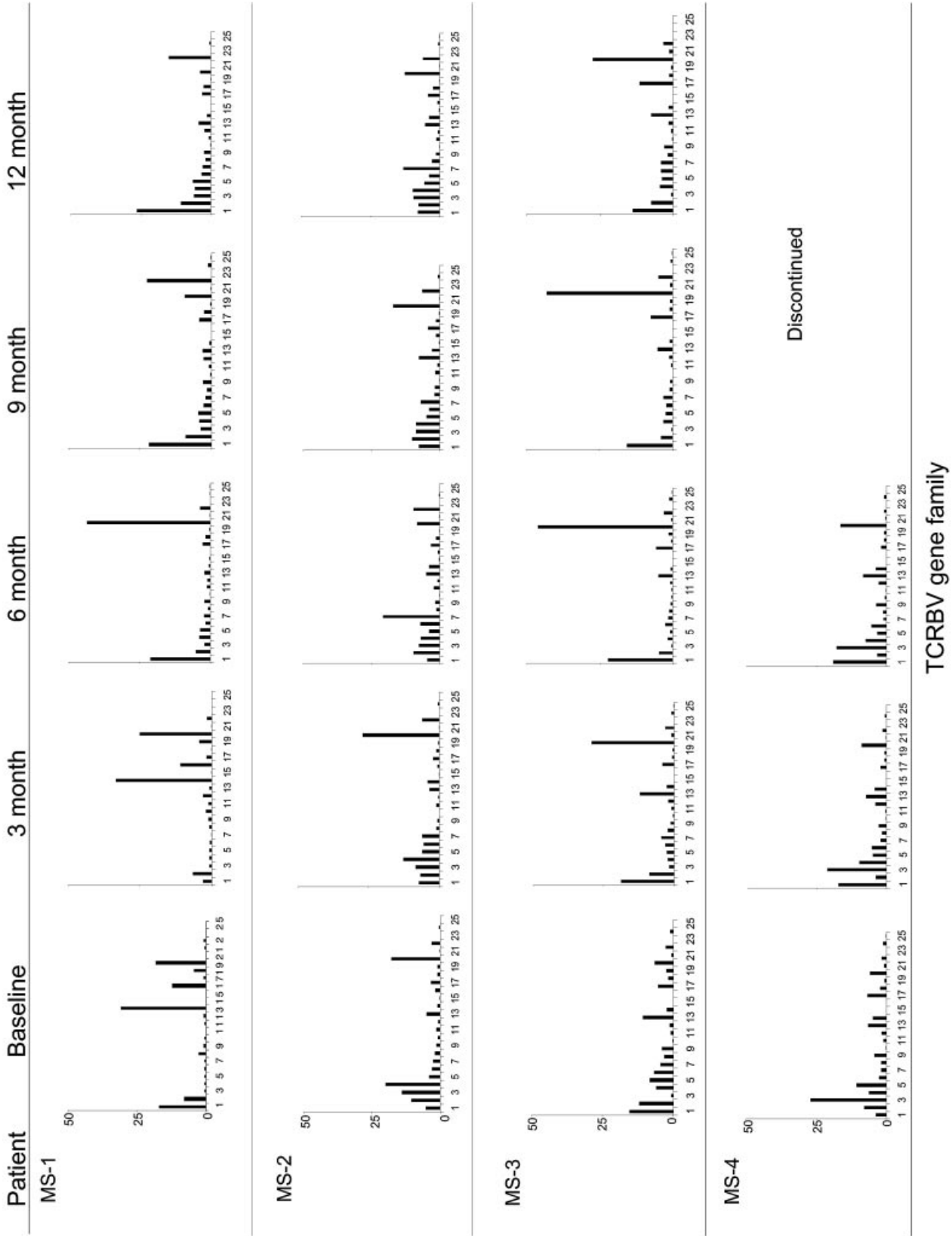


Fig. 4 Changes in the TCR BV gene distribution profile in relation to HSCT. TCR BV gene expression was analysed quantitatively in PBMC specimens collected at the indicated time-points by real-time PCR using specific primers for 25 BV genes. BV gene distribution is presented as the mean percentage expression of each BV gene relative to BC expression on the y axis.

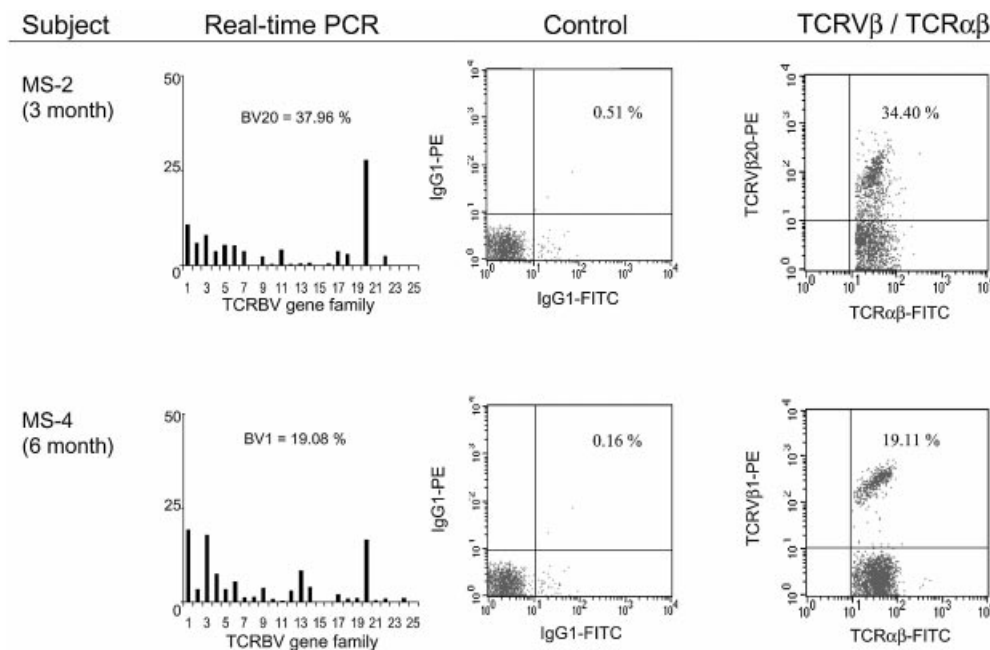


Fig. 5 Surface TCR BV expression of peripheral blood T cells by flow cytometry. PBMCs were analysed by flow cytometry using mouse anti-human V β antibodies (TCR $\alpha\beta$ -FITC and V β 1-PE or V β 20-PE). The analysis was performed in MS-2 and MS-4 at month 3 and month 6 after HSCT, at which time overexpression of BV1 and BV20 was apparent.

sclerosis of 6 years. Patient MS-4 was stable at his 6-month evaluation but declined further follow-up. In the other three patients, the EDSS remained unchanged over the period of 12 months. None of the patients demonstrated any enhancing lesions on follow-up MRI of the brain or spine. MS-3 had several new T2-hyperintense lesions on her follow-up brain MRI at 12 months. No other patient showed any increase in the number of T2 hyperintense lesions on follow-up imaging. CSF was obtained in all patients at baseline and 3 months after HSCT. All samples were abnormal at baseline, showing the presence of oligoclonal bands separate from those present in serum. At follow-up CSF examinations, the number of oligoclonal bands decreased in two patients (MS-2 and MS-3); oligoclonal bands disappeared in MS-4 and persisted in MS-1. None of the patients still being followed has required any further immunosuppressive therapy.

Complete immune ablation was achieved in all patients, and was followed by reconstitution of the immune system from mobilized haematopoietic stem cells. As shown in Fig. 2, the white blood cell count reached the pretreatment level ~1 month after HSCT and the percentages of monocytes, neutrophils and total lymphocytes remained largely unchanged (Fig. 2A and B). However, although the percentage of CD3⁺ total T-cell population recovered to the pretreatment level after HSCT, the ratio of CD4⁺ to CD8⁺ T cells was altered because of sustained depression of CD4⁺ T cells (Fig. 2C). Serum levels of immunoglobulin (Ig) G, IgA, IgM and IgE were examined at baseline and every 3 months after HSCT and did not change significantly compared with

baseline (data not shown). Furthermore, serum cytokine levels, including interleukin (IL)-12, γ -interferon, tumour necrosis factor α , IL-10 and IL-4, fluctuated variably following HSCT and returned to pretreatment low levels at 12 months (Fig. 3). There was significant elevation of serum γ -interferon, tumour necrosis factor α and IL-10 between 3 and 6 months, whereas serum IL-12 production decreased persistently after HSCT in all patients.

Dynamic changes in the TCR variable gene repertoire and thymic T-cell output

Diversity and comprehensiveness of the T-cell immunity can be reflected in the TCR variable gene repertoire. In a series of real-time quantitative PCR analyses, PBMCs derived from patients at baseline and every 3 months following HSCT were examined for the TCR BV gene distribution pattern using 25 TCR BV-specific primers. The results revealed a highly significant BV skewing for BV20 in all four patients, whereas biased expression of BV1 and BV14 was noted in some but not all multiple sclerosis patients (Fig. 4). The skewed BV gene expression persisted after HSCT for several months before the TCR BV gene distribution returned to a more heterogeneous pattern in 12 months. The overexpression of TCR variable genes (BV20 and BV1) was confirmed in parallel experiments by flow cytometry using conjugated monoclonal antibodies to V β 20 and V β 1. Representative experiments are shown in Fig. 5. Furthermore, PCR-amplified TCR BV transcripts were subject to CDR3 length analysis

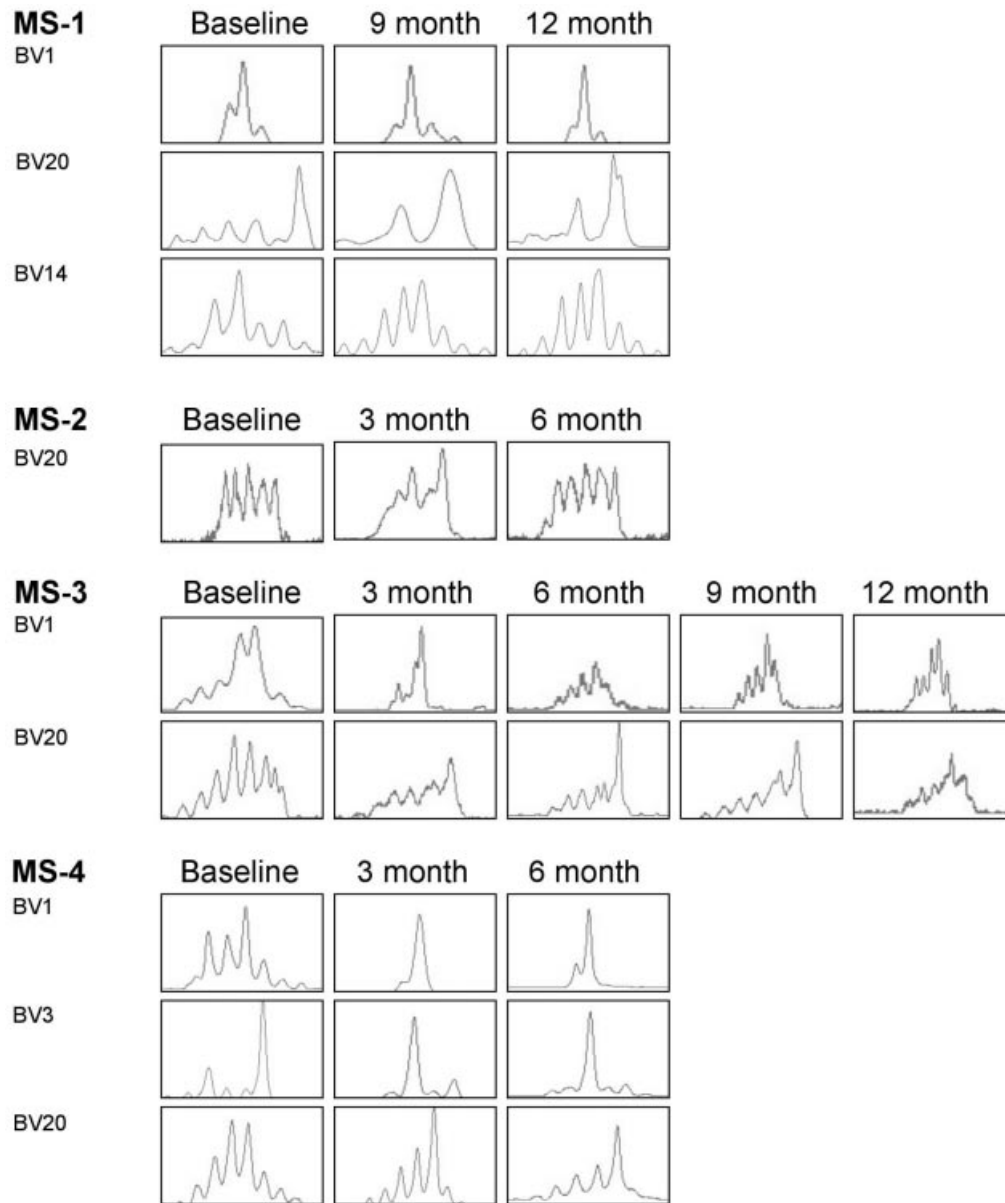


Fig. 6 CDR3 length analysis of 5' BV-3' BC region of the overexpressed BV transcripts. TCR transcripts of the overexpressed BV genes identified in each individual (BV1/BV20/BV14 for MS-1; BV20 for MS-2, BV1/BV20 for MS-3 and BV1/BV3/BV20 for MS-4) were analysed for CDR3 length (the V-D-J junctional region) by immunoscope using specific primers. The CDR3 size distribution pattern is expressed as peak areas (x axis). The y axis represents arbitrary units of fluorescence intensity.

using immunoscope technique to address whether the overexpression of BV genes represented clonal expansion. As illustrated in Fig. 6, the overexpressed BV20, BV1 and BV14 predominantly exhibited a Gaussian pattern of CDR3 length, suggesting polyclonal distribution. There were a few exceptions that exhibited characteristics of an oligoclonal pattern (e.g. MS3-BV1) (Fig. 6). These findings demonstrated that T-cell populations commonly expressing BV20 and, to a lesser degree, BV14 and BV1, underwent selective expansion and dynamic changes in clonal composition following HSCT, as evident by CDR3 length profiling.

The observed characteristic changes in TCR variable gene repertoire are consistent with selective expansion of pre-existing T cells through a thymus-independent pathway during the first several months (Bomberger *et al.*, 1998; Douek *et al.*, 2000). The TCR repertoire that was subsequently reconstituted from haematopoietic stem cells had a more heterogeneous distribution pattern that was relatively similar to the pattern at baseline. This is supported by the profile of thymic T-cell output in these patients. In parallel experiments, the thymic output of T cells was measured quantitatively by analysing the numbers of TCRC in

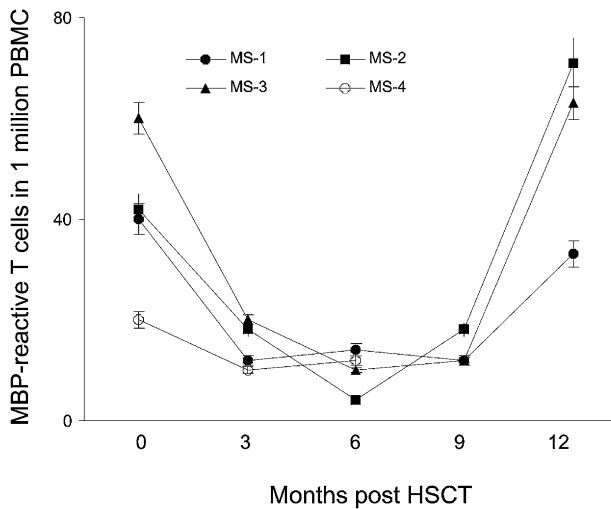


Fig. 7 Changes in the precursor frequency of MBP-reactive T cells in relation to HSCT. The precursor frequency of MBP-reactive T cells was analysed by ELISPOT in PBMCs obtained from four multiple sclerosis patients (MS-4 was discontinued at months 9 and 12). The results are presented as the number of γ -interferon-secreting T cells in response to MBP stimulation (40 μ g/ml) per million PBMCs at the indicated time-points.

peripheral T cells using real-time PCR (Douek *et al.*, 2000). The relative expression of TREC was 27.87 ± 10.97 at baseline and declined drastically to 1.03 ± 1.27 at month 6 after HSCT. The TCREC expression had recovered to 24.76 ± 9.37 by 12 months after HSCT, indicating recovery of thymus-dependent T-cell output within the period between 9 and 12 months.

Precursor frequency and recognition pattern of MBP-reactive T cells recapitulated from autologous haematopoietic stem cells

One of the most important issues to be addressed in this study was related to the recapitulation of autoreactive T cells from autologous haematopoietic stem cells. In this study, MBP was selected for its potential relevance to the pathogenesis of multiple sclerosis. First, we examined the precursor frequency of MBP-reactive T cells using ELISPOT at baseline and every 3 months after HSCT. As shown in Fig. 7, the precursor frequency of MBP-reactive T cells ranged from 2 ± 10^{-5} to 6×10^{-5} in baseline PBMC specimens of multiple sclerosis patients. The observed frequency of MBP-reactive T cells was higher than that routinely measured in multiple sclerosis patients (Olsson *et al.*, 1992; Hellings *et al.*, 2001). MBP-reactive T cells were depleted by a conditioning regimen and recovered at very low precursor frequency for ~9 months after HSCT in all multiple sclerosis patients. However, MBP-reactive T cells reconstituted from autologous haematopoietic stem cells underwent rapid expansion and reached high precursor frequencies that were similar to or

exceeded the baseline levels. During that period (from month 9 to month 12), there was no significant change in clinical progression and treatment regimen in these patients.

Further analyses were carried out to determine whether the epitope recognition pattern of MBP-reactive T cells occurring 12 months after HSCT was comparable to that of MBP-reactive T cells present at baseline. To this end, PBMC specimens were primed with a set of overlapping 15-mer peptides of MBP and the reactivity to each of the overlapping peptides was measured by ELISPOT based on γ -interferon secretion. The results revealed that, at 12 months, the epitope recognition pattern of MBP-reactive T cells was considerably more heterogeneous than that of MBP-reactive T cells present at baseline (Fig. 8). It was noted that the skewed reactivity of MBP-reactive T cells to certain predominant epitopes (e.g. residues 41–59, 81–99, 111–129, 131–149 and 151–170) seen at baseline in these patients persisted at 12 months, though additional epitopes were detected at that time. These experiments concluded that, following sustained suppression after HSCT, MBP-reactive T cells appeared to reconstitute from autologous haematopoietic stem-cell grafts and occurred at frequencies comparable to or higher than pretreatment levels, with a relatively heterogeneous epitope recognition pattern.

Discussion

The study described here has revealed, for the first time, a number of important observations that have direct relevance to the current effort to evaluate the role of HSCT in the treatment of multiple sclerosis. First, the real-time PCR analyses of the TCR variable gene repertoire indicated selective expansion of certain T-cell subsets, as characterized by skewed expression of BV20, BV1 and BV14. Overexpression of BV20 occurred in all individuals. The observed T-cell expansion is likely to have resulted from pre-existing T cells that survived the conditioning regimen. There is evidence indicating that rapid but transient recovery of limited T-cell immunity during the first several months following autologous HSCT is mediated by the expansion of a small population of pre-existing memory T cells—the thymus-independent pathway (Douek *et al.*, 2000). Consistent with previous reports (Bomberger *et al.*, 1998; Douek *et al.*, 2000) is our finding that the TCR repertoire gradually shifted to a more heterogeneous pattern as new naive T cells were generated from the reconstituted immune system. Although skewed expression of other TCRVB genes has been reported in previous studies unrelated to multiple sclerosis, this is the first description of a common overexpression of BV20 in multiple sclerosis patients after HSCT. The significance of the finding is that the TCR repertoire may be shaped towards selective expansion of BV20⁺ T cells by the *in vivo* environment characteristic of multiple sclerosis. For example, a microbial superantigen(s) associated with multiple sclerosis may drive certain T-cell subsets expressing specific BV gene(s) to expand. The CDR3 length analysis of

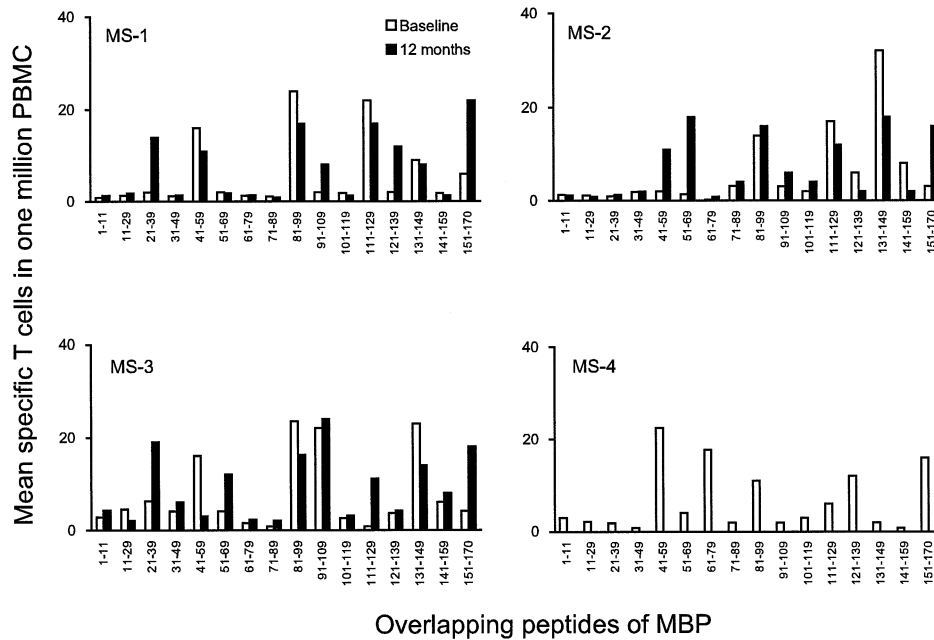


Fig. 8 Peptide recognition of MBP-reactive T cells at baseline and month 12. MBP-reactive T cells present at baseline and at month 12 were analysed for reactivity with a panel of overlapping peptides of MBP by ELISPOT. PBMC specimens were stimulated with peptides of MBP (20 $\mu\text{g/ml}$), respectively. The T-cell frequency is expressed as the number of γ -interferon-secreting T cells to each peptide per million PBMCs.

the overexpressed BV transcripts seems to support this possibility.

One of the most important findings described here is related to the occurrence and characteristics of myelin-reactive T cells during reconstitution of the immune system following HSCT. This study shows, for the first time, that MBP-reactive T cells recapitulate from autologous haematopoietic stem cells in multiple sclerosis patients. Following a conditioning regimen, MBP-reactive T cells were initially depleted and recovered subsequently at very low precursor frequencies for several months before they underwent rapid expansion between 9 and 12 months. The finding supports the notion that the T-cell repertoire that initially expanded from pre-existing memory T cells is limited and does not contain sufficient MBP-reactive T cells to mount T-cell responses to MBP, whereas complete reconstitution of a comprehensive T-cell repertoire from autologous haematopoietic stem cells leads to expansion and responsiveness of MBP-reactive T cells. This is further supported by the observation that the thymic output of T cells drastically decreased following HSCT and that it recovered 9–12 months after HSCT, which signals T-cell reconstitution and expansion from stem-cell grafts in the thymus-dependent pathway (Douek *et al.*, 2000). Furthermore, the reconstituted MBP-reactive T cells seem to have a broad epitope recognition repertoire. It is interesting to note that the repertoire of reconstituted MBP-reactive T cells has the imprint of epitope recognition of MBP-reactive T cells present at baseline. That is, they maintained the skewed

reactivity towards certain regions of MBP also seen at baseline and broadened the reactivity to additional epitopes of MBP that were not detected before HSCT in these patients. The findings are particularly important as these autoreactive T cells are implicated in the disease processes of multiple sclerosis, and they may also apply to other autoreactive T-cell responses that are potentially involved in multiple sclerosis.

Taken together, the findings described here indicate that, following HSCT, the T-cell component of the immune system seems to recover through two phases, which are characterized by initially limited T-cell immunity as a result of selective T-cell expansion from pre-existing T cells (thymus-independent pathway) and the gradual development and maturation of comprehensive T-cell immunity as new T cells expand from autologous haematopoietic stem cells in a thymus-dependent pathway. The two phases have distinct characteristics in terms of the TCR repertoire, cytokine milieu and autoreactive T cells. Thus, although the immune system is significantly altered during the first phase following HSCT for several months, the functional and structural/phenotypic properties and clonal composition of the reconstituted T cells do not seem to differ significantly from and carry the imprint of the original immune system that was destroyed by autologous HSCT in these multiple sclerosis patients. This is not particularly surprising as haematopoietic stem cells of autologous origin carry all the same genes and have the same lymphocyte ontogeny, and they are not altered *per se* to develop a different immune system in the same *in vivo*

environment. The findings would indicate that the source of haematopoietic stem cells is important in determining whether the clinical benefit of HSCT will be sustained over time, and would support further investigation to compare immunological properties and the clinical effect of allogeneic versus autologous stem-cell grafts in future studies. It is likely that the immune system reconstituted from a healthy allogeneic source of haematopoietic stem-cell graft may retain some functional and phenotypic characteristics and the cellular composition of the original healthy immune system and may recapitulate the regulatory T-cell component that is critical to sustaining the effect of HSCT.

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