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ORIGINAL ARTICLE

T-cell clones persisting in the circulation after autologous hematopoietic SCT are undetectable in the peripheral CD34+ selected graft

AN Dubinsky[1,2], RK Burt[3], R Martin[1,4] and PA Muraro[1,2]

[1]*Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA;* [2]*Division of Neuroscience and Mental Health, Department of Cellular and Molecular Neuroscience, Imperial College London, London, UK;* [3]*Division of Immunotherapy, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, USA and* [4]*Institute for Neuroimmunology and Clinical Multiple Sclerosis Research, Hamburg, Germany*

In addition to its established hematological indications, autologous hematopoietic SCT (HSCT) can ameliorate the course of severe autoimmune disorders through a reconditioning of the immune system. We have shown earlier that HSCT determines extensive renewal of the TCR repertoire in multiple sclerosis patients. However, the observed persistence post-therapy of some pre-existing T-cell clones suggested the potential for disease recapitulation. Here, we investigated whether TCRs that reappear after a myeloablative conditioning regimen and HSCT were reintroduced with the autologous, CD34-selected hematopoietic stem cell (HSC) graft. In all, we cloned and sequenced 2237 TCR clones from peripheral blood and HSC grafts from four patients who underwent autologous HSCT for severe multiple sclerosis. Surprisingly, in-frame TCR sequences were detectable in only one of four patient grafts and no TCR sequences were found to be shared between the graft and pre- or post-HSCT samples. These findings provide the first evidence from extensive sequencing analysis to suggest that T cells in autologous HSC grafts that have been mobilized with CY + G-CSF and CD34-selected have limited survival capacity and are therefore unlikely to be a major source of carryover of T-cell expansions potentially involved in autoimmune disease.

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Correspondence: Dr PA Muraro, Division of Neuroscience and Mental Health, Department of Cellular and Molecular Neuroscience, Imperial College Hammersmith Hospital Campus, Du Cane Road, Burlington Danes Building, Room E415, London W12 0NN, UK.
E-mail: p.muraro@imperial.ac.uk
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Introduction

Autologous hematopoietic SCT (HSCT) can control severe autoimmune disease in high-risk patients who fail to respond to immunomodulatory and conventional immunosuppressive treatments. One mechanism through which this beneficial action is thought to occur is by executing a large-scale depletion of pathogenic memory T cells and by promoting the regeneration of a new and tolerant immune repertoire.[1] This notion termed 'immune resetting' has been corroborated by the demonstration of a massive renewal of the TCR repertoire post-HSCT in patients with multiple sclerosis (MS)[2] and by the evidence of recovery of regulatory T cells in individuals with juvenile idiopathic arthritis.[3]

Currently, there is a debate as to whether patients with autoimmune disease should be treated with maximum intensity myeloablative conditioning regimens or with less intensive non-myeloablative schemes.[4] Myeloablative regimes are expected to be more effective in ablating pretransplant disease-related clones, but are complicated by treatment-related toxicities.[5–9] In our previous study, we found that a myeloablative conditioning regimen resulted in extensive immune renewal, yet a minority of the T-cell clones populating the peripheral blood pre-therapy was also detected in the blood post-transplantation in some patients.[2] Although the frequency of these clones was decreased post-transplant and their persistence was not associated with a different clinical outcome during the follow-up, an incomplete immune renewal even after a myeloablative regimen raised the concern of potential recapitulation of autoimmunity and prompted us to investigate the source of the remnant cells.

We undertook this analysis to clarify whether persisting T-cell clones could have arisen from the reinfused CD34-selected hematopoietic stem cell (HSC) autograft. To understand the origin of the persisting clones, we carried out extensive TCR repertoire sequencing in four patients with severe MS undergoing autologous myeloablative HSCT. After methodological optimization, TCR sequences were analyzed from pre- and post-transplantation samples, as well as from the CD34-selected HSC graft to evaluate their potential reintroduction. Low yields of in-frame TCR

rearrangements recovered from the HSC graft samples suggest extensive T-cell death in the HSC grafts obtained after CY mobilization and did not show the presence of any TCRs that persisted post-transplantation in the peripheral blood. These findings suggest that persisting T-cell clones are unlikely to have arisen from the infused autograft.

Patients and methods

Patients and HSCT regimen

Patients with MS received autologous HSCT as part of an investigational protocol that has been previously reported.[2,10] The study had received the Institutional Review Board (local and NIH 02-N-N196) and the US Food and Drug Administration approval (IDE 6440). HSCs were collected by therapeutic mobilization using 2 g/m^2 of CY and 5 μg/kg/day of G-CSF beginning 96 h later. When the WBC count rebounded to more than 1×10^9/l, leukapheresis was performed using a Cobe Spectra (Lakewood, CO, USA) continuous flow blood cell separator. Products were enriched for CD34+ HSC using the Isolex (Baxter, Chicago, IL, USA) stem cell separation device. The myeloablative conditioning regimen began after adequate collection of CD34+ HSCs and consisted of 120 mg/kg of CY and 1200 cGy of TBI.[10]

Clinical specimens

PBMCs were obtained from leukapheresis before mobilization (baseline) or at the indicated time points after transplant. From each peripheral autologous graft, an aliquot constituting 1/100th of the purified graft was obtained after release from regulatory requirements. All samples were cryopreserved according to standard techniques. PBMC samples were screened for inclusion in this study using TCR Vβ-specific Ab staining by FACS and complementarity-determining region 3 (CDR3) spectratyping analysis confirming significant changes in the post-HSCT repertoire compared with baseline.[2,11] Pre-mobilization and post-transplantation PBMC samples and autologous hematopoietic graft aliquots from four patients (MS-1, MS-2, MS-3 and MS-4) were selected for in-depth TCR sequencing analysis. CD4+ or CD8+ T-cell subsets were immunomagnetically sorted as described.[2] A comprehensive list of persisting TCR clones present in the TCR BV repertoire for both the pre- and post-HSCT samples of these patients was then obtained by sequencing large numbers of clones (100–300 per patient time point) using described methods.[2] TCR BV gene family primers used to sample the T-cell repertoire in this study included BV5, BV9, BV13 and BV19.

TCR BV-BJ amplification

TCR BV-BJ rearrangements were PCR amplified from genomic DNA using BIOMED-2 multiplex primers as described.[12] Two BIOMED-2 multiplex primer sets were prepared: multiplex primer tube 1 containing 23 TCR BV and six JB1 gene-specific primers; and tube 2 contained the same TCR BV and seven JB2 gene-specific primers.[12] Final PCR conditions were optimized to: 1 × ABI buffer II, 1.5 mM $MgCl_2$ (multiplex primer tube 1), 2 mM $MgCl_2$ (multiplex primer tube 2), 10 pmol of each primer, 200 uM dNTP and 2 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were: 95 °C for 7 min; 40 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min. PCR amplicons were then cloned and sequenced as described.[13]

Table 1 Characteristics of CD34+ selected hematopoietic stem cell grafts

Patient	*% CD34+*	*CD34+ Infused*[a]	*% CD3+*	*CD3+ Infused*[b]
MS-1	91.2%	4.07×10^8	0.20%	8.54×10^5
MS-2	71.8%	4.88×10^8	0.10%	7.00×10^5
MS-3	70.4%	1.83×10^8	0.60%	1.61×10^6
MS-4	70.5%	2.81×10^8	0.35%	1.37×10^6

[a]The number of CD34+ cells infused in each patient was calculated by multiplying the total number of cells infused by the percentage of CD34+ cells in the autologous graft as determined by FACS staining.
[b]The number of infused CD3+ cells was calculated as described above for CD34+ cells.

Results

Characteristics of the TCR repertoire in the HSC grafts and peripheral T-cell subsets

The composition of the HSC graft is provided in Table 1. CD3+ cells had low frequencies in the CD34+ selected autologous HSC grafts, comprising 0.1–0.6% of total cells (Table 1). The calculated total number of CD3+ cells infused in each patient ranged from 7.00×10^5 to 1.61×10^6.

Amplification of the selected TCR BV genes from CD4+ and CD8+ T-cell subsets sorted from PBMCs obtained before mobilization and after autologous HSCT resulted in 2237 in-frame TCR B rearrangements. An average of 177 sequences were obtained for each patient time point (Table 2), providing an accurate representation of TCR diversity within each sample, as shown by our previous methodological work.[13] Longitudinal comparisons of pre-mobilization and post-transplantation TCR sequences from patients MS-1, MS-2 and MS-3 identified oligoclonal species persisting in baseline (pre-HSCT), 6 months and 1–2 year post-HSCT samples (data not shown). No persisting TCR sequences were found to be shared between pre-mobilization and post-HSCT samples for MS-4. Oligoclonal expansions detected by TCR sequencing of patient MS-1, BV 9 pre-mobilization samples (Figure 1a), were also detected, though more prominently, by CDR3 spectratyping (Figure 1b). Overall, we observed a clear trend toward increased CDR3 diversity for MS-1, BV 9 samples at both 6 months and 2 years post-therapy using both techniques. Similar results were also obtained from MS-1, BV 19 CD4+ T cells by TCR sequencing (Figure 1c), and spectratyping (Figure 1d), where skewing of the CDR3 repertoire diversity observed early post-therapy at 6 months showed increased diversity by 2 years post-transplantation. The representation of CDR3 length diversity was overall very similar with only minor differences between the two techniques, confirming that

the sequencing analysis provided an accurate representation of the actual TCR repertoire.

BIOMED-2 primer optimization

To overcome potential limitations of reverse transcription and PCR on the limited graft samples, we chose to amplify TCR BV-BJ rearrangements from the HSC graft DNA using BIOMED-2 multiplex primers.[12] As HSC graft specimens were extremely limited in quantity, we first optimized PCR conditions to ensure robust amplification and cloning efficiency. Two pHN1 plasmids, Q334 and F6, containing in-frame TCR rearrangements were used in positive control spike-in experiments for the BIOMED-2 multiplex primers. The Q334 plasmid containing an in-frame TRBV6-3*01, TRBJ1-2*01, TRBD1*01 rearrangement was used as a positive control for BIOMED-2 multiplex primer tube 1; and for multiplex primer tube 2, an F6 plasmid containing a TRBV13*01, TRBJ2-7*01, TRBD2*02 TCR rearrangement was used as a positive control.

Using optimized PCR conditions described above in conjunction with Q334 or F6 plasmids, we observed robust amplification for both multiplex primer tubes 1 and 2 in the presence of 10 or more copies of plasmid template per reaction, as determined by agarose gel electrophoresis (Figure 2a). Subsequent cloning and sequencing of the gel-purified products confirmed the correct TCR insert in 8/10 colonies for Q334 and tube 1 and in 6/10 colonies for F6 and tube 2 (data not shown). Serial dilutions of both the Q334 and F6 plasmids down to approximately one copy plasmid template per reaction resulted in 2/10 correct in-frame TCR sequences for both F6 and Q334 (data not shown).

As CD3+ cells were detected at low frequencies within the CD34+ selected HSC graft aliquots, we next wanted to determine the sensitivity of the optimized BIOMED-2 multiplex primers and TCR sequencing techniques in recovering in-frame TCRs from live T cells mixed in a heterogeneous cell population. To test this, two CD3+ T-cell clones of known TCR rearrangements were used as positive control 'spikes' into a 'background' of non-TCR-

Table 2 Analysis of TCR repertoire diversity in pre-mobilization and post-HSCT transplantation samples, for selected BV genes of interest

Samples			*TCR repertoire diversity in PBMC No. of TCR clones (% unique)*[a]		
			Pre-mobilization	*Post-HSCT*	
Patient	*Subset*	*BV gene*[b]	*Baseline*	*6–12 months*	*2 years*
MS-1	CD4	BV 9	157 (43%)	114 (52%)	110 (86%)
MS-1	CD4	BV 19	108 (71%)	100 (35%)	109 (80%)
MS-2	CD8	BV 19	246 (94%)	ND[c]	255 (67%)
MS-3	CD8	BV 5	240 (87%)	ND[c]	213 (64%)
MS-4	CD8	BV 13	312 (58%)	236 (57%)[d]	ND[c]

[a]No. of TCR clones describes the absolute number of in-frame TCR rearrangements obtained for each patient/time point; % unique describes the proportion of the TCR repertoire, which is comprised of sequences found as a single copy and are not found in any other sample time points.
[b]Indicates the TCR BV gene selected for analysis based on TCR FACS staining and CDR3 spectratyping data. Nomenclature is given according to IMGT TCR BV designation.
[c]ND = not done (specimen unavailable, or insufficient).
[d]1-year post-HSCT.

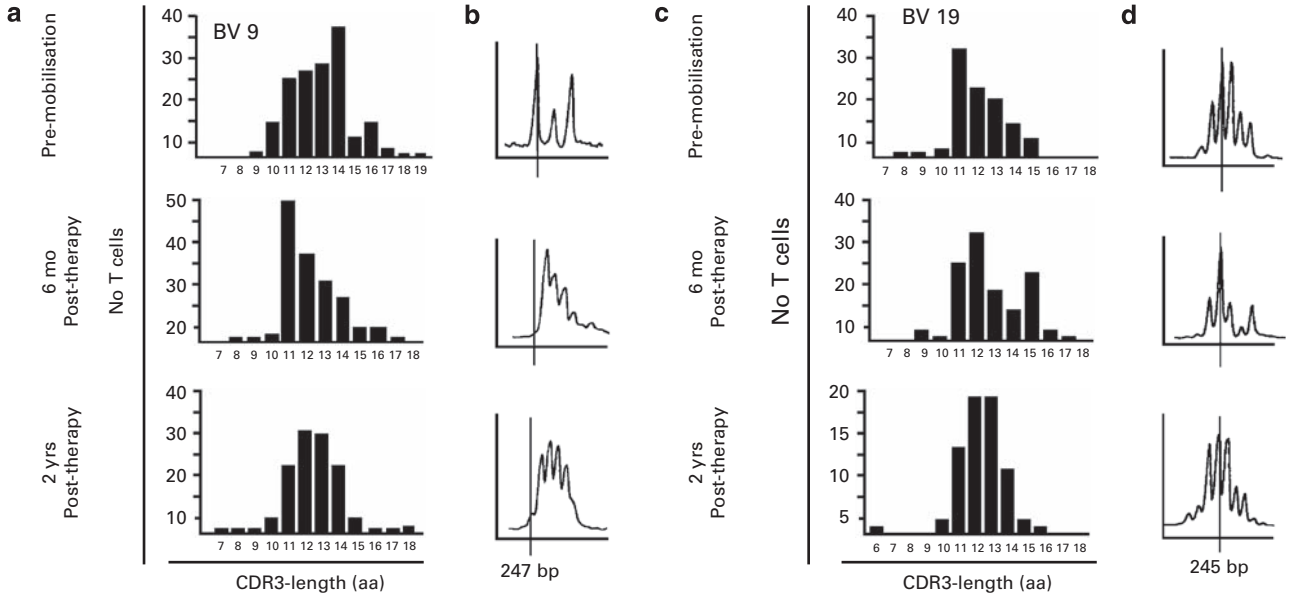

Figure 1 Complementarity-determining region 3 (CDR3) length analysis from TCR B sequencing and spectratyping MS-1 of BV-9 and BV-19 amplified samples. The CDR3 length in amino acids (aa) is shown for baseline (pre-mobilization) and 6 month (mo), and 2 year (yr) post-therapy samples. TCR B diversity for BV 9 amplified samples from (**a**) TCR sequencing showing the total number of T-cell sequences (no T cells) obtained with similar CDR3 lengths; and (**b**) high-resolution TCR BV CDR3 spectratyping analysis. A DNA size marker (vertical line) is shown at 247 bp and serves as a reference for comparative analysis between samples. As in (**a**) and (**b**), TCR B diversity is shown for BV 19 amplified samples using (**c**) TCR B sequencing techniques and (**d**) spectratyping. A reference line at 245 bp is provided in the spectrogram.

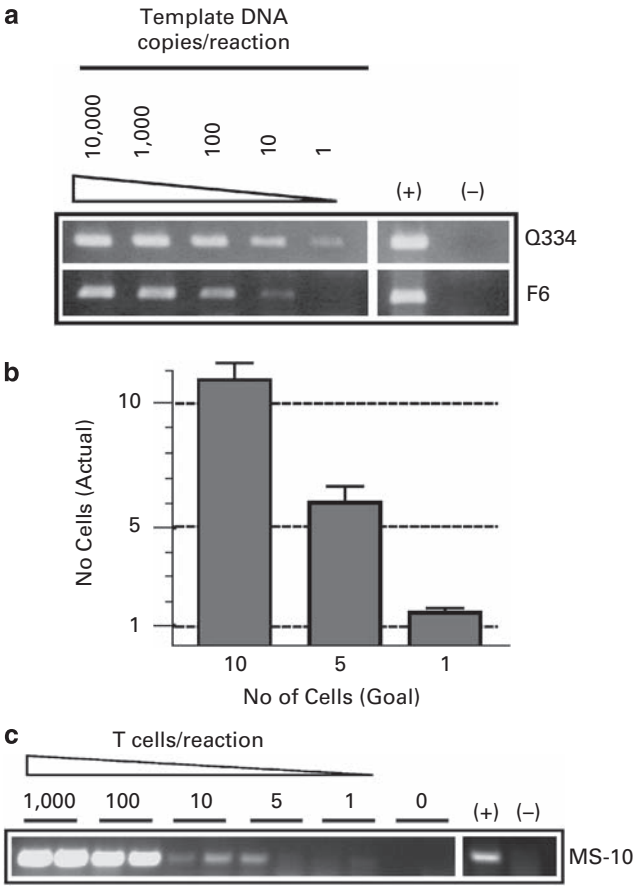

Figure 2 BIOMED-2 multiplex primers sensitively amplify TCR BC-BJ rearrangements. PCR amplification conditions for BIOMED-2 multiplex primers were optimized for multiplex primer tubes 1 and 2. (**a**) Control plasmids containing in-frame TCR BC-BJ rearrangements (Q334 and F6) were serially diluted 10-fold (10 000–1 copy template per reaction) and used as template DNA to optimize PCR and TCR sequencing conditions. In-frame TCR sequences were reliably obtained from amplicons from 1 to 10 copies template per reaction. (**b**) Validation of techniques to isolate low numbers of T cells for positive-control 'spike' experiments. Cells were serially diluted on a glass slide and 1, 5 and 10 cells were removed using a fine gauge tip. The total number of cells intended to be transferred (goal) was then plotted against the number of cells collected (actual) as counted by a different individual. $n=6$ replicates for each cell count. Error bars show ± s.d. (**c**) The indicated number of live TCR-positive T cells from clone MS-10 were transferred into 1×10^6 live TCR-negative cells. Cells were then PCR amplified with BIOMED-2 multiplex primers and cloned and sequenced as described. Representative amplicons obtained from MS-10 PCR products by agarose gel electrophoresis are shown. In-frame TCR rearrangements were recovered from PCR amplicons cloned from 1000, 100, 10, 5 and 1 T cells per reaction condition (see text).

positive cells: MS-10 containing an in-frame TRBV18*01, TRBJ1-1*01, TRBD1*01 rearrangement; and a Jurkat T-cell line containing a TRBV12-3*01, TRBJ1-2*01, TRBD1*01 rearrangement.

After optimization of techniques to reliably transfer low numbers of T cells (Figure 2b), 1000, 100, 10, 5 and 1 TCR-positive 'spike' cells were then transferred into 1×10^6 TCR-negative 'background' cells (HEK 293 or B cells). Genomic DNA was then extracted and PCR amplified (Figure 2c). Although PCR amplification products from low cell numbers were below the limits of detection of agarose gel electrophoresis and ethidium bromide staining, after cloning and sequencing we were able to successfully recover in-frame TCR sequences for all samples, even in the presence of low numbers of T-cell 'spikes.' Cloning and sequencing of PCR amplicons obtained starting from ten T cells per reaction resulted in the correct in-frame rearrangement for 2/4 bacterial colonies sequenced for MS-10, and 1/4 for the Jurkat T cells; starting from five T cells per reaction, we obtained in-frame rearrangements from 5/9 colonies for MS-10, and 2/5 colonies for the Jurkat T cells; and finally, PCR amplicons obtained from a single T-cell 'spike' for both MS-10 and Jurkat T-cell lines resulted in 3/11 and 2/13 in-frame TCR rearrangements, respectively. These results confirmed the capability of our techniques to sensitively amplify in-frame TCR rearrangements from genomic DNA of a single TCR-positive cell, even in the presence of a majority of TCR-negative cell populations.

TCR amplification of HSC grafts

Hematopoietic stem cell graft aliquots from all patients were then amplified with BIOMED-2 multiplex primer tubes 1 and 2. A total of 467 HSC-derived colonies were selected and submitted for sequencing, from which 37 in-frame TCR sequences were obtained from the MS-1 graft specimen (Figure 3, and data not shown). Multiple comparative analyses of these sequences confirmed that 40% (15/37) of the TCR sequences were found as a single copy; the remaining repeated sequences were present at a frequency comprising 2–4% of the total population (Figure 3, and data not shown). Analysis of the CDR3 length diversity of the TCR sequences amplified from the HSC graft showed a near-Gaussian distribution (Figure 3). Surprisingly, no TCR sequences were amplified from MS-2, 3 and 4 graft samples other than those of plasmid DNA from clones Q334 and F6, which served as an internal positive control confirming the high efficiency of amplification.

TCR sequences present in baseline and post-HSCT samples are not found in the autologous CD34+ selected HSC graft

In-frame TCR rearrangements from the HSC graft, as well as from all pre-mobilization and post-HSCT peripheral blood samples for MS-1, were then assessed to determine whether persisting clones were detectable in the autologous HSC graft. A total of 698 in-frame TCR sequences from MS-1 CD4+ T-cell subset samples (pre-mobilization and post-transplantation) were visually compared with the 37 in-frame rearrangements from the HSC graft. However, none of the persisting TCR sequences detected in CD4+ T cells from PBMCs at baseline (pre-) or post-HSCT were found in the sequences obtained from the graft (Figure 3, and data not shown).

Discussion

One aspect of the rationale for using autologous myeloablative HSCT to treat autoimmune disease is the opportunity to eradicate all mature T cells with myeloa-

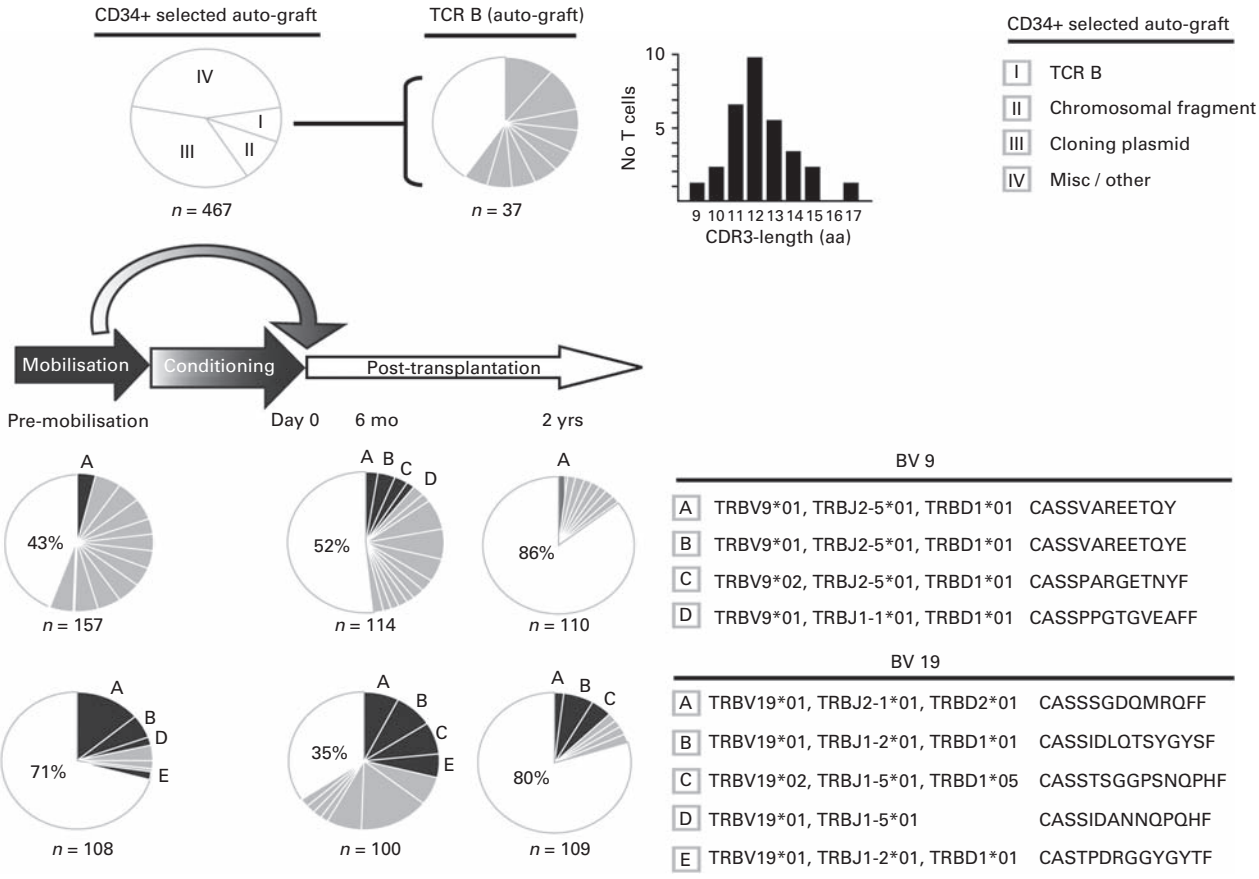

Figure 3 Expanded TCR sequences in pre-mobilization and post-HSCT samples are not detected in the hematopoietic stem cell graft. HSCT transplantation scheme (center); pre-mobilization samples were collected at baseline before HSC mobilization; auto-graft samples for CD34+ enrichment were obtained after HSC mobilization. Day 0 constitutes the first day after conditioning, during which the CD34+ selected autograft was infused. Representative analysis is shown for MS-1 TCR B sequencing of the CD34+ selected HSC autograft (top), and for BV 9 and BV 19 amplified pre- and post-transplantation samples (below). Pie charts show the relative composition of sequences obtained from the CD34+ selected autograft (top, I, II, III and IV; $n = 467$). In-frame TCR B sequences obtained from the autograft ($n = 37$) had diverse complementarity-determining region 3 (CDR3) lengths (top right) and do not reveal the presence of oligoclonal biasing within the sample. Corresponding TCR B analysis from BV 9 and BV 19 amplified pre- and post-transplantation samples (below). Pie charts show the proportion of the repertoire, which constitutes TCR sequences detected: as a single copy at a single time point (unique, white); in multiple copies at a single time point (expanded, gray); or recurring at two or more time points (A–E, black). The total number of in-frame TCR sequences (n) analyzed for each sample/time point is shown below. Legend (bottom, right) provides TCR BV rearrangements and CDR3 sequences for expanded sequences detected in BV 9 or BV 19 amplified samples. No TCR sequences that were found in any pre- and post-HSCT samples were found in the purified autologous HSC graft.

blative conditioning. It is therefore clinically relevant to consider whether the reinfusion of T cells 'contaminating' the autologous hematopoietic graft could cause or contribute to the persistence of individual CD4 and CD8 T-cell clones (potentially including disease-mediating clones) after autologous HSCT. Lymphocyte depletion can be achieved by graft manipulations such as CD34+ cell selection, or *in vivo* using anti-thymocyte globulin or monoclonal antibodies. CD34 selection results in efficient depletion of CD3+ T cells, which remain within the HSC autologous graft only at low frequencies. In this study, we used state of the art TCR sequencing methods to ascertain whether individual T-cell clones that re-emerged or persisted post-transplant in patients with MS who underwent HSCT had originated from the autologous CD34-selected graft.

As aliquots of the HSC grafts were limited in size, we first carried out PCR optimization experiments using positive-control spike-in experiments on plasmids containing in-frame TCR rearrangements to determine the minimum number of sequences that must be present for detection by TCR sequencing. Serial dilutions of the TCR-positive plasmid DNA template down to 10 or fewer copies per cloning reaction confirmed our ability to detect TCR sequences with high sensitivity. Next, we tested the optimized PCR conditions on live T-cell clones 'spiked' into a TCR-negative 'background' cell population to closely mimic amplification and cloning conditions applicable to the HSC grafts, and to allow us to determine the sensitivity of our methodologies in detecting viable T cells of low number with intact genomic DNA from a heterogeneous cell population. Even in the presence of a single TCR-positive T cell, we were able to successfully clone and sequence in-frame TCRs from the T-cell 'spike,' confirming the capabilities of our amplification and cloning techniques to sensitively amplify TCR BV rearrangements from low numbers of target cells.

Next, HSC graft samples were analyzed from all patients. A total of 467 HSC-derived colonies were selected and

sequenced for in-frame TCR rearrangements using BIOMED-2 multiplex primers, from which a total of 37 in-frame TCR sequences were obtained from MS-1. Remarkably none of the TCR sequences from the MS-1 graft were detected in any other samples from the patient, suggesting that the clones contained in the graft were not represented at high frequency in the peripheral blood either before or after HSCT. Surprisingly, no TCR sequences were amplified from the other three patients' grafts in spite of robust control TCR amplification. As we used a sensitive methodology, and MS1 had the second lowest percentage of T cells among the four grafts, further reassuring that the sensitivity of our system was not the limiting factor, we interpret the low yields as a consequence of the effects of CY treatment ($2\,g/m^2$) used for HSC mobilization before graft collection. CY is a potent alkylating agent that induces DNA fragmentation and apoptosis in lymphocytes, which have low levels of aldehyde dehydrogenase, the enzyme that converts the active alkylating compound phospharamide mustard into the inert compound carboxyphosphamide.[14,15] Common protocols for the evaluation of hematopoietic graft composition include flow cytometric enumeration of CD3+ cells but do not assess their apoptotic status. We hypothesize that T cells contained in HSC grafts obtained with mobilization schemes not using CY or other lymphotoxic agents may have greater engraftment potential, and it would be important to test this hypothesis in future studies.

Our results provide important first evidence against the potential reintroduction of clonally expanded T cells from within CY (+G-CSF)-mobilized, CD34-selected hematopoietic grafts. Two alternative explanations for the persistence of these clones in the patients' post-HSCT are: (a) *de novo* rearrangement and selection of the clones post-transplantation; or (b) survival of the pre-existing T clones despite a myeloablative conditioning regimen. Although persistent viral infections can exert selective pressure on thymic generation of naive CD4+ cells post-transplant,[16] *de novo* generation of identical TCR sequences is expected to be rare (especially at the nucleotide level), given the vast combinatorial diversity of TCR B chain rearrangements.[17] Therefore, persistence of T-cell clones post-HSCT more likely reflects their survival through the conditioning chemoradiotherapy. This notion has implications concerning the rationale for using maximally intensive myeloablative conditioning regimens for treatment of autoimmune disease. In this context, if the goal is complete immune ablation, CD34+ selection would be theoretically appropriate so long as the graft represents the sole source of carryover of mature lymphocytes. A study comparing CD34-selected versus unmanipulated HSCT in rheumatoid arthritis (RA) detected no differences in clinical outcomes.[18] However, the possibilities that incomplete immunoablative conditioning or an intrinsically poor response to autologous HSCT in RA could render CD34 selection irrelevant could not be excluded. Several factors contribute to determining chemo- and radioresistance of lymphoid cells. These include cell-dependent factors such as lineage, maturation and cell cycle status. In general, the cytotoxicity of alkylating agents is greatest during the S phase of the cell cycle, resulting in the more effective killing of cells that are rapidly dividing compared with resting cells. Such property has long been known and exploited for therapeutic targeting of cancer cells. When the target is a non-malignant autoimmune T-lymphocyte population and the objective of cytotoxic chemotherapy is immunosuppressive conditioning, the T-cell state of activation needs to be considered. Naive and, to a lesser extent, resting (central) memory cells are more likely to survive cytotoxic chemotherapy than activated memory/effector cells. This differential susceptibility attenuates but does not eliminate the concern of potential autoimmune disease recapitulation. Strategies to minimize this risk may include dose intensification (limited by toxicities), multiple immunosuppressive drugs with additive or synergistic effects (also limited by toxicities) and *in vivo* lymphocyte depletion with poly- or monoclonal antibodies. Interestingly, it has been suggested that polyclonal anti-T-lymphocyte globulins (ATGs) efficacy at preventing GVHD relies not only on its capacity to deplete T cells but also on immunomodulatory properties.[19] ATG (or alemtuzumab, which has also shown immune modulating effects)[20] has been included in conditioning regimes of varying intensity used in clinical trials of HSCT in MS, as recently discussed[21] or reported.[22]

The approach and methodological improvements to studying the human TCR repertoire described here could facilitate further studies addressing how mobilization schemes, graft manipulations, conditioning regimes and *in vivo* T-cell depletion affect the carryover of mature T cells in hematopoietic transplantation for autoimmune and malignant disorders.

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