

B-Cell Reconstitution After Autologous Hematopoietic Stem Cell Transplantation in Multiple Sclerosis

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Abstract

Background and Objectives

Autologous hematopoietic stem cell transplantation (aHSCT) is increasingly used to treat aggressive forms of multiple sclerosis (MS). This procedure is believed to result in an immune reset and restoration of a self-tolerant immune system. Immune reconstitution has been extensively studied for T cells, but only to a limited extent for B cells. As increasing evidence suggests an important role of B cells in MS pathogenesis, we sought here to better understand reconstitution and the extent of renewal of the B-cell system after aHSCT in MS.

Methods

Using longitudinal multidimensional flow cytometry and immunoglobulin heavy chain (IgH) repertoire sequencing following aHSCT with BCNU + Etoposide + Ara-C + Melphalan anti-thymocyte globulin, we analyzed the B-cell compartment in a cohort of 20 patients with MS in defined intervals before and up to 1 year after aHSCT and compared these findings with data from healthy controls.

Results

Total B-cell numbers recovered within 3 months and increased above normal levels 1 year after transplantation, successively shifting from a predominantly transitional to a naive immune phenotype. Memory subpopulations recovered slowly and remained below normal levels with reduced repertoire diversity 1 year after transplantation. Isotype subclass analysis revealed a proportional shift toward IgG1-expressing cells and a reduction in IgG2 cells. Mutation analysis of IgH sequences showed that highly mutated memory B cells and plasma cells may transiently survive conditioning while the analysis of sequence cluster overlap, variable (IGHV) and joining (IGHJ) gene usage and repertoire diversity suggested a renewal of the late posttransplant repertoire. In patients with early cytomegalovirus reactivation, reconstitution of naive and memory B cells was delayed.

Discussion

Our detailed characterization of B-cell reconstitution after aHSCT in MS indicates a reduced reactivation potential of memory B cells up to 1 year after transplantation, which may leave patients susceptible to infection, but may also be an important aspect of its mechanism of action.

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Glossary

aHSCT = autologous hematopoietic stem cell transplantation; **ATG** = anti-thymocyte globulin; **BEAM** = BCNU (carmustine) + Etoposide + Ara-C (cytarabine) + Melphalan; **CMV** = cytomegalovirus; **DMT** = disease-modifying treatment; **EBV** = Epstein-Barr virus; **HC** = healthy control; **IgH** = immunoglobulin heavy chain; **IGHJ** = immunoglobulin heavy chain joining gene; **IGHV** = immunoglobulin heavy chain variable gene; **IQR** = interquartile range; **MBC** = memory B cell; **MS** = multiple sclerosis; **PBMC** = peripheral blood mononuclear cell; **PC** = plasma cell; **RRMS** = relapsing-remitting multiple sclerosis; **SSc** = systemic sclerosis.

High-dose chemotherapy with autologous hematopoietic stem cell transplantation (aHSCT) has first been applied to patients with advanced multiple sclerosis (MS) over 25 years ago and has since been performed on several thousand patients.^{1,2} During the last decade, mortality and adverse events have steadily declined, and evidence from an increasing number of studies shows that its efficacy is considerably higher than the most effective approved MS therapies.²⁻⁶ The underlying rationale for aHSCT in patients with MS is the depletion of autoreactive lymphocytes by the conditioning regimen and subsequent immune restoration with a new and tolerant adaptive immune system. Studies from the past decade indicate that such an immune resetting is indeed possible to some extent.⁷⁻⁹ Based on the current understanding of MS pathogenesis, previous research has mainly focused on T-cell reconstitution and demonstrated that T-cell repertoire renewal is more thorough for CD4⁺ T cells while CD8⁺ T-cell repertoire renewal was less complete.^{10,11}

Besides the well-documented role of autoreactive CD4⁺ T cells in MS, numerous observations now support a central role for B cells in the pathogenesis. The success of B cell-directed therapies in blocking disease activity (anti-CD20, but also anti-CD52 and cladribine) is probably the most important evidence,^{12,13} and the early onset of their effects indicates that other B-cell functions, rather than antibody secretion, may be relevant in MS pathogenesis. The most important functions are their ability as antigen-presenting cells to transport and present antigens to T cells,^{14,15} production of proinflammatory cytokines,^{16,17} or even their regulatory function.^{18,19} The various roles of B cells in MS pathology have recently been reviewed in detail elsewhere.²⁰⁻²²

B-cell reconstitution following aHSCT has been studied in B cell-/antibody-mediated autoimmune diseases, most notably systemic sclerosis (SSc) and systemic lupus erythematosus. Phenotypical analyses in these settings have established that there is a quick recovery of total B-cell numbers within 2 to 6 months after transplantation with an early domination of a naive phenotype and a persistent reduction in the memory compartment.²³⁻²⁶ Transitional B cells have also been described as important intermediates in B-cell reconstitution.^{24,27} Several studies have confirmed these general B-cell reconstitution dynamics in the context of MS.^{7,28-30} However, there is only limited information about B-cell subpopulation recovery and no data on immunoglobulin repertoire characteristics.

Here, we aimed at analyzing the B-cell reconstitution, both phenotypically and temporally, with a focus on the abovementioned

missing aspects. Twenty patients with MS were included and underwent clinical and immunologic evaluation before transplantation and at regular intervals up to 1 year after therapy. By including early analysis time points, we were able to precisely dissect the reconstitution dynamics. Using extensive phenotyping marker sets, we confirmed previous findings and further extended the characterization of B-cell subsets to the level of isotype subpopulations. This immunophenotypic analysis was combined with immunoglobulin heavy chain (IgH) repertoire sequencing to estimate the extent of B-cell system renewal after aHSCT.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

Patients were enrolled in a national aHSCT in MS registry study, approved by the Cantonal Ethics Committee (BASEC 2018-01854), and informed written consent was obtained from each patient. Ablative conditioning was performed according to the BCNU (carmustine) + Etoposide + Ara-C (cytarabine) + Melphalan anti-thymocyte globulin (BEAM-ATG) protocol. Details of the clinical aHSCT study protocol and sample collection are provided in the eMethods, links.lww.com/NXI/A743, section in this article's supplement or can be found in the sister study.³¹

Patient Samples

Peripheral blood samples were collected from 20 patients with MS at defined study visits including baseline before stem cell mobilization and up to 1 year post-aHSCT (demographic and clinical features listed in eTable 1, links.lww.com/NXI/A743). For immunophenotypic analysis, 14 age-matched healthy controls (HCs) were included (see Table 1 for key characteristics). Healthy donor IgH repertoire data used here have been

Table 1 Description of the Cohort

	n	Sex	Age (y)
		% Female	Median (range)
Patients	20	50	42 (25-53)
Healthy donors			
Flow cytometry	14	57	39 (26-56)
IgH repertoire sequencing	25	60	30 (18-51)

described previously.³² For adequate comparison, only data from unsorted peripheral blood samples from donors aged 18 years or older were used in the present study, resulting in a healthy control data set of 25 individuals as summarized in Table 1.

Immunophenotyping

Immunophenotyping on peripheral blood mononuclear cells (PBMCs) was performed with 2 EuroFlow flow cytometry panels as previously described^{33,34}: the 8-color PID orientation tube (Cytognos) and the 12-color IgH-isotype B-cell tube (Cytognos). For details of staining protocol, antibodies (eTable 2, links.lww.com/NXI/A743) and gating strategy (eFigure 1 and eTable 3, links.lww.com/NXI/A743) refer to the eMethods, links.lww.com/NXI/A743, section in the supplement. In accordance with EuroFlow nomenclature, we refer to the CD19⁺CD5⁻CD27⁺CD38⁺⁺ subpopulation as plasma cells (PCs), which include both short-lived plasmablasts and long-lived PCs.³³

IgH Library Preparation and Sequence Processing

A total of 3×10^6 viable PBMCs (exact input cell numbers in eTable 4, links.lww.com/NXI/A743) were used for RNA extraction and IgH library preparation as previously described.^{32,35} Details of library preparation, raw sequence processing, sequence clustering, sequence specificity matching, and repertoire diversity calculations are outlined in the eMethods, links.lww.com/NXI/A743, section. The number of raw and processed sequences for each sample is detailed in eTable 4, links.lww.com/NXI/A743.

Statistical Analysis

Detailed data processing and statistical analysis can be found in the eMethods, links.lww.com/NXI/A743, section.

Data Availability

Raw sequence data used for analysis in this study are available at the NCBI Sequencing Read Archive (ncbi.nlm.nih.gov/sra) under BioProject number PRJNA763367 including metadata meeting MiAIRR standards.³⁶ The processed and annotated final data set is available in Zenodo (doi.org/10.5281/zenodo.5513967) along with the protocol describing the exact processing steps with the software tools and version numbers. All other data as well as codes used for repertoire data analysis in this study will be made available from the corresponding author on reasonable request.

Results

Patient Characteristics

Twenty patients with MS were included whose demographic and clinical features are summarized in Table 1. None of these patients had relapses during the follow-up period. Cytomegaloviral (CMV) reactivation occurred in 4 patients within the first weeks after aHSCT, 2 of whom had concomitant clinically

asymptomatic and transient Epstein-Barr virus (EBV) reactivation. All of our patients were EBV seropositive pre-aHSCT, and this low proportion of patients with EBV reactivation after the BEAM-ATG protocol is in contrast to a recently described cohort in which EBV reactivation was detected in 80% of patients after a cyclophosphamide-ATG conditioning protocol.³⁷

Diverging Reconstitution Dynamics in Global Lymphocyte Subsets Following aHSCT

By flow cytometry, we assessed the reconstitution of lymphocyte subpopulations longitudinally. Recovery of total lymphocyte counts to baseline levels took on average 12 months (Figure 1A). Major lymphocyte subsets showed very different kinetics, resulting in the following pattern (Figure 1B): recovery was fastest for NK cells, with cell counts at normal levels at all sampling time points and even showing some excess 1 month after transplantation as described earlier.³¹ B-lymphocyte numbers were second to recover, returning at 3 months post-aHSCT and exceeding normal levels at the last time point measured. T lymphocytes were the slowest to reconstitute, with most but not all patients reaching normal levels only at 12 months after transplantation.

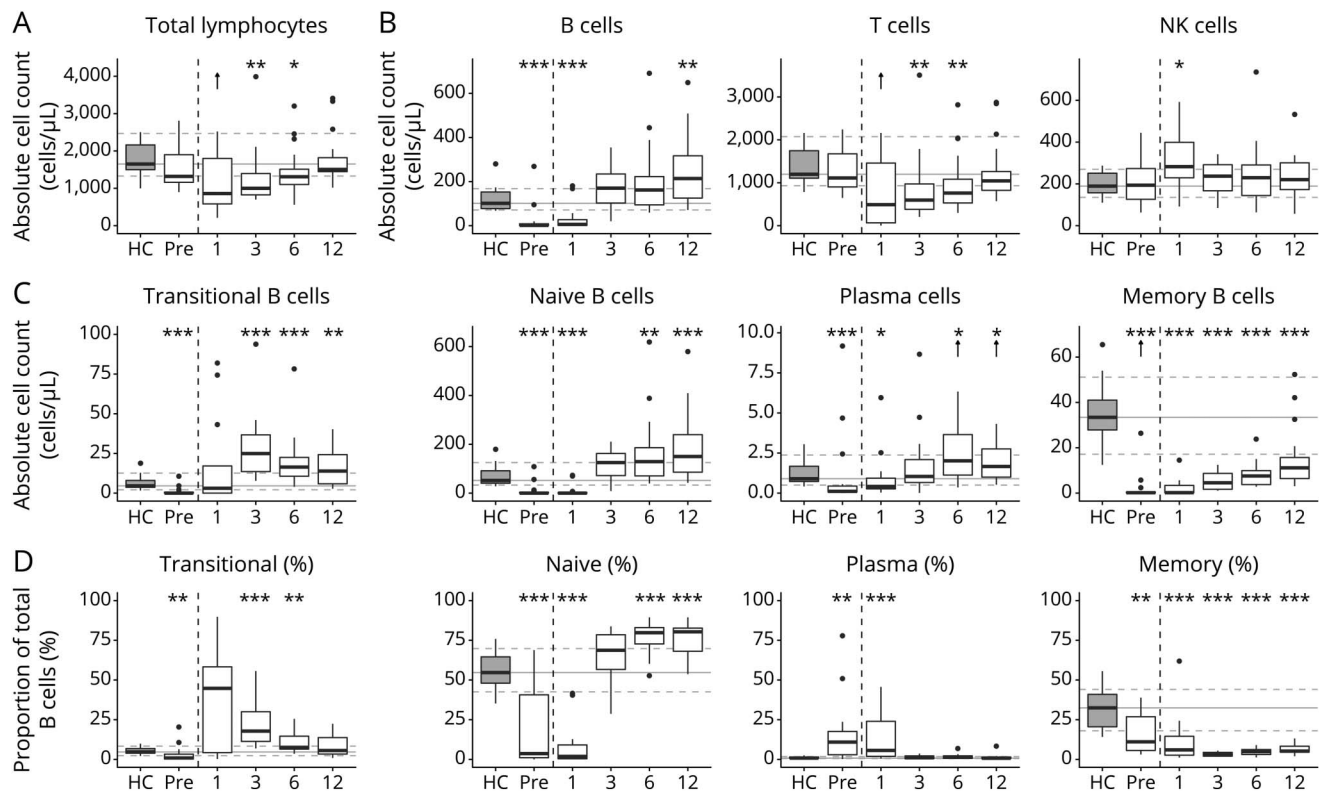
Naive and Transitional B Cells Recover Quickly While Memory Subsets Remain at Subnormal Levels

B cell–directed anti-CD20 treatment pre-aHSCT in 16 of 20 patients with MS eliminated B cells at almost every stage of differentiation, leading to very low absolute B-cell numbers before transplantation (Figure 1, B–D). Two patients treated with natalizumab had increased total B-cell numbers before therapy compared with healthy controls, as described,³⁸ but their reconstitution was not different from patients treated with anti-CD20 therapy. Although there was no increase in absolute PC numbers early after aHSCT, we found that a substantial proportion of early B cells were PCs (median 10.8%; interquartile range [IQR], 2.9–17.6, Figure 1D). Transitional immature B cells were the earliest B cells to repopulate and accounted for the largest B-cell population 1 month post-aHSCT (median 44.7% of all B cells; IQR, 4.2–58.3). The excess of these precursors is also reflected in absolute numbers 3 months following aHSCT. The relative frequency of transitional B cells decreased over the following months while the proportion of mature naive B cells rose already above the reference at 3 months post-aHSCT and remained elevated throughout the study period (Figure 1D). This sustained generation of high numbers of mature naive B cells resulted in increased absolute numbers of peripherally circulating naive B cells from 3 to 12 months post-aHSCT compared with healthy age-matched controls (Figure 1C). The memory B-cell (MBC) compartment was slowest to recover after transplantation. Absolute numbers were still below normal levels at the 1-year follow-up (Figure 1C).

Isotype Subclass Usage Is Shifted Toward IgG1 in Posttransplant B Cells

Within the memory compartment, switched MBCs were overrepresented compared with HC at the pretransplant and

Figure 1 Longitudinal Immunophenotyping of Major Lymphocyte Subpopulations Before and After aHSCT



(A) Total lymphocyte numbers. (B) Cell counts of main lymphocyte subsets: B, T, and NK cells. (C) Absolute numbers of major B-cell subpopulations and (D) their proportions out of total B cells. Reference band (HC; n = 14) showing median and 10th and 90th percentiles. Arrows indicate single outliers that have been removed for visualization (total lymphocytes, 1 m: 11'950 cells/ μ L; T cells, 1 m: 11'162 cells/ μ L; plasma cells, 6 m: 18.5 cells/ μ L and 12 m: 19.2 cells/ μ L; memory B cells, pre: 102 cells/ μ L). Comparison of each time point to the HC group was performed using the Wilcoxon test. * p < 0.05, ** p < 0.01, *** p < 0.001. Number of samples per time point: pre = 19, 1 m = 14, 3 m = 11, 6 m = 20, and 12 m = 20. aHSCT = autologous hematopoietic stem cell transplantation.

early posttransplant visits, with normalization observed at 3 months when IgD isotypes started to be proportionally increased compared with baseline (Figure 2A). The proportion of switched PC remained constantly high throughout the entire first year (Figure 2A). At the isotype subclass level, we found that only IgG1, IgG3, and IgD memory cells started to approach the normal healthy control range at the last visit (Figure 2B, eFigure 2A, links.lww.com/NXI/A743). PCs reached normal levels earlier than MBCs with a considerable variation among PCs expressing different Ig isotypes (Figure 2B, eFigure 2B, links.lww.com/NXI/A743). Overall, the reconstitution dynamics by isotype was similar for MBCs and PCs with IgG1-, IgG3-, IgA1-, and IgD-expressing B cells recovering earlier than IgA2-, IgG4-, IgG2-, and IgM-expressing cells (Figure 2B). Proportionally, there was a significant shift in PC isotype subclass distribution after transplantation, with higher usage of IgG1 at the expense of IgG2 and IgG4, whereas IgA subclass usage remained stable over time (eFigure 3A, links.lww.com/NXI/A743). In the MBC compartment, this shift was less pronounced and occurred gradually. Analysis of the isotype distribution on a sequence level confirmed the dynamics observed at the cellular level: IgG1 sequences were proportionally overrepresented in the reconstituting repertoire at the expense of IgG2 sequences,

reflecting the earlier reconstitution of cells that express this isotype (eFigure 3B, links.lww.com/NXI/A743).

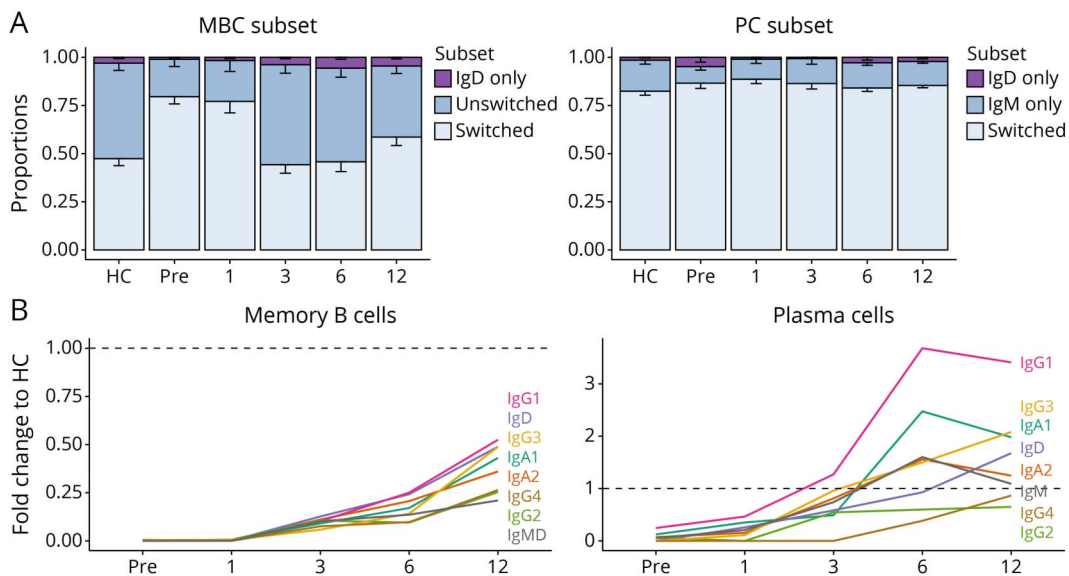
T-Cell Reconstitution Is Faster for CD8⁺ Cells vs CD4⁺ Cells

T-cell regeneration has been analyzed in detail in a sister study, and here, we only assessed recovery of major T-cell populations. T-cell reconstitution was slow, and it took 12 months for the majority of patients to reach near-normal numbers (Figure 1B). CD8⁺ T cells recovered rapidly despite the protracted reconstitution of naive CD8⁺ T cells, whereas CD4⁺ T cells remained below normal at the 12-month follow-up visit, particularly naive CD4⁺ T cells (eFigure 4A, links.lww.com/NXI/A743). The CD4⁺/CD8⁺ ratio was significantly decreased throughout the entire first year post-aHSCT. This shift toward predominance of CD8⁺ T cells in the new immune system can also be seen in the proportional analysis of T-cell subpopulations (eFigure 4B, links.lww.com/NXI/A743).

B-Cell Immune Reconstitution Is Negatively Correlated With CMV Reactivation Post-aHSCT and Progressive MS

We explored statistical associations between regeneration of B-cell numbers and relevant clinical characteristics and found

Figure 2 Longitudinal Immunophenotyping of Memory B Cells and Plasma Cells by Isotype and Subclass

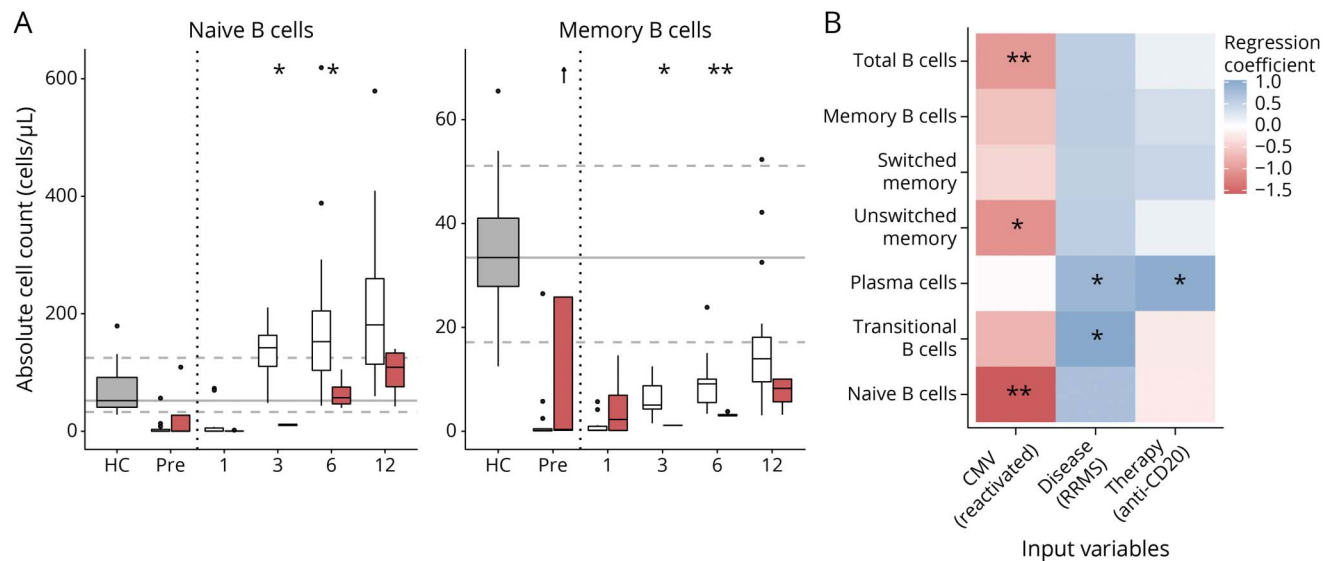


(A) Proportions of switched, unswitched, and IgD+ only subsets within total memory B cells and proportions of switched, IgM+ only, and IgD+ only subsets within total plasma cells. (B) Reconstitution dynamics of memory B-cell and plasma cell isotype subpopulations. Kinetics are shown as the ratio of the median cell count per time point compared with the healthy control data set. The dashed line indicates where the patient median cell count corresponds to the HC median value. Number of samples per time point: pre = 19, 1 m = 14, 3 m = 11, 6 m = 20, and 12 m = 20.

that B cells, and in particularly the naive subpopulation, reconstitute slower in patients with a CMV reactivation (Figure 3). CMV reactivation is a common complication following B and T cell-depleting therapies such as conditioning

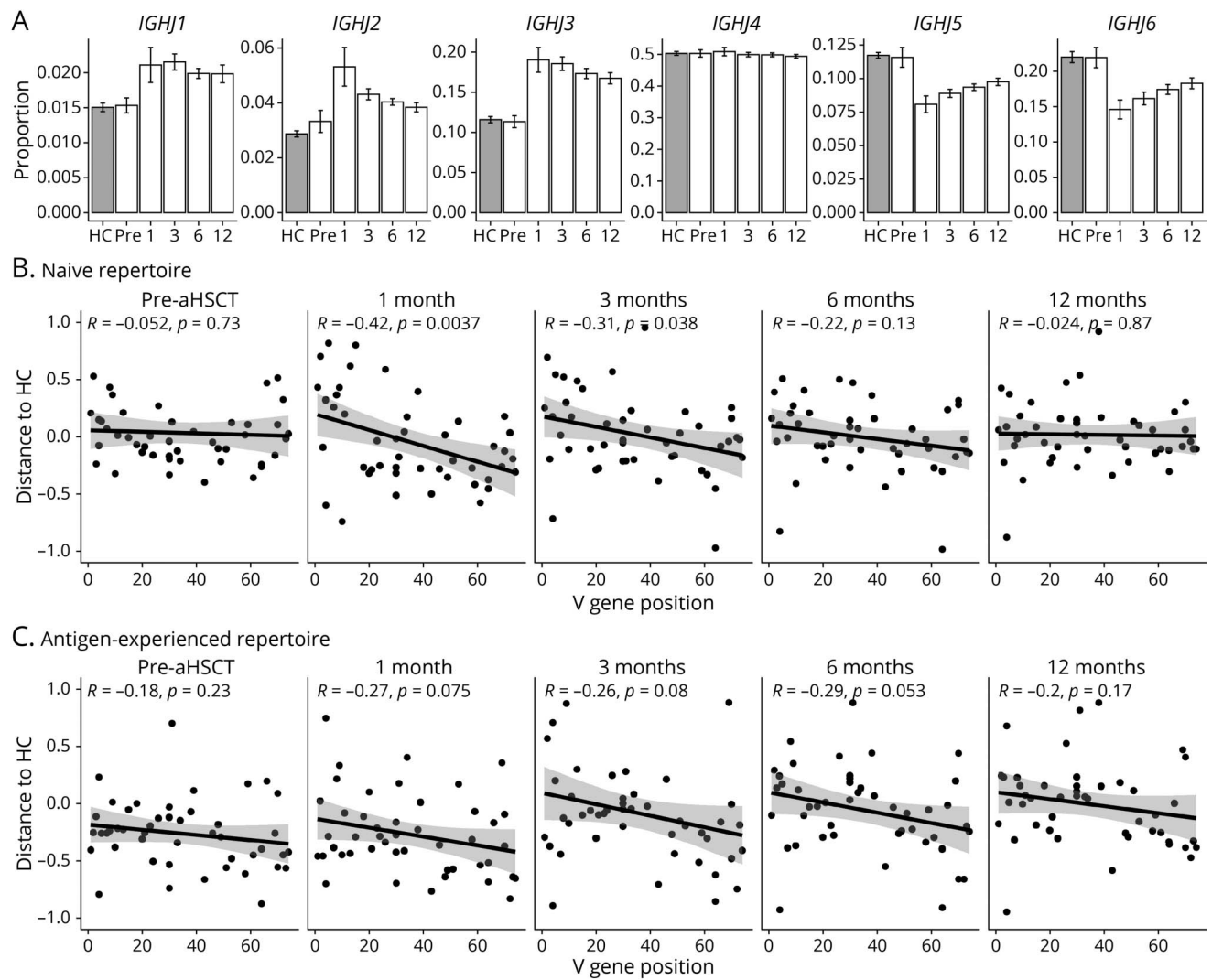
for stem cell transplantation, particularly in combination with ATG.³⁹ In MS, CMV is not considered a disease trigger, and even protective effects of CMV infection have been reported, although CMV reactivation has been correlated with worse

Figure 3 Delayed B-Cell Reconstitution in Patients With CMV Reactivation



(A) CMV reactivation is correlated with a delayed B-cell reconstitution in naive and memory B-cell subsets. The reference group is displayed in gray (HC; n = 14) including the median and 10th and 90th percentiles. Absolute cell counts of patients with early CMV reactivation (n = 4) are shown in red. Comparison between CMV status by time point was performed using the Wilcoxon test. * $p < 0.05$, ** $p < 0.01$. The arrow indicates a single outlier removed for visualization (memory B cells in a patient with CMV reactivation pre-aHSCT: 102 cells/ μ L) (B) Heat map regression coefficient matrix showing the statistical association of B-cell subset cell counts and the following variables: CMV reactivation (yes/no), disease type (RRMS/progressive MS), and last therapy before transplantation (anti-CD20/other). A mixed-effect model was used, with participants as random effect and CMV status, disease type, last therapy, and time points posttransplant as fixed effects. * $p < 0.05$, ** $p < 0.01$. CMV, cytomegalovirus; RRMS, relapsing-remitting MS.

Figure 4 Longitudinal Analysis of Joining (IGHJ) and Variable (IGHV) IgH Gene Usage



(A) IGHJ gene proportions during B-cell reconstitution. Healthy control reference proportions are shown in gray ($n = 25$). Number of samples from patients with MS per time point: pre = 19, 1 m = 14, 3 m = 11, 6 m = 20, and 12 m = 19. IGHV gene usage on a single gene level shown separately for the naive repertoire (B) and the antigen-experienced repertoire (C). IGHV genes are ordered based on their order in relation to the IgH locus starting from the gene closest to the recombination site (position 0). Distance to health is calculated as the median of the normalized ratios of patients to control proportions per time point. Orphan and nonlocated IGHV genes as well as IGHV genes with a proportion <0.001 in HC were excluded from this analysis (eFigure 7, links.lww.com/NXI/A743). At 1 month posttransplant, IGHV genes closer to the recombination site were significantly overrepresented within the reconstituting naive B-cell repertoire ($p = 0.0037$). A similar, but less pronounced trend was observed for the antigen-experienced repertoire. MS = multiple sclerosis.

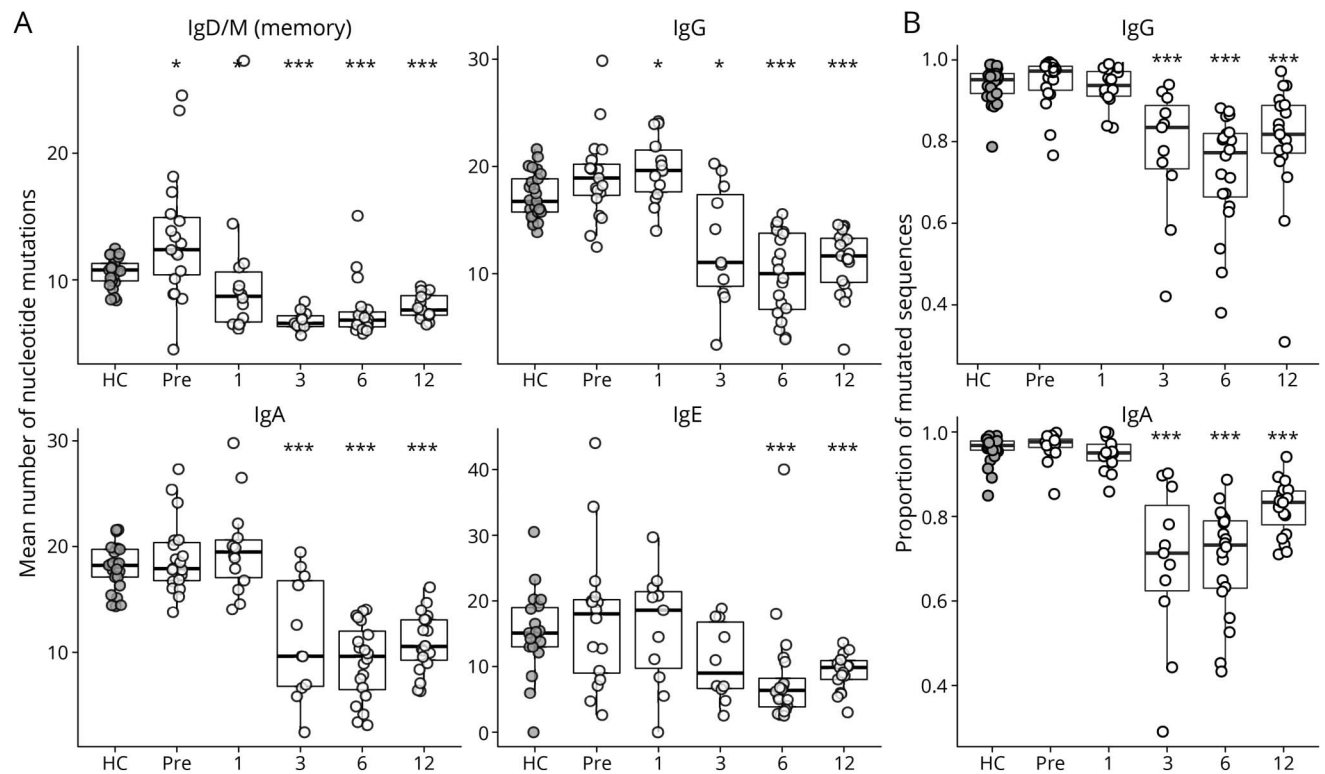
disease outcome (reviewed in Ref. 40). In our cohort, 4 patients developed CMV reactivation within the first weeks post-aHSCT (with the onset reported between 16 and 48 days post-aHSCT). Particularly, the repopulation of naive and unswitched MBC subsets was significantly delayed in these patients and remained below the level of patients without CMV reactivation throughout follow-up (Figure 3A). There were no major differences among T-cell subpopulations but slightly elevated numbers of CD8⁺ T cells in patients after CMV reactivation (eFigure 5A, links.lww.com/NXI/A743). We found that B cells reconstituted more rapidly in patients with relapsing-remitting multiple sclerosis (RRMS) than in patients with a progressive form of MS, particularly transitional B cells

(Figure 3B). We additionally included the type of last therapy before transplantation into our model, by forming a group of patients who had received anti-CD20 therapy (rituximab and ocrelizumab) and a group with another last therapy (natalizumab and fingolimod) (Figure 3B). The type of last prior therapy correlated significantly only with PC reconstitution. Patients who received anti-CD20 therapy before transplantation had a faster reconstitution of PCs than patients with other highly effective disease-modifying treatments (DMTs).

IgH Repertoire Sequencing Following aHSCT

IgH repertoire sequencing allowed us to explore the reconstitution of the B-cell system with respect to diversity and

Figure 5 Longitudinal Mutation Analysis



(A) Mean number of nucleotide mutations by individual and isotype. (B) Proportion of mutated sequences (defined as having 3 or more mutations) within the IgG and IgA repertoires. Comparison of each time point to the HC group was performed using the Wilcoxon test. * $p < 0.05$, *** $p < 0.001$.

composition. We obtained 120.4 million raw sequences from 83 samples from the 20 patients with MS. Processing, filtering, and unique molecular identifier collapsing resulted in a final data set of 11.3 million unique IgH sequences. Sample information, input cell numbers, and sequence numbers can be found in eTable 4, links.lww.com/NXI/A743.

IGHV and IGHJ Gene Usage Depends on Chromosomal Location

To track the recombination machinery and selection mechanism of the reconstituting B-cell compartment, we explored IGHJ and IGHV gene usage over time. The proportion of IGHJ genes was comparable to that of the reference group before transplantation. Following aHCT, IGHJ genes closer to the recombination site (IGHJ1, IGHJ2, and IGHJ3) were overrepresented in the initial posttransplantation period, followed by a slow return to balanced levels. In contrast, B cells using IGHJ5 and IGHJ6 that are farther from the recombination site reconstituted later and still did not reach baseline levels at the 1-year follow-up (Figure 4A).

Similarly, IGHV genes that are located closer to the recombination site were significantly overrepresented in the early reconstituted naive repertoire compared with the healthy reference repertoire (Figure 4B). This imbalance favoring recombination-close genes normalized during the first year post-aHCT. In contrast, such a bias was not

observed in the early reconstituted antigen-experienced repertoire (Figure 4C). A direct comparison can be found in eFigure 6, links.lww.com/NXI/A743.

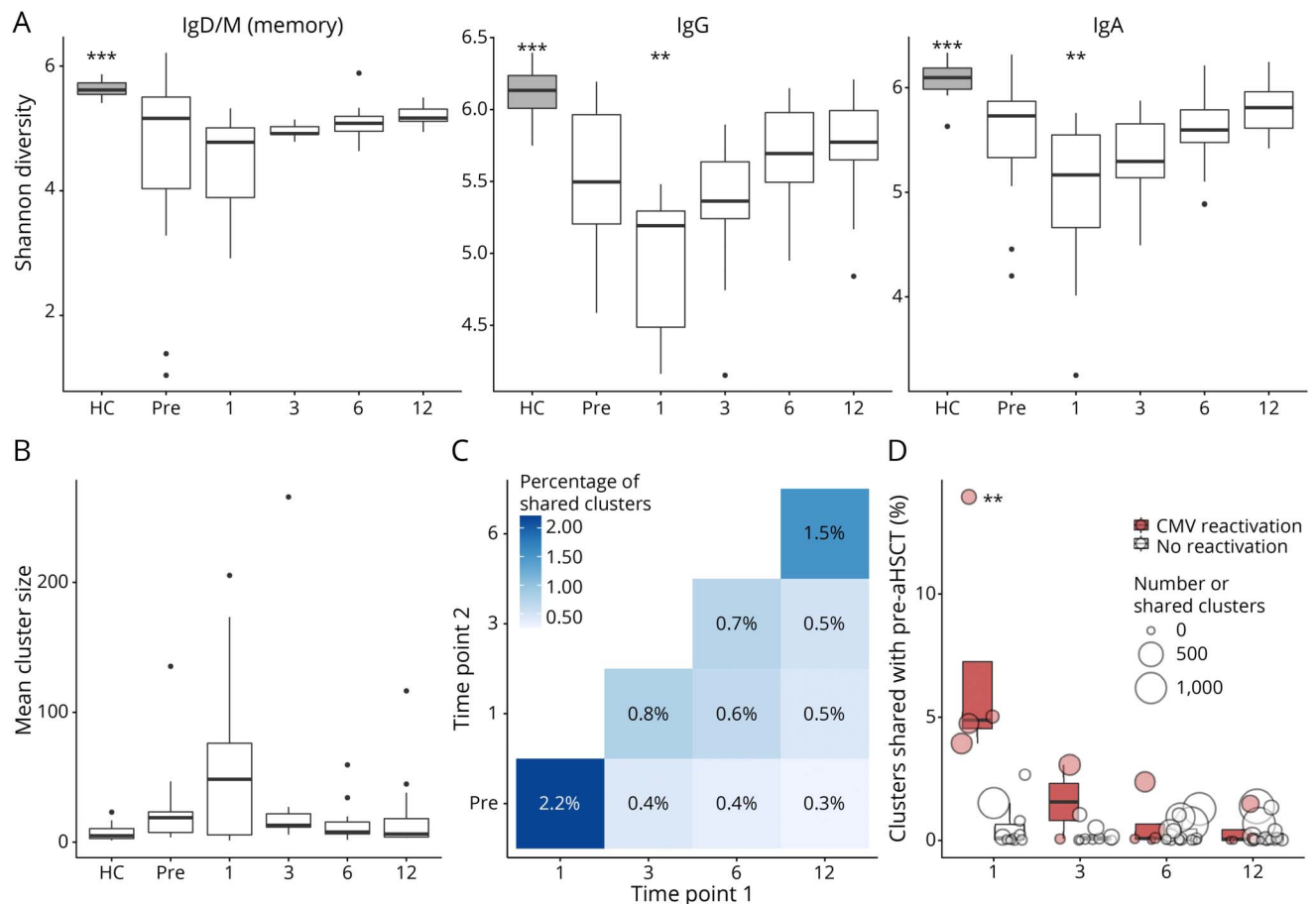
Mutation Analysis Suggests the Survival of Antigen-Experienced Memory Populations

Ig repertoires are highly diverse due to nucleotide changes introduced during V(D)J recombination and somatic hypermutation. Assuming that a new B-cell repertoire emerges after aHCT, mutation analyses should allow to analyze the degree of antigen exposure and reformation of a diverse B-cell repertoire. By longitudinally assessing the mean number of mutations by memory cell type and patient, we observed a consistent and significant decrease post-aHCT (Figure 5A). Of interest, at the 1 month follow-up, all switched isotypes showed normal or slightly elevated mean number of mutations, and the decrease was not apparent until 3 months post-aHCT. Similarly, the proportion of mutated sequences in switched IgG and IgA isotypes remained at a normal level until 3 months post-aHCT, followed by a decline and finally a resurgence at the 12-month follow-up visit (Figure 5B).

Repertoire Diversity Returns to Pretransplant Levels Within 6 Months

Assessing the diversity in IgH repertoires has proven difficult⁴¹ because the results are biased by several factors, in particular the input cell numbers, sampling depth, and differential

Figure 6 Repertoire Diversity and Sequence Cluster Overlap



(A) Shannon diversity index calculated on the proportions of mutation and junction length distributions for the different memory repertoires. For IgD/M (memory), only sequences with 3 or more mutations were considered. Pretherapy time point was used as the reference group for statistical comparison using the Wilcoxon test. (B) Mean size of sequence clusters in the antigen-experienced repertoire. (C) Mean percentage of cluster overlap between time points. (D) Proportion and number of clusters shared with the pretransplant repertoire for each time point after transplantation in patients with and without CMV reactivation. Wilcoxon test between groups of different CMV reactivation status. $**p < 0.01$, $***p < 0.001$. CMV, cytomegalovirus.

expression patterns. To partially account for some of these issues, we calculated the Shannon diversity index based on the distribution of sequences with specific combinations of mutation numbers and junction lengths. Using this approach, we found significant reductions in diversity in all memory repertoires and at all time points following aHSCT (Figure 6A). Diversity was already reduced in pre-aHSCT repertoires of patients with MS compared with healthy controls and further decreased in the early reconstituted IgG and IgA repertoires before returning to pre-transplant levels within 6 months. However, repertoire diversity remained below that of the healthy control population throughout the observation period and across all isotypes.

Decreasing Sequence Cluster Overlap Indicates Renewal of B-Cell Repertoire

Sequences were clustered based on their IGHV gene family and IGHJ gene identity, junction length, and sequence similarity. The mean cluster size in the antigen-experienced repertoire was increased at the earliest time point posttransplant before rapidly returning to HC levels

(Figure 6B). For each patient, we calculated the number of overlapping sequence clusters between time points to better assess novelty of the reconstituted repertoire (Figure 6C). The average cluster overlap before and 1 month after transplantation was 2.2%. Over time, the proportion of cluster overlapping with earlier time points decreased to 0.4% after 3 and 6 months and to 0.3% 12 months after transplantation. At the same time, new overlapping clusters formed during reconstitution: on average 0.3% of clusters at 12 months were shared with those before transplantation, 0.5% with those at 1 and 3 months, and 1.5% with those at 6 months post-aHSCT.

We found that CMV-reactivated patients showed a significantly higher proportion of overlapping clusters with the pretransplant repertoire in the early reconstitution phase (Figure 6D). After 1 month, an average of 6.91% ($\pm 2.35\%$) of the clusters was shared with the pretransplant repertoire (compared with an average of 0.55% in patients without CMV reactivation).

Discussion

In this study, longitudinal immunophenotyping and immunoglobulin repertoire sequencing demonstrated rapid immune reconstitution of major B-cell lymphocyte populations. Detailed analysis of the peripheral B-cell compartment showed a predominantly naive and oligoclonal immune phenotype with the probable persistence of some highly mutated MBCs in the early recovery phase. This was followed by a very slow production of antigen-experienced B cells. CMV reactivation after aHSCT was associated with a delayed B-cell reconstitution.

Our results confirmed findings about general immune reconstitution dynamics observed in other settings, namely diverging kinetics among major subsets in which innate immunity typically precedes adaptive immunity⁴² and the differentially affected T-cell subsets leading to an inverted CD4/CD8 ratio during the first year after transplantation.^{7,10,29} Naive T-cell subsets were still below normal levels up to 1 year after aHSCT, whereas early circulating B cells were dominated by transitional and naive phenotypes, as shown before in the context of HSCT.²⁷ Because interaction between primed CD4⁺ T cells and naive B cells is essential for B-cell activation and differentiation, the delayed regeneration of the CD4⁺ compartment may be a key factor in slowing down the recovery of MBC subsets. Previous studies have also started to establish a coarse timeline of recovery, with total B cells recovering between months 1 and 6 after transplantation.^{7,28,29} A dominating transitional phenotype in early reconstituting B cells after HSCT has been described in SSc²⁴ and in leukemia,²⁷ and we confirm the same pattern of repopulation in the context of MS. Whether the early excess of transitional immature B cells exerts a regulatory function remains to be assessed.

Isotype subclass distribution analysis revealed an overrepresentation of IgG1-expressing plasma and to a minor extent memory cells during reconstitution at the expense of IgG2- and IgG4-expressing cells. This shift was also reflected in our sequencing data and appeared to be driven by memory cells, which clearly outnumber PCs. Whether this is a long-lasting change or merely reflects developmental processes during reconstitution in which upstream genes (IgG3 and IgG1) are class switched earlier than downstream genes (IgG2 and IgG4)⁴³ remains to be investigated by analysis of later time points. The slow reconstitution and persistently low numbers of IgG2 cells after transplantation could specifically impair immune responses against polysaccharides⁴⁴ and thus be clinically relevant.

Consistent with the fact that PCs are not targeted by anti-CD20 therapy, we detected an elevated proportion of PCs pretransplant. More interestingly, this pattern persisted shortly after transplantation, indicative of a partial carryover of these cells. Nevertheless, absolute PC numbers were greatly reduced after transplantation, and their fast recovery indicates functional and effective B-cell differentiation early after immune restoration. In contrast, de novo generation of MBCs was slow and not completed even 12 months after aHSCT. MBC subset analysis showed that DMT-surviving memory

cells were predominantly class switched, and the persistence of this pattern into the early posttransplant system suggests that not only PCs but also memory subsets survive conditioning. Further evidence for the carryover of residual populations of antigen-experienced plasma and memory cells comes from the mutational IgH repertoire analysis. The amount of somatic hypermutation can be seen as sign of repeated antigen exposure and hence also as a proxy for antigen experience and maturational age of B cells. Early after transplantation, the proportion of highly mutated sequences remained at normal levels, and only after 3 months was there a marked decrease in the number of mutated sequences and a shift toward a naiver repertoire. Increased mean cluster size at the 1-month follow-up and significantly decreased repertoire diversity in the early memory repertoire suggest that these memory cells were clonally expanded. Whether these cells are functional and involved in host defense mechanisms in the early reconstitution phase remains unclear, but a significantly higher sequence cluster overlap in patients with CMV reactivation implies that they might be. Repertoire diversity recovered to pretransplant levels within the first year, indicating the reconstitution of a newly formed diverse repertoire. Decreasing overlap with older sequence clusters, combined with the accumulation of new persisting clusters, also points toward repertoire renewal. However, these data should be interpreted carefully because we do not have comparable longitudinal data showing baseline overlap in healthy individuals.

To further clarify the developmental history of the reconstituting repertoire, we longitudinally analyzed IGHJ and IGHV gene usage and the relationship to their position on the IgH locus.⁴⁵ It has long been proposed that the distance to recombination site on the IgH locus determines the recombination frequency of variable, diversity, and joining genes, with more proximal genes used more frequently than more distal genes during early cell development.⁴⁶ Consistent with this postulate, we found an overrepresentation of proximal IGHJ genes in the early reconstitution phase, followed by a slow convergence to normal distribution patterns during the first year post-aHSCT. Similarly, there was a significant negative correlation between IGHV gene usage and distance to the variable-diversity recombination site in the naive repertoire during the first month after aHSCT that leveled off over the following months. These findings suggest that V(D)J recombination early during B-cell reconstitution depends mainly on IgH locus positioning, whereas regular V(D)J usage patterns develop slowly, possibly driven by antigen experience. In addition to low MBC numbers and a reduced repertoire diversity, these findings suggest a nondirected incomplete B-cell repertoire at the 12-month follow-up, which may leave patients with suboptimal immune responses to pathogen encounter.

By linking clinical with cellular and sequencing data, we found greatly reduced naive and MBC formation at 3 and 6 months post-aHSCT in patients with early CMV reactivation. It is possible that in patients with an early CMV reaction, the transient expansion of (CMV-specific) MBCs disrupts the

newly developing B-cell compartment and thereby subsequently leads to a delay in overall immune reconstitution. Of interest, these patients also showed a significantly higher sequence cluster overlap between their pre- and early post-transplant repertoires, suggesting expansion of persistent MBC clones. However, sequence matching to known CMV-specific sequences showed neither an increased proportion of specific sequences in patients with CMV reactivation nor an increase of CMV-specific sequences over time (eFigure 8A, links.lww.com/NXI/A743). Therefore, it remains unclear whether these clusters contain CMV-specific clones. The diversity of the early repertoire was slightly but not significantly lower in these patients (especially in IgA, eFigure 5B, links.lww.com/NXI/A743), possibly due to higher clonality. Our data suggest that early posttransplantation monitoring and suppression of possible CMV reactivation is important for rapid B-cell reconstitution. A previous study has shown expansion of CD4⁺CD28^{null} T cells in CMV-seropositive patients,⁴⁷ whereas in our study, CMV reactivation was associated with higher CD8⁺ T-cell counts rather than CD4⁺ expansion, possibly indicating the strong CD8⁺ T-cell stimulus that CMV reactivation can trigger. In 2 of the patients with early CMV reactivation, EBV reactivation occurred simultaneously, making it difficult to separate their effects on the developing immune repertoire. Alignment with EBV-specific sequences did not reveal an increased proportion of these sequences in patients with EBV reactivation (eFigure 8B, links.lww.com/NXI/A743).

Of interest, we saw a faster reconstitution in patients with a relapsing-remitting form of MS compared with patients with progressive MS, especially for PCs and early transitional cells. Although the data set is well balanced in respect to sex and disease duration, this variation in reconstitution dynamics could be partially influenced by the age of patients, with patients with progressive MS being slightly older (mean age: 42.1 years) than patients with RRMS (mean age: 38.6 years). Importantly, an atypically large proportion of our cohort consisted of patients with progressive MS. This might possibly hamper some comparisons with other studies despite careful inclusion of this parameter into our modeling. Similarly, the cohort was dominated by patients receiving anti-CD20 treatment before aHSCT, leading to very low pretransplant B-cell counts. This might affect the translatability of our findings to other pre-treatments. In addition, incomplete immune reconstitution was still observed at 12 months posttransplant, advocating a longer follow-up period. The limited number of patients restricted the application of more comprehensive statistical modeling. Using the methods of immune phenotyping and repertoire analysis, we are lacking information about molecular and functional aspects and thus are limited in assessing the immune tolerance restoration. Subsequent studies are therefore needed to better understand functional aspects such as antigen presentation, antibody specificity, or regulatory functions.

Regarding the functional involvement of B cells in the pathogenesis of MS, recent data show that both their secretion of proinflammatory cytokines like interleukin 6 and granulocyte

macrophage colony-stimulating factor¹⁷ and their antigen-presenting function play important roles.^{48,49} Regarding the latter, the increased autoprolieration of T and B cells and the homing of autoreactive CD4⁺ T cells to the brain involve activated, highly DR-expressing switched MBCs⁴⁸ and likely also their immunopeptidome, which is remarkable for its high proportion of human leukocyte antigen DR-derived self-peptides.⁴⁹ The B-cell reconstitution dynamics after aHSCT show that switched MBCs are proportionally increased pretransplant but less abundant during most of the posttransplant period and then gradually return to healthy control levels. It will be interesting to examine in the future if these newly generated switched MBCs have lost the ability to engage in autoprolieration and/or other functional properties that may be important for their interaction with T cells. Conversely, if the autoprolieration were primarily T cell dependent, the slow recovery of naive and subsequently central memory T cells may be related to the delayed return of switched MBCs. The fact that the majority of patients remain disease-free post-aHSCT indicates that whatever processes are involved in the B-T interactions, they are effectively stopped. Regarding the latter, it is noteworthy that B cells are found not only in parenchymal lesions in MS but particularly also in the so-called tertiary lymphoid follicle-like structures in the meninges.⁵⁰ It is assumed that these tissue-resident B cells contribute to chronic inflammation in MS by both antigen presentation and release of proinflammatory cytokines.⁵⁰ Because the drugs used in the BEAM regimen (i.e., BCNU, melphalan, and to a limited extent also etoposide and cytarabine) can enter the CNS compartment, it would be very interesting to know whether tissue-resident B cells are affected by the conditioning regimen of aHSCT. Monoclonal antibodies like anti-CD20 do not cross the blood-brain barrier to any significant degree, and these pharmacokinetic differences may play a role in the high efficacy of aHSCT.

Taken together, we propose the following scheme of reconstitution for B cells: highly mutated, antigen-experienced memory and PCs survive DMTs as well as aHSCT and form a substantial part of the earliest repertoire, partly through clonal expansion. Repopulation with a new and naive B-cell system starts early and rapidly, in line with the normal maturation process in development, which means that recovery of antigen-experienced memory cells is slow and still incomplete after 1 year. Several meta-analyses have confirmed that aHSCT is a highly effective therapy for MS, with event-free survival rates that exceed those of the best available pharmacotherapies.²⁻⁵ To further optimize aHSCT as an MS therapy and, for example, to identify less invasive but also highly effective therapeutic regimens, it is crucial to improve our understanding of the molecular and cellular processes that promote the immunotolerant state after treatment, particularly with regard to B-cell functions of antigen presentation, proinflammatory cytokine secretion, and involvement in autoreactive T-cell activation.

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Josefine Ruder, PhD	Neuroimmunology and MS Research Section, Department of Neurology, University Hospital Zurich, University of Zurich	Drafting/revision of the manuscript for content, including medical writing for content; study concept or design; and analysis or interpretation of data
Marie Ghraichy, PhD	Division of Immunology and Children's Research Center, University Children's Hospital Zurich, University of Zurich	Analysis or interpretation of data
Ilijas Jelcic	Neuroimmunology and MS Research Section, Department of Neurology, University Hospital Zurich, University of Zurich	Drafting/revision of the manuscript for content, including medical writing for content
Antonia Maria Müller	Department of Medical Oncology and Hematology, University Hospital Zurich	Drafting/revision of the manuscript for content, including medical writing for content
Urs Schanz	Department of Medical Oncology and Hematology, University Hospital Zurich	Study concept or design

Appendix (continued)

Name	Location	Contribution
Roland Martin	Neuroimmunology and MS Research Section, Department of Neurology, University Hospital Zurich, University of Zurich	Drafting/revision of the manuscript for content, including medical writing for content; study concept or design; and analysis or interpretation of data
Johannes Trüch	Division of Immunology and Children's Research Center, University Children's Hospital Zurich, University of Zurich	Drafting/revision of the manuscript for content, including medical writing for content; study concept or design; and analysis or interpretation of data

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