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A recent study of AHSCT for active relapsing-remitting multiple sclerosis (RRMS) showed efficacy in preventing disease worsening. However, the immunologic basis for efficacy remains poorly defined. MS pathology is known to be driven by inflammatory T cells that infiltrate the central nervous system (CNS). Therefore, we hypothesized that the pre-existing T cell repertoire in the intrathecal compartment of active RRMS participants was ablated, and replaced with new clones following AHSCT. T cell repertoires were assessed using high-throughput TCRβ chain sequencing in paired cerebrospinal fluid (CSF) and peripheral blood CD4+ and CD8+ T cells from participants that underwent AHSCT, before and up to 4 years following transplantation. >90% of the pre-existing CSF repertoire in participants with active RRMS was removed following AHSCT, and replaced with clonotypes predominantly generated from engrafted autologous stem cells. Of the pre-existing clones in CSF, ~60% were also detected in pre-therapy blood, and concordant treatment effects were observed for clonotypes in both compartments following AHSCT. These results indicate that replacement of the pre-existing TCR repertoire in active RRMS is a mechanism for AHSCT efficacy, and suggest that peripheral blood could serve as a surrogate for CSF to define mechanisms associated with efficacy in future studies of AHSCT.

Extensive intrathecal T cell renewal following hematopoietic transplantation for multiple sclerosis

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Laurence Turka is employed by, and holds equity grants in, Rheos Medicines. Harlan Robins is employed by and holds equity in Adaptive Biotechnologies. The other authors have no conflicts of interest.

Abstract

A recent study of AHSCT for active relapsing-remitting multiple sclerosis (RRMS) showed efficacy in preventing disease worsening. However, the immunologic basis for efficacy remains poorly defined. MS pathology is known to be driven by inflammatory T cells that infiltrate the central nervous system (CNS). Therefore, we hypothesized that the pre-existing T cell repertoire in the intrathecal compartment of active RRMS participants was ablated, and replaced with new clones following AHSCT. T cell repertoires were assessed using high-throughput TCRβ chain sequencing in paired cerebrospinal fluid (CSF) and peripheral blood $CD4^+$ and $CD8^+$ T cells from participants that underwent AHSCT, before and up to 4 years following transplantation. >90% of the pre-existing CSF repertoire in participants with active RRMS was removed following AHSCT, and replaced with clonotypes predominantly generated from engrafted autologous stem cells. Of the pre-existing clones in CSF, ~60% were also detected in pre-therapy blood, and concordant treatment effects were observed for clonotypes in both compartments following AHSCT. These results indicate that replacement of the pre-existing TCR repertoire in active RRMS is a mechanism for AHSCT efficacy, and suggest that peripheral blood could serve as a surrogate for CSF to define mechanisms associated with efficacy in future studies of AHSCT.

Introduction

Autologous hematopoietic stem cell transplantation (AHSCT) has been investigated in clinical trials for aggressive autoimmunity as a therapeutic strategy for replacing the autoreactive immune system with a new, potentially self-tolerant immune repertoire (1). HALT-MS was a phase II study of AHSCT for patients with treatment-resistant relapsing-remitting multiple sclerosis (RRMS), which showed that AHSCT without maintenance disease-modifying therapy was effective for inducing durable remissions of active RRMS for at least 5 years (2). In the cohort of 24 participants that underwent AHSCT, 17 achieved durable complete remission from active disease, 3 experienced clinical relapse, 2 showed disease progression by increased Expanded Disability Status Scale (EDSS), and 2 had recurrence of CNS inflammation detected at MRI through the 60 months follow-up (2). Notably, all study participants showed an attenuation of the disease activity after AHSCT compared to pre-therapy.

In 2014, we used high throughput TCR sequencing to analyze how AHSCT modified the peripheral blood repertoire in patients enrolled in the HALT-MS trial. This study showed that while the CD4 repertoire was largely replaced as a result of the procedure, the CD8 repertoire was less profoundly affected, as many pre-existing CD8 T cell clones “survived” the procedure, and expanded at month 12 post-transplant(3). While providing novel information about the effects of AHSCT on the immune repertoire, this interim analysis was limited to what was seen in peripheral blood at 12 months post-transplant and provided no insight into what was happening in the target organ of the disease, the CNS.

MS pathology is known to be driven by inflammatory T cells that enter and infiltrate the central nervous system (CNS) (4). T cells that access the CNS recirculate in the cerebrospinal fluid (CSF) (5), thus presenting a more direct point of access to

the site of disease than peripheral blood (6). However, the sampling limitations and the low number and instability of T cells in CSF have hampered the ex vivo characterization of these cells (7). To investigate one mechanism potentially explaining the strong mitigating effect on CNS inflammation seen in HALT-MS, we hypothesized that the T cell repertoire in the intrathecal compartment pre-therapy, likely including autoreactive T cells, was ablated and replaced with new clones post-AHSCT therapy. To test this, we have compared the reconstitution of T cell composition in peripheral blood to paired reconstitution in the target disease organ using cerebrospinal fluid (CSF) from HALT-MS participants analyzed by high-throughput deep sequencing of T cell receptor beta (TCRβ) complementary determining region 3 (CDR3) chains (7, 8).

The data reported in this manuscript support the hypothesis that AHSCT removes the majority of T cell clones present in the intrathecal compartment of patients with active RRMS, and enables the generation of a new immune repertoire in both intrathecal and peripheral blood compartments. In addition, our findings suggest that peripheral blood could serve as a surrogate for CSF to define mechanisms associated with efficacy of AHSCT in active RRMS. These novel and important insights identify new avenues of investigation for future studies of AHSCT and other immunomodulatory intervention strategies for MS.

Results

Persistent ablation of pre-existing T cell repertoire in peripheral blood following AHSCT therapy.

We previously reported that AHSCT had distinctive effects on $CD4^+$ and $CD8^+$ T cell repertoires in peripheral blood of HALT-MS participants (3). In circulating $CD4^+$ T cells, participants largely developed a new repertoire, whereas the reconstituted $CD8^+$ T cell repertoire was created by clonal expansion of pre-existing cells that were not effectively removed by AHSCT therapy (3). We have extended upon this analysis to evaluate the longer-term reconstitution of TCR repertoires in blood $CD4^+$ and $CD8^+$ T cell compartments using an approach that could be applied to the corresponding CSF samples. The percent TCR overlap was used to determine the sharing between T cell repertoires from identical tissues (blood or CSF) at different time points. This metric is preferred because it ensures a method robust to differences in sampling depth by weighting the relative frequencies of shared clones between time points (8).

Consistent with our previous report (3), most of the pre-existing $CD4^+$ T cell repertoire in blood pre-therapy was undetectable by ultra-deep sequencing in blood following transplantation at months 12, 24 and 48 (mean 81.4% ±7.4 SD at month 12; mean 83.8% ±5.5 SD at month 24; mean 88.7% ±4.7 SD at month 48) (Figure 1). In blood, ablation of the pre-existing $CD8^+$ T cell repertoire was significantly less extensive compared to the $CD4^+$ T cell compartment ($p< 0.001$, $p<0.001$ and <0.01 at months 12, 24 and 48, respectively) and was highly variable among participants (mean 68.2% ±12.8 SD at month 12; mean 70.9% ±11.9 SD at month 24; mean 71.2% ±15.2 SD at 48) (Figure 1). The striking changes we observed in the TCR repertoire of transplanted patients were almost certainly due to AHSCT therapy itself, and not the interval between sample time points given the repertoire stability we previously

reported for paired samples of T cells collected from healthy individuals 12 months apart (3). These data show that the effects of AHSCT on the elimination of pre-existing T cell repertoire in peripheral blood are durable through 48 months follow-up.

AHSCT therapy removes most of the pre-existing T cell repertoire in CSF.

T cells recirculating in the intrathecal compartment provide a more direct point of access to the CNS than peripheral blood (5, 6), and may therefore contain a higher frequency of disease-causing clonotypes than peripheral blood. Thus, we asked whether elimination of T cell clones from the CSF of MS patients could be one of the mechanisms by which AHSCT effectively treated RRMS in HALT-MS (2). To determine the sharing between T cell repertoires in CSF pre-therapy versus time points post-therapy, the percent TCR overlap was used because it ensures a method robust to differences in sampling depths between time points (Supplemental Tables 1 and 3) by weighting the relative frequencies of shared clones between time points (8). The majority of T cells detected in CSF pre-therapy were undetectable in CSF by deep sequencing at months 24 and 48 post-transplant (Figure 2A). On average, 91.8% (±7.2 SD) and 93.5% (±6.9 SD) of the pre-existing CSF repertoire was undetectable in CSF at 24 and 48 months, respectively. Abundance of pre-existing clones in CSF pre-therapy was not associated with whether or not clonotypes were detected or undetectable in CSF at month 24 post-therapy (p=0.4) (Figure 2B), but indeed some clones were highly persistent following AHSCT. These data indicate that most of the pre-existing TCR repertoire in CSF was removed by AHSCT irrespective of the clones' individual frequency, and show that the effects are durable through 48 months follow-up.

We next examined the relationship between clones shared between CSF and peripheral blood compartments in active RRMS pre-therapy and at month 24 post-AHSCT therapy. On average, 62.3% (±6.1% SD) of clones in CSF pre-therapy were also detected in blood pre-therapy (Figure 2C). The degree of clonal sharing between CSF and peripheral blood compartments was significantly reduced following AHSCT at month 24 post-therapy to 40.6% (±7% SD) ($p<0.0001$, Figure 2C). Given the efficacy of AHSCT in this study cohort, we asked whether AHSCT removed clones exclusively detected in CSF pre-therapy. Our rationale was that clonotypes enriched or exclusively detected in CSF of patients with active RRMS (as per trial inclusion criteria) may comprise a higher frequency of T cells with specificity to CNS antigens, likely including disease-causing T cells, than would be detected in peripheral blood. We found that 40.5% (±11.8 SD) of pre-existing clones that were removed from the CSF following AHSCT were exclusively detected in CSF (Figure 2D gold vs. red bars), whereas clones that persisted in CSF were predominantly shared between compartments pre-therapy (Figure 2D grey vs. blue bars). Notably, very few clones that were detected in CSF and not in blood pre-therapy persisted at 24 months, which indicates that T cell clones detected in CSF post-transplant originate from precursors in peripheral blood.

T cell renewal in CSF following AHSCT therapy.

Having determined that >90% of the T cell repertoire in CSF pre-therapy was undetectable at months 24 or 48 post-AHSCT therapy (Figure 2A), we wanted to determine the proportion of new T cell clones arising in the reconstituted CSF repertoire following AHSCT therapy. The percent TCR overlap analysis showed that the majority (80.3% ±14.2 SD) of T cells detected in CSF at month 24 were

undetectable by deep sequencing in the CSF pre-therapy (Figure 3A), indicating that most of the reconstituted repertoire in the intrathecal compartment comprised new species of T cell clones. To ensure the robust changes observed were not due to differences in sampling depths between time points (Supplemental Tables 1 and 3), randomized, re-sampling was performed 1000 times on pre-therapy CSF TCRβ sequences to match with the corresponding sampling of CSF TCRβ sequences at month 24 post-transplant. The mean percentage of clones that overlapped in smaller post-transplant sampling from the larger pre-therapy samples was determined to be 92.9% (±6.5 SD) (Supplemental Figure 1). These results rule out sampling bias, and strengthen the claim that AHSCT replaced >90% of the pre-existing T cell repertoire in CSF with new clones at month 24 post-transplant (Figure 2A).

Clonotypes identified as new in the reconstituted CSF could arise from mature T cells that survived the immunoablative procedure and were pre-existing in blood but not in CSF, or from differentiation of engrafted autologous hematopoietic stem cells (aHSCs). To address this, the percent of new T cell clones in CSF at month 24 post-transplant that were detected versus undetectable by ultra-deep sequencing in pre-therapy blood samples was determined for individual participants. The rationale was that new clones in reconstituted CSF that were detected in blood pre-therapy were generated from pre-existing T cells that survived immunoablation, whereas new clones arising in CSF that were undetectable in blood pre-therapy likely originated from new differentiation. Most (mean 72% ±13.3 SD) of the new T cell repertoire in CSF at month 24 post-transplant comprised clonal species that were undetectable in blood pre-therapy for all participants evaluated with the exception of P4, and thus, likely generated from T cell differentiation of engrafted aHSCs (Figure 3B). As new T cell clones differentiate from engrafted aHSCs and repopulate the peripheral blood

repertoire, and the CSF is populated by continuous trafficking of blood-circulating cells, one might expect that the proportion of new clones arising in CSF at month 24 post-transplant that are also detected in blood should increase over time. Consistent with this notion, the percent of T cell clones identified as new in CSF at month 24 post-transplant that were also detected in blood was significantly higher at months 12 (mean 40.9% ±17.3 SD) and 24 (mean 56.5% ±16.4 SD) post-transplant as compared to month 0 pre-therapy (mean 28% ±13.3 SD) (Figure 3C and Supplemental Figure 2).

*Longitudinal evaluation in blood of clone*s detected in both CSF and peripheral blood T cell *compartments.*

Having observed extensive removal of pre-existing clonotypes in both CSF (Figure 2A) and CD4 T cells in blood (Figure 1) following hematopoietic transplantation, together with the notable degree of clonal sharing between CSF and blood compartments pre-therapy (Figure 2C), we wanted to determine if the treatment effects observed in CSF were reflected in T cells in blood. To assess this, clones that were classified as removed, new or persistent in CSF at month 24 post-transplant, which were also detected in blood pre-therapy, were evaluated as the cumulative proportion within $CD4^+$ or $CD8^+$ T cells in blood over time. Clones that were identified as removed from CSF (i.e. pre-existing in blood and CSF that were undetectable in CSF at month 24 post-transplant) formed a significantly reduced proportion of $CD4^+$ and $CD8^+$ T cell repertoires circulating in blood at months 12, 24, and 48 post-transplant as compared to month 0 pre-therapy (Figure 4A and Supplemental Figure 3A). This contrasted sharply with clones that were identified as new in CSF at month 24 post-transplant (i.e. pre-existing in blood pre-therapy and undetectable in CSF pre-therapy), which formed a significantly greater proportion of $CD4^+$ and $CD8^+$ T cell

repertoires circulating in blood at months 12, 24, and 48 post-transplant as compared to month 0 pre-therapy (Figure 4B Supplemental Figure 3B). Most of the clones that persisted in CSF at month 24 post-transplant were detected in blood pre-therapy (Figure 2D grey vs. blue bars), which transiently increased then persisted within $CD4^+$ and $CD8^+$ T cell repertoires in blood post-transplant (Figure 4C and Supplemental Figure 3C). These results show concordant effects of AHSCT therapy on clones present in both intrathecal and peripheral blood compartments.

Diversification of T cell repertoires in peripheral blood and CSF following AHSCT.

We next set out to determine the effects of AHSCT therapy on the degree of TCR complexity, termed TCR entropy, within and between compartments using the Shannon entropy index (8). In accordance with previous studies of hematopoietic transplantation (9), we observed earlier recovery of TCR entropy of $CD4^+$ T cells in blood compared to their CD8 counterparts, with return to baseline entropy levels at month 24 post-transplant in $CD4^+$ but not $CD8^+$ T cells (Figure 5A). Consistent with the reduced TCR entropy of $CD8^+$ T cells compared to $CD4^+$ T cells in blood at month 24 post-transplant (Figure 5A and Supplemental Figure 4A), the ratio of effector memory (EM):naïve T cells was significantly increased from month 0 pre-therapy at month 24 post-transplant in the $CD8^+$ compartment, and not in the $CD4^+$ compartment (Figure 5B and Supplemental Figure 4B).

Further participant stratification revealed an association between CMV infection post-transplant and the reduced recovery of TCR entropy in circulating $CD8^+$ T cells at month 24 post-transplant ($p=0.003$) (Figure 5A and Supplemental Figure 4A). This could be explained by oligoclonal expansion of CMV-reactive $CD8^+$ T cells (10), and potentially cytokine-mediated bystander activation of memory T cells (11). In support

of these possibilities, CMV-positivity post-transplant was associated with higher ratios of CM:naïve for $CD8^+$ T cells at months 12 and 24 and EM:naïve for both $CD8^+$ and $CD4^+$T cells at months 12, 24 and 36 post-transplant (Figure 5B and Supplemental Figure 4B).

TCR entropy was lower in the CSF repertoire (mean 10.3 ±2.9 SD) pre-therapy (Figure 6A and Supplemental Figure 5) as compared to $CD4^+$ and $CD8^+$ repertoires in blood pre-therapy (mean 15.7 ±0.8 SD for $CD4^+$ and mean 13.2 ±1.5 SD for $CD8^+$). Similar to what we observed for TCR entropy of $CD8^+$ T cells at month 24 post-transplant, TCR entropy of CSF was not fully reconstituted at month 24 post-transplant as compared to pre-therapy (Figure 6B). However, the reduced diversity of the reconstituted CSF repertoire at month 24 was not associated with CMV infection, EBV infection, age, or clinical events related to MS worsening (data not shown).

Discussion

AHSCT therapy has been shown to be effective in patients with treatment-resistant RRMS (2), yet the neuroimmunologic therapeutic mechanisms to potentially explain the strong mitigating effect on CNS inflammation seen in HALT-MS remain poorly defined. To investigate one possibility, we tested the hypothesis that the pre-existing T cell repertoire in the intrathecal compartment pre-therapy was ablated and replaced with new clones post-therapy. Using high-throughput TCRβ chain sequencing, we have compared the reconstitution of T cell repertoires in the target disease organ using CSF to paired reconstitution in peripheral blood from HALT-MS participants with active RRMS that underwent AHSCT therapy. In support of our hypothesis, we show that AHSCT therapy leads to the generation of a new T cell

repertoire within the intrathecal compartment, providing primary evidence that immunoablation followed by AHSCT facilitates immune resetting in the CNS.

We found a >90% degree of clonal ablation in the T cell repertoire in CSF that persisted out to 4 years post-transplantation (Figure 2A). The reconstituted repertoires in CSF comprised predominantly new T cell clonotypes differentiated from engrafted aHSCs, and a smaller population of mature T cells from the pre-existing repertoire in blood that survived immunoablation (Figure 3). The robust changes we observed in relation to the CSF pre-therapy sharply contrasts with the stability of the T cell repertoire in CSF previously reported for MS patients that underwent two lumbar punctures 14 months apart, where 91%-100% of sequences found in the first CSF sample were also found in the second sample (12). Our findings suggest that the extensive replacement was a consequence of AHSCT therapy, and not simply due to physiological T cell turnover in CSF over time.

Interestingly, the extent of repertoire replacement was significantly greater in CSF compared to $CD4^+$ T cells in blood at month 24 ($p<0.001$), and in CSF compared to $CD8^+$ T cells in blood at both month 24 ($p<0.001$) and month 48 ($p<0.01$). We speculate that the increased $CD4^+$ T cell replacement seen in peripheral blood at 48 months (no longer significantly different from T cell renewal in the CSF) may reflect enlargement of the pool of new clones continuing to differentiate after year 2. This notion is consistent with the significant increase of recent thymic emigrants (RTEs) we observed in the pool of $CD4^+$ T cells in blood at month 36 post-transplant compared to pre-therapy (13).

We found that >40% of the removed CSF repertoire consisted of clonotypes that were exclusively detected in CSF (i.e., which were undetectable in peripheral blood T cells) of active RRMS patients pre-therapy. It is intriguing to postulate that

pre-existing clones enriched in CSF may be relevant to disease. Elegant studies in mice indicate that under physiologic conditions, the choroid plexus (CP), a unique neuro-immunological interface that produces CSF and regulates immune surveillance in the CNS (14), maintains a broad repertoire of CNS-specific memory $CD4^+$ T cell clones that can remain in the CP, or enter the CSF and change effector function depending on the context of antigen presentation in the microenvironment (15). Unfortunately, the autoantigens in MS remain poorly defined, and the autoreactive repertoire may be unique among individuals with MS. Therefore, evaluation of CNS-specific T cell clones in CSF and peripheral blood specimens was beyond the scope of our current study. Our hope is that in time, the autoantigens and/or TCR specificity of disease-causing T cells in MS will be revealed, so that the database of TCRβ sequencing from this study available at https://www.itntrialshare.org/HALTMS_JCIinsight.url, and archived PBMC specimens remaining, will enable sufficient investigation of this possible mechanism underlying the efficacy of AHSCT.

In contrast to what was previously reported (12), our analysis demonstrated that ~60% of pre-existing clones in CSF were detected in blood pre-therapy (Figure 2C). This discrepancy (mean 62% vs. 29%) may be explained by the differences in sampling depth (ultra-deep versus survey level) and methods (sorted CD4+ and CD8+ T cells versus whole blood) used for evaluating T cells in blood and/or differences in the study cohorts (12). The notable percent overlap of CSF clones detected in blood pre-therapy, together with the concordant effects of AHSCT observed on clonotypes shared between compartments pre-therapy (Figure 4), suggest that peripheral blood could serve as a surrogate for CSF to interrogate TCR repertoire as a biomarker associated with efficacy in future studies of AHSCT therapy in active RRMS.

During the steady-state, circulating leukocytes can readily enter the CP, but few T cells other than CD4 T cells with a central memory phenotype enter the CSF (16). In contrast, leukocyte migration into the CSF increases as a result of decreased barrier function and augmented chemokine production during neuro-inflammation (14). Therefore, we anticipated enhanced sharing of pre-existing clones between immune compartments pre-therapy in active RRMS, and postulated that the degree of sharing and repertoire complexity would be reduced following the attenuation of inflammation and normalization of leukocyte trafficking into the CNS through the CSF post-therapy. Several observations support this possibility. First, the percent of CSF clones detected in blood was significantly decreased at month 24 post-transplant compared to pre-therapy (Figure 2C). Second, clones removed from CSF were present in blood, but at reduced frequencies at months 12, 24, and 48 post-transplant compared to pre-therapy (Figure 4A). Third, diversification of the TCR repertoire in CSF was not fully reconstituted to pre-therapy levels at month 24 post-transplant (Figure 6). This could be explained by reduced trafficking of T cells into the CNS as a result of restored blood-brain barrier function and/or restoration of T cell homing programs after immunoablation and reconstitution. Alternatively, the reduced complexity of CSF repertoire could result from increased clonal dominance in association with recurrent autoimmunity, however, we believe this is less likely given the positive long-term outcome of most participants in the HALT-MS trial (2).

Our interim analysis suggested that improved clinical outcomes were associated with higher TCR diversity of T cells in peripheral blood at month 2 post-transplant (3). Given that this association was not observed at month 12 post-transplant, we did not expect to find an association between clinical outcome and diversification of the TCR repertoire in blood T cells at months 24 and 48 analyzed

here. While none of our TCR repertoire analyses revealed associations with disparate long-term clinical outcomes, we accept the limitations of the study for identifying biomarkers of response as reported previously (13).

Taken together, our studies support the hypothesis that AHSCT therapy removes the majority of T cells existing in the intrathecal compartment of patients with active RRMS, likely including pathogenic T cells, and induces the generation of a newly differentiated immune repertoire that persists in both intrathecal and peripheral blood compartments. Of the pre-existing clones in CSF, ~60% were also detected in pre-therapy blood, and concordant treatment effects were observed for clonotypes in both compartments following AHSCT. These results indicate that replacement of the pre-existing TCR repertoire in active RRMS is a mechanism for AHSCT efficacy, and suggest that peripheral blood could serve as a surrogate for CSF to define mechanisms associated with efficacy in future studies of AHSCT. Our novel findings identify new avenues of investigation for future studies of AHSCT therapy and other immunomodulatory intervention strategies for MS.

Methods

Further information can be found in the Supplemental Methods.

Research Specimens. Of the 24 transplanted HALT-MS participants that had paired CSF and peripheral blood specimens for evaluation pre-therapy, 18 had paired CSF and peripheral blood specimens for evaluation at month 24 post-transplant, and 7 of these participants had specimens for evaluation at month 48 post-transplant. Our analyses included specimens from a subgroup of 7 participants who did not meet the trial definition of success of therapy. However, no blood or CSF samples were obtained at the time of MS reactivation (i.e. at the time of relapse, new MRI activity or worsening of disability post-transplantation).

Cell Preparation and DNA Isolation. PBMCs frozen in 20% DMSO/human AB serum were thawed and then separated by sequential positive selections for $CD4^+$ cells (Miltenyi Biotec; Catalog # 130-045-101) followed by $CD8^+$ cells (Miltenyi Biotec; Catalog # 130-045-201) per manufacturer's instructions. Frozen cellular pellets from 20-30 mL CSF were thawed for genomic DNA isolation. DNA was isolated using the Qiagen QIAmp DNA mini kit (Qiagen; Catalog # 51306) per manufacturer's instructions except the elution volume was increased to 100 µL for use in TCR sequencing.

TCR sequencing. Due to the multi-center logistics of the HALT-MS trial, together with the limited number and stability of cells in CSF *ex vivo*, the decision was made to use the entire cell fraction for molecular studies. Cell counts were obtained by the clinical labs, but T cell counts were not, and could not be estimated from the version of the

Adaptive TCRβ sequencing platform that was used for this project. Input DNA from the cellular fraction (mean 154,000 total leukocytes) from 20-30 mL CSF collected pre-therapy and at months 24 and 48 post-transplant was ~1 µg per sample, and the input DNA for 1 million sorted $CD4^+$ and $CD8^+$ T cells from blood pre-therapy and at months 12, 24 and 48 post-transplant was 5 µg. Rearranged TCRβ chains from all specimens were amplified, sequenced and processed using the immunoSEQ® Assay (17). This sequencing strategy is sufficient to achieve at least 4-fold coverage of every original template, which is sufficient to prevent a sampling effect (18). Survey level TCRβ sequencing was used to evaluate the full complexity of the CSF repertoire (12), which is limited in T cell numbers and richness compared to blood. This produced an average of 447,387 sequence reads distributed among an average of 4,275 unique TCRB CDR3 rearrangements for each CSF cellular pellet sample (Supplemental Table 1). Ultra-deep level TCRβ sequencing was used to enable accurate description of richer repertoires and detection of low-frequency clones in the millions of $CD4^+$ and $CD8^+$ T cells sorted from blood of individual participants for longitudinal evaluation (19). This produced an average of 6,216,316 and 5,911,245 sequence reads distributed among an average of 210,455 and 112,709 unique TCRB CDR3 rearrangements for each sample of one million $CD4^+$ or $CD8^+$ T cells (Supplemental Table 2). For known autoreactive clones in psoriasis, their abundance in the peripheral blood is near or above 1/million cells (19). We sequenced 1 million T cells from each of the $CD4^+$ and $CD8^+$ compartment, reaching levels far below 1/million PBMCs (19). Sequence comparisons were based on annotated TCRβ variable (TCRBV) and joining (TCRBJ) gene segments and CDR3 amino acid (AA) sequence. For each repertoire, the frequencies of clones with identical TCRBV, TCRBJ and CDR3 AA sequences, but different DNA sequences were merged. The assumption is that T cell clones with

identical TCRBV, TCRBJ and CDR3 AA sequences recognize the same antigen. CDR3 AA sequences were favoured over DNA sequences to better reflect changes in antigen recognition of the reconstituted T cell repertoire between time points. Shared clones between two repertoires are defined as having identical TCRBV and TCRBJ segments and CDR3 AA sequence.

To determine the overlap between T cell repertoires from identical tissues at different time points, we used the TCR overlap percentage (8). The percent overlap is weighted by the relative frequencies of shared clones between time points ensuring that this method is robust to differences in sampling depth. The massive difference in numbers of T cell clones sampled from peripheral blood compared to the CSF precluded the use of this method to compare the overlap of these tissues. Instead, the proportion of specific clonotypes was tracked from one compartment to the other pre-therapy or over time. The Shannon entropy was used as an index of TCR repertoire diversity. This method weights clones by relative frequency favoring neither dominant or rare clones disproportionately.

Statistics. For longitudinal assessment between time points, data were analyzed using mixed model for repeated measures. Pairwise comparisons were made between time points and p-values were adjusted using Tukey's multiple testing corrections. R 3.5.1 was used for all analyses. Statistical significance was set at 5% ($p < 0.05$).

Data and materials availability. Data sets for these analyses are accessible through TrialShare, a public website managed by the Immune Tolerance Network (https://www.itntrialshare.org/HALTMS_JCIinsight.url), which allows the user to filter

the underlying data and generate figures and results of analysis in addition to those submitted as part of the manuscript.

Study approval. The HALT-MS study (a Phase II Study of High-Dose Immunosuppressive Therapy [HDIT] and Autologous CD34+ Hematopoietic Stem Cell Transplant [HCT] for the Treatment of Poor Prognosis Multiple Sclerosis) was sponsored by NIAID and conducted by the ITN (ITN033AI) (ClinicalTrials.gov NCT00288626). The protocol was reviewed and approved by the IRB at each of the clinical sites, and all subjects provided informed consent prior to their participation. This prospective, open-label, single-arm, multicenter clinical trial was designed to determine the 5-year durability of disease stabilization in MS subjects after HDIT and autologous HCT (2).

Author Contributions

Clinical study design, conduct, supervision and coordination: R.A.N., L.M.G., and P.A.M. Experimental design: K.M.H., N.L., P.L., H.R., L.A.T, and P.A.M. Data presentation and analysis: K.M.H., N.L., P.L., H.R., L.A.T, and P.A.M. Data interpretation: K.M.H., N.L., P.L., H.R., L.A.T, and P.A.M. Drafted the manuscript: K.M.H and P.A.M. All authors contributed critical revision of the manuscript for important intellectual content.

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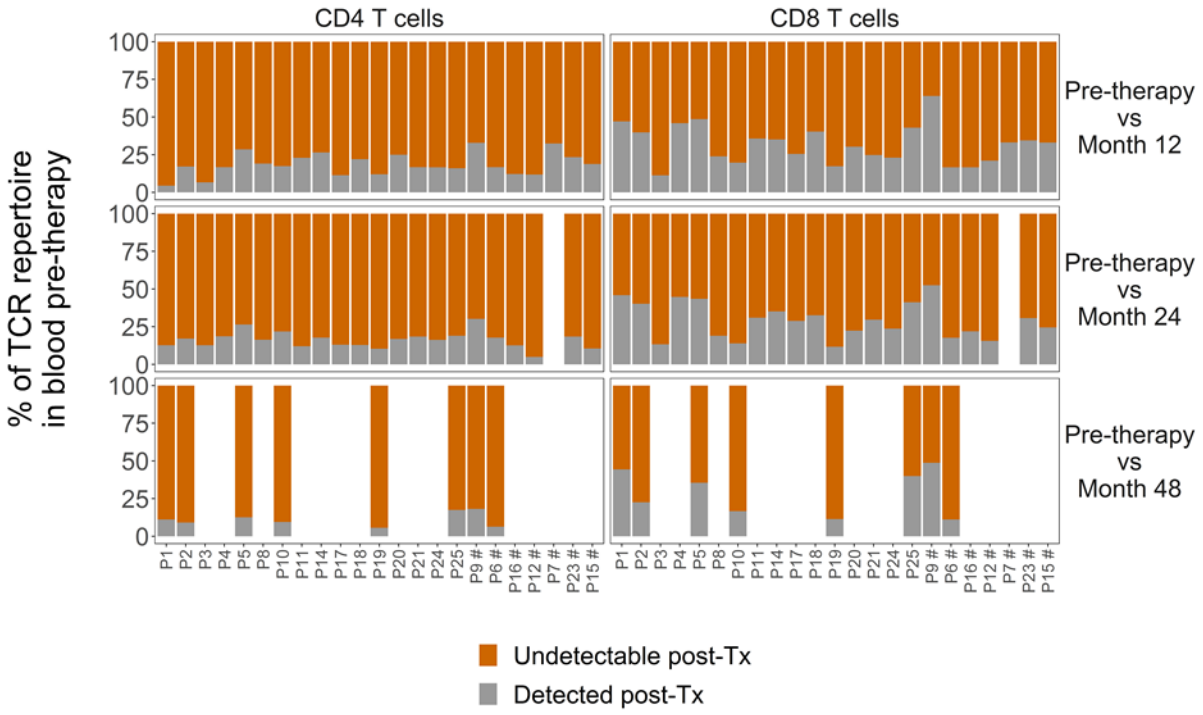

Figure 1. Effects of AHSCT therapy on ablation of pre-existing T cell repertoires in peripheral blood are durable through 48 months. TCR overlap percentage was used to determine the percent of clones that were undetectable (orange) versus detected (grey) by ultra-deep sequencing of TCR repertoires in $CD4^+$ T cells and $CD8^+$ T cells isolated from peripheral blood, comparing pre-therapy to months 12, 24 or 48 for each participant. # participants that met the primary endpoint for the HALT-MS study. All other participants remained in remission from active MS until the last follow-up.

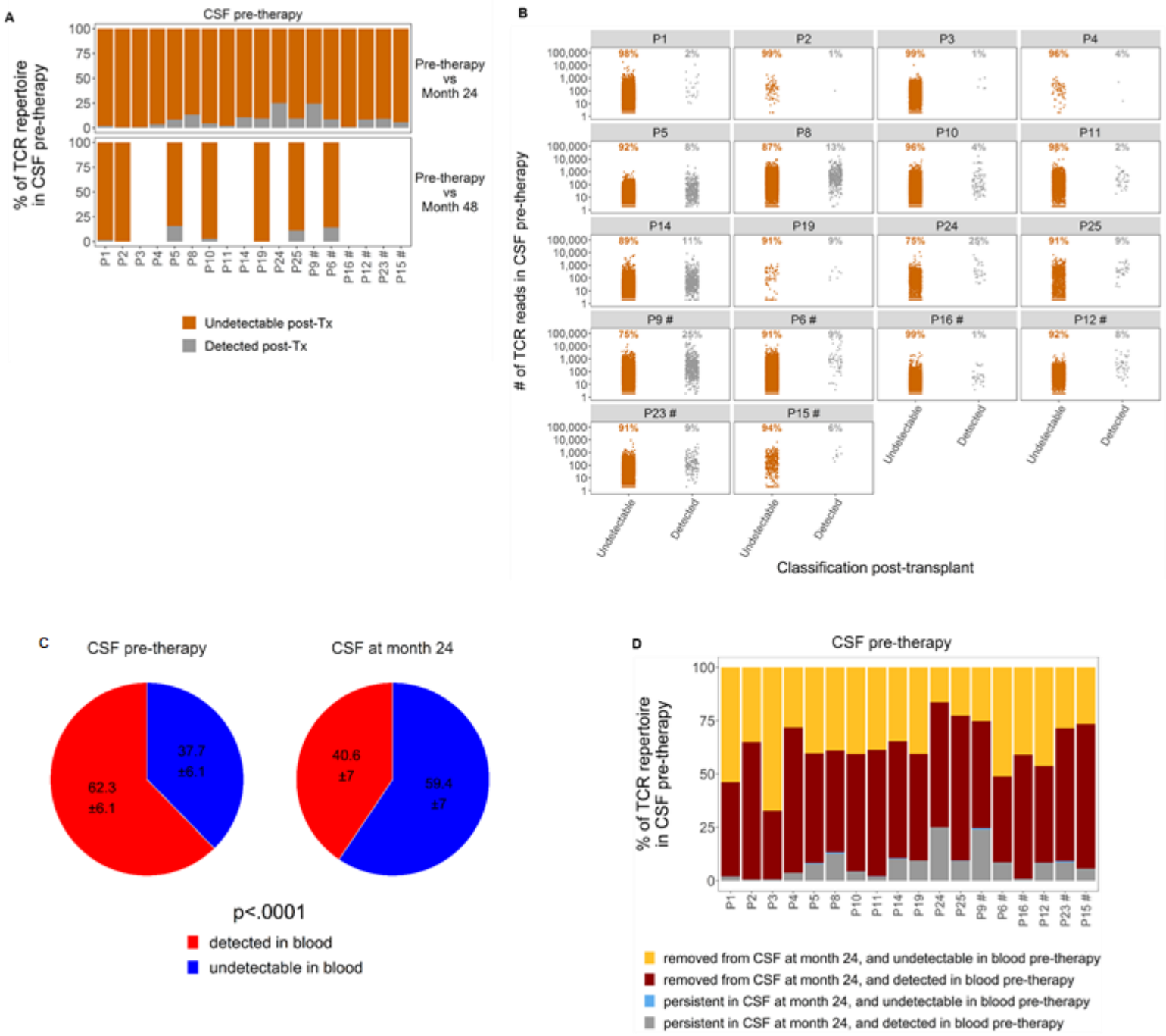

Figure 2. The pre-existing CSF T cell repertoire was substantially removed following AHSCT therapy. (A) TCR overlap percentage was used to determine the percent of clones that were undetectable (orange) versus detected (grey) by deep sequencing of TCR repertoires in CSF, comparing pre-therapy to months 24 or 48 for each participant. (B) Number of TCR reads for each clone in CSF pre-therapy that was undetectable (orange) or detectable (grey) in CSF at month 24 for each participant.

Percentages correspond to the relative proportion of the TCR repertoire in CSF pre-therapy in panel A. (C) Percent of clones in CSF that were either detected (red) or undetectable (blue) in peripheral blood $CD4^+$ or $CD8^+$ T cells pre-therapy versus at month 24 post-transplant. *$p<0.0001$ % CSF clones detected in blood at month 0 pre-therapy versus % CSF clones detected in blood at month 24 post-transplant using paired T-test. (D) TCR overlap analysis was used to determine the percent of clones that were undetectable versus detected in the TCR repertoire in CSF pre-therapy compared to month 24 post-transplant as in Figure 2A. The percent of these clones that were detected versus undetectable in pre-therapy peripheral blood $CD4^+$ or $CD8^+$ T cells was determined by ultra-deep sequencing, and clones in CSF pre-therapy were classified into one of four categories: removed from CSF at month 24, and either undetectable in blood pre-therapy (gold) or detected in blood pre-therapy (red); persistent in CSF at month 24 and either undetectable in blood (blue) or detected in blood pre-therapy (grey). # participants that met the primary endpoint for the HALT-MS study. All other participants stayed in remission from active MS until the last follow-up.

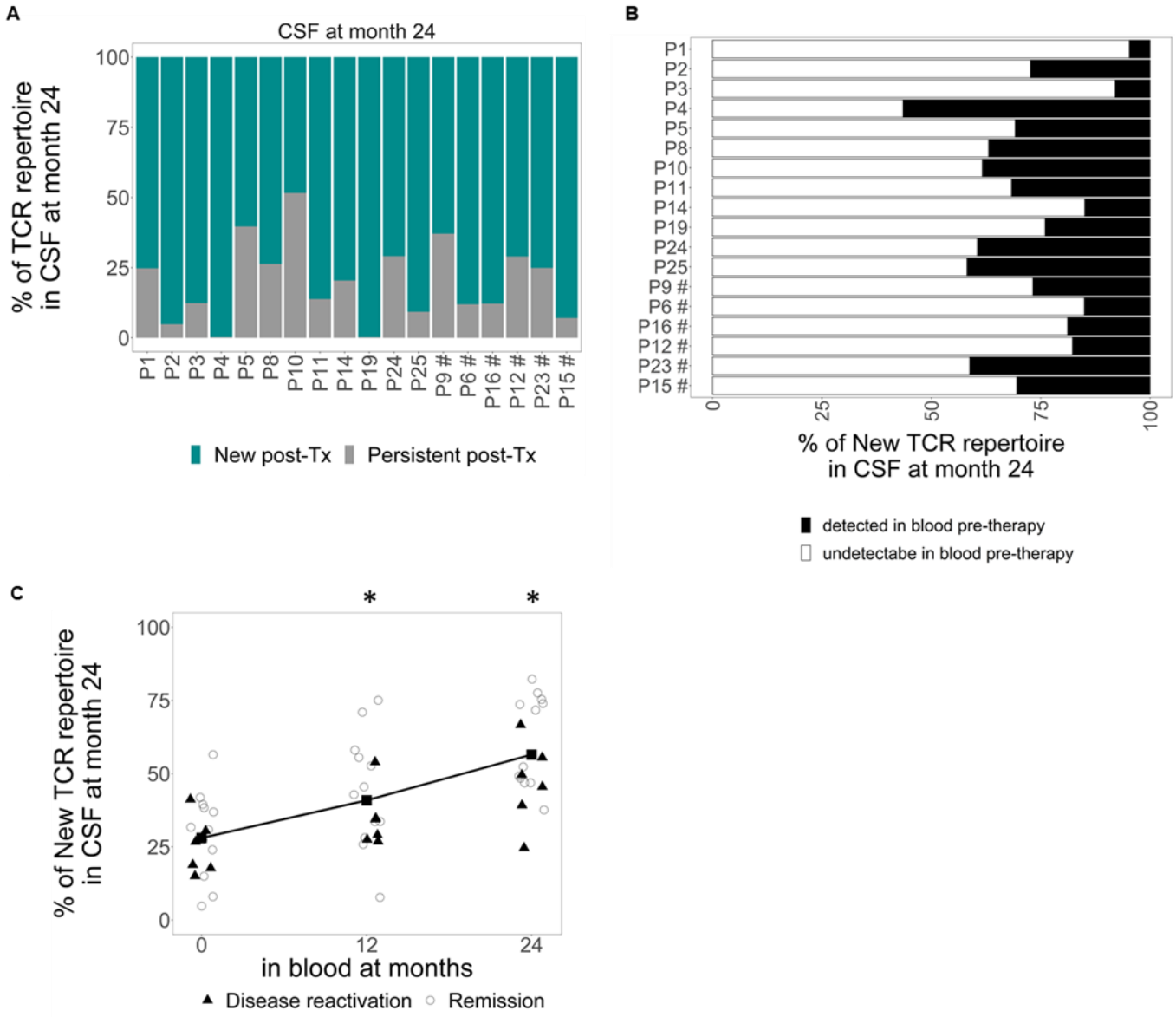

Figure 3. The CSF T cell repertoire was substantially replaced following AHSCT therapy with clonotypes originating predominantly from the graft. (A) TCR overlap percentage was used to determine the percent of clones that were undetectable, termed new (teal) versus detected, termed persistent (grey) in the CSF repertoire at month 24 compared to pre-therapy for each participant. (B) The percent of new clones in CSF at month 24 that were detected (black) versus undetectable (white) by ultra-deep sequencing in pre-therapy peripheral blood $CD4^+$ or $CD8^+$ T cells was determined. # participants that met the primary endpoint for the HALT-MS study.

All other participants stayed in remission from active MS until the last follow-up. (C) Longitudinal evaluation of new clones in CSF at month 24 as the percentage detected by ultra-deep sequencing in peripheral blood $CD4^+$ or $CD8^+$ T cells at the indicated time points. Filled triangles are participants that met the primary endpoint for the HALT-MS study before month 60 post-transplant, and open circles are participants that stayed in remission from active MS until the last follow-up. The line represents the mean of participants evaluated. *$p<0.05$ between month 0 pre-therapy versus months 12 and 24 post-transplant using mixed model for repeated measures.

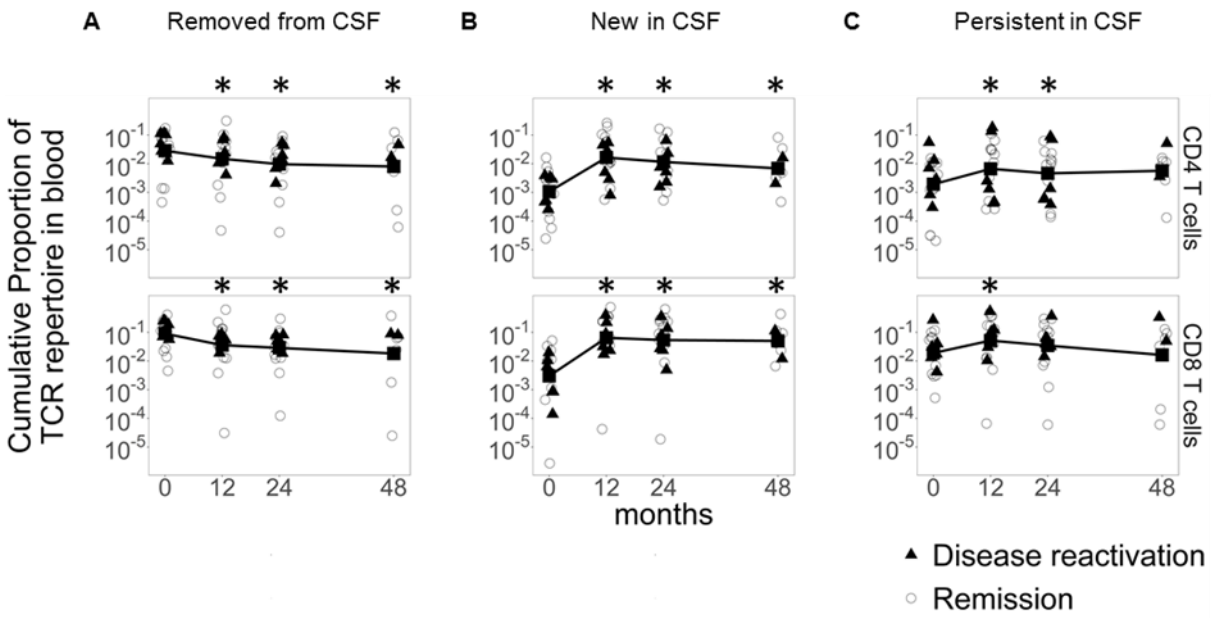

Figure 4. Longitudinal evaluation in blood of clonotypes detected in both CSF and CD4+ or CD8+ T cells in blood. Clonotypes classified as (A) pre-existing in CSF pre-therapy and undetectable in CSF at month 24, termed removed from CSF, (B) new in CSF at month 24 post-transplant, and (C) persisting in CSF at month 24 post-transplant. Proportions were aggregated per subject within circulating $CD4^+$ or $CD8^+$ T cell repertories pre-to-post transplant and then log-transformed. Filled triangles are participants that met the primary endpoint for the HALT-MS study before month 60 post-transplant, and open circles are participants that stayed in remission from active MS until the last follow-up. The line represents the mean of participants evaluated. *$p<0.05$ between month 0 pre-therapy versus months indicated post-transplant using mixed model for repeated measures.

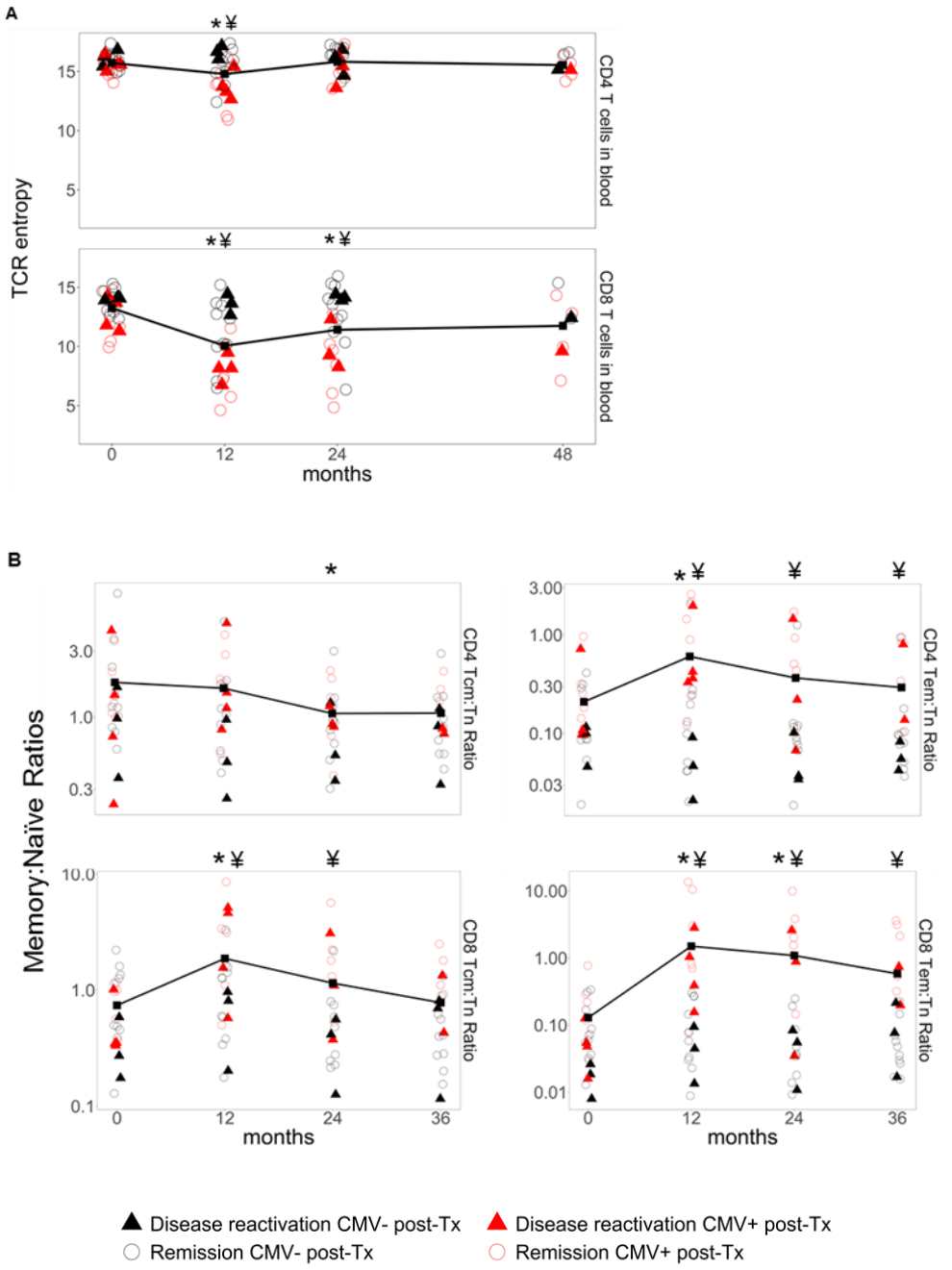

Figure 5. CMV infection post-AHSCT therapy is associated with impaired TCR diversification and an increased ratios of memory to naïve cell subsets in circulating T cells. (A) TCR repertoire diversity was analyzed using the Shannon entropy index in blood $CD4^+$ and $CD8^+$ T cells before therapy and at months 12, 24 and 48 post-transplant. (B) Ratios of circulating $CD4^+$ (top) and $CD8^+$ (bottom) central memory (Tcm) and effector memory (Tem) to naïve (Tn) cells were analyzed by flow

cytometry as previously reported (13). Filled triangles are participants that met the primary endpoint for the HALT-MS study before month 60 post-transplant, and open circles are participants that stayed in remission from active MS until the last follow-up. Participants were further stratified based on CMV infection status post-transplant. Black shapes are participants that were CMV negative, and red shapes are participants that were CMV positive, by PCR post-transplant. The line represents the mean of all participants evaluated at each time point. *$p<0.05$ between month 0 pre-therapy versus months indicated post-transplant using mixed model for repeated measures in panels A and B. ¥$p<0.05$ between CMV^+ vs. CMV^- groups post-transplant at each visit using mixed model for repeated measures in panels A and B.

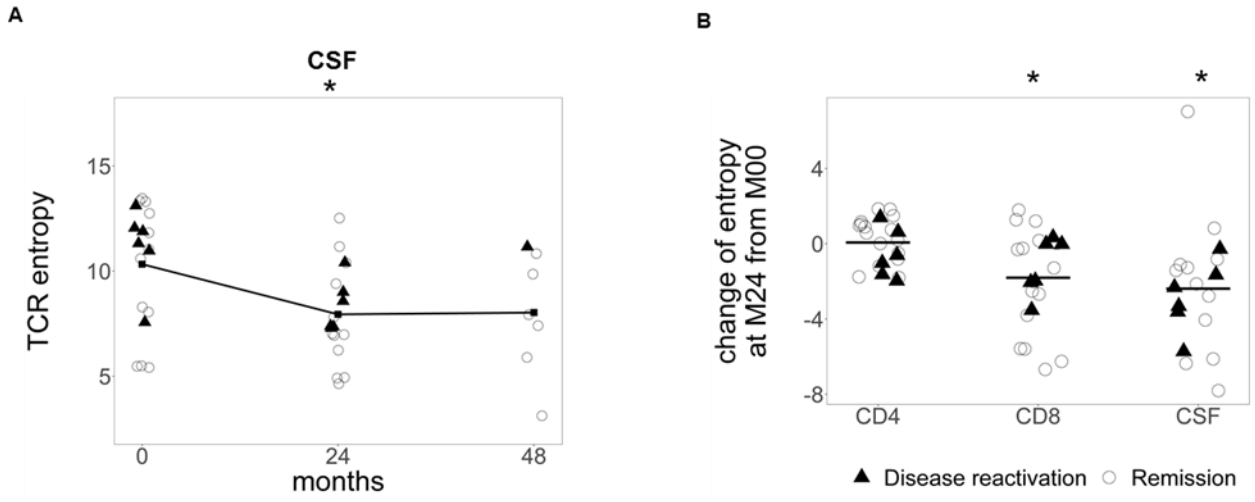

Figure 6. TCR diversification in CSF is reduced at month 24 post-AHSCT therapy. (A) TCR repertoire diversity was analyzed using the Shannon entropy index in CSF before therapy and at months 24 and 48 post-transplant. (B) The absolute change in TCR entropy at month 24 post-transplant from baseline was calculated for $CD4^+$ and $CD8^+$ T cells in blood (Figure 5A) and CSF. The line represents the mean change from baseline at month 24 for all participants evaluated. Filled triangles are participants that met the primary endpoint for the HALT-MS study before month 60 post-transplant, and open circles are participants that stayed in remission from active MS until the last follow-up. *$p<0.05$ between month 0 pre-therapy versus at month 24 post-transplant in panel A, and between the absolute change in TCR entropy at month 24 (M24) from month 0 (M00) for $CD4^+$ T cells in blood compared to $CD8^+$ T cells in blood or CSF cells in panel B.