Molecular monitoring of T-cell kinetics and migration in severe neurotoxicity after real-world CD19-specific chimeric antigen receptor T cell therapy

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Abstract

CD19-specific chimeric antigen receptor (CD19-CAR) T-cell therapies mediate durable responses in late-stage B-cell malignancies, but can be complicated by a potentially severe immune effector cell-associated neurotoxicity syndrome (ICANS). Despite broad efforts, the precise mechanisms of ICANS are not entirely known, and resistance to current ICANS-directed therapies (especially corticosteroids) has been observed. Recent data suggest that inflammatory cytokines and/or targeting of cerebral CD19-expressing pericytes can disrupt the blood-brain barrier and facilitate influx of immune cells, including CAR T cells. However, specific tools for CD19-CAR T-cell analysis within often minute samples of cerebrospinal fluid (CSF) are not broadly available. Here, we applied our recently developed digital polymerase chain reaction assays to monitor CD19-CAR T-cell kinetics in CSF and blood in real-world patients with neurotoxicity. Consistently, we observed a CAR T-cell enrichment within CSF in ICANS patients with further progressive accumulation despite intense corticosteroid-containing immuno-chemotherapies in a subset of patients with prolonged and therapy-resistant grade 3-4 neurotoxicity. We used next-generation T-cell receptor-β sequencing to assess the repertoire of treatment-refractory cells. Longitudinal analysis revealed a profound skewing of the T-cell receptor repertoire, which at least partly reflected selective expansion of infused T-cell clones. Interestingly, a major fraction of eventually dominating hyperexpanded T-cell clones were of non-CAR T-cell derivation. These findings hint to a role of therapy-refractory T-cell clones in severe ICANS development and prompt future systematic research to determine if CAR T cells may serve as 'door openers' and to further characterize both CAR-positive and non-CAR T cells to interrogate the transcriptional signature of these possibly pathologic T cells.

Introduction

CD19-specific chimeric antigen receptor (CD19-CAR) T-cell therapy mediates durable remission of relapsed and/or refractory (r/r) B-cell malignancies, which prompted approval of several licensed CAR T-cell products, including axicabtagene ciloleucel (axi-cel) and tisagenlecleucel (tisa-cel).[1-8] However, this promising treatment modality may be associated with serious toxicities, including a unique immune effector cell-associated neurotoxicity syndrome (ICANS).[9-12] The precise mechanisms mediating this potentially life-threatening condition have not been entirely elucidated, but accumulating evidence suggests that the cytokine release upon CAR T-cell activation and/or direct targeting of CD19-expressing cerebral pericytes induce vascular injury, which may facilitate migration of immune effectors, including CAR T cells, across the blood-brain-barrier.[9,11,13,14] High-dose corticosteroids are the first choice for ICANS-directed therapies, but fail in some patients.[10,11] Interleukin (IL)-1 pathway blockade (anakinra) or intrathecally (IT)-administered cytotoxic chemotherapies are used as second-line therapies, but optimal treatment of severe therapy-refractory ICANS has not been established.[10,11,15] ICANS monitoring largely relies on assessment of clinical status, imaging, and analysis of serum biomarkers.[10,11,16] However, to date systematic data on CAR T-cell kinetics within the cerebrospinal

fluid (CSF) are missing, which partly reflects the lack of broadly available diagnostic CAR-specific monitoring tools to easily examine the frequently scanty CSF specimen in the non-trial setting.[17,18] In our prior work, we developed and validated digital polymerase chain reaction (dPCR) assays for CD19-CAR T-cell tracking.[19-22] Here, we applied these assays to assess the CD19-CAR T-cell kinetics in diagnostic CSF samples and corresponding peripheral blood specimen from patients experiencing severe grade ≥3 neurotoxicity of CD19-CAR T-cell therapy. We found a preferential CAR T-cell enrichment within the CSF with further accumulation under ICANS-directed therapies. We used a previously established T-cell receptor-β (TRB) next-generation sequencing (NGS) assay[23] to examine the clonal composition and longitudinal evolution of the refractory cells in patients with prolonged ICANS despite intense steroid-containing immune-chemotherapies.

Methods

Patient treatment

We examined CAR T-cell kinetics in peripheral blood mononuclear cells (PBMC) and CSF in 48 consecutive patients with r/r B-cell malignancies treated with licensed CAR T-cell products (axi-cel or tisa-cel) after a cyclophosphamide- and fludarabine-based lymphodepleting regimen (described in the *Online Supplementary Appendix*).[8,21] The local ethic committee approved the study (PV7081). Written informed consent for the schedule of sample collection was obtained from all patients. The sample collection schedule is described in detail in the *Online Supplementary Appendix*. Cytokine release syndrome (CRS) and ICANS were graded according to guidelines of the American Society for Transplantation and Cellular Therapy (ASTCT) and managed as described in the *Online Supplementary Appendix*.

Digital polymerase chain reaction-based analysis of patient samples

Genomic DNA (gDNA) was isolated from aliquots of patients' PBMC, CSF, and the infusion product (IP) as described.[19,20,22] Briefly, PBMC were isolated from peripheral blood by density-gradient centrifugation using SepMate (Stem Cell Technologies, Cologne, Germany) following the manufacturer's instructions, washed, and resuspended in phosphate-buffered saline (PBS, Gibco, Thermo Fischer Scientific, Waltham, USA). Samples of CSF or the IP were centrifuged and the mononuclear cells (MNC) resuspended in phospate-buffered saline. Genomic DNA (gDNA) was extracted using QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Typically, aliquots of 100–120 ng gDNA, corresponding to 15,000–18,000 diploid genomes (cells), were subjected to the dPCR analysis using the previously described axi-cel-specific[19] or "universal" (axi-cel and tisa-cel-specific) dPCR assays.[20] All dPCR runs were performed as duplex reactions using the diploid hematopoietic cell kinase gene as reference.[19,20] The final concentrations of primers (900 nM) and probes (250 nM) followed Bio-Rad dPCR guidelines. In order to reduce sample viscosity and improve target accessibility, 5 units EcoRI (Thermo Fischer Scientific) were added for 5 minutes at room temperature to the reaction. The dPCR was carried out with the QX100 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, USA). Droplets were analyzed with the QX100 droplet reader and data processed with QuantaSoft_v1.7 software (Bio-Rad). Data were analyzed and visualized using Graphpad PRISM Software 8.4.3. (GraphPad Software, San Diego, USA). All reported *P* values are two-sided, and values <0.05 were considered statistically significant.

Fluorescent-activated cell sorting

Aliquots of cryopreserved PBMC or the IP were thawed and stained with a CD19-CAR reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions. Briefly, cells were stained for 10 minutes with the CAR reagent, washed twice with fluorescent-activated cell sorting (FACS) buffer (PBS containing 2% fetal bovine serum, Sigma-Aldrich GmbH, Taufkirchen, Germany), and then stained for 15 minutes with a fluorochrome-conjugated anti-CD3 antibody and anti-biotin reagent (Miltenyi Biotec). T cells were sorted on a FACSAria_IIIu (BD Biosciences, San Jose, USA) into CAR-positive and CAR-negative subsets, washed with PBS, and used for gDNA isolation.

T-cell receptor-β amplification and Illumina-based next-generation sequencing

Aliquots of gDNA obtained from samples of PBMC, CSF, or the IP underwent unbiased amplification of the TRB rearrangement using BIOMED2-TRB primer pools as described[23] and detailed in the *Online Supplementary Appendix*. Amplicons were barcoded, sequenced on an Illumina MiSeq sequencer (San Diego, USA) and analyzed using the MiXCR software as described previously,[23] and as described in the *Online Supplementary Appendix*.

Results

In our prior work, we treated 21 consecutive r/r B-cell lymphoma patients with axi-cel after a cyclophosphamide- and fludarabine-based lymphodepleting therapy in a non-trial setting (March 2019-July 2020).[12,21] Since then (until May 2022), additional 27 consecutive patients with advanced B-cell malignancies received axi-cel (n=14) or tisa-cel (n=13). In the entire cohort, the overall incidence of grade ≥1 CRS

after axi-cel treatment was 85.7% (grade ≥3 in 14.3%) and 84.6% (grade ≥3 in 15.4%) after tisa-cel. The incidence of ICANS (≥1) was 57.1% (grade ≥3 in 20%) in patients receiving axi-cel, and 38.5% (grade ≥3 in 23.1%) after tisa-cel (Table 1). The median day of ICANS grade 3-4 onset was day 4.5 (range, 0-10), and the median duration was 13.5 days (range, 5-60 days).

Proposed biomarkers associated with high risk of ICANS include measures of tumor burden such as baseline lactate dehydrogenase (LDH), inflammatory markers (IL-6 peak levels), and/or CAR T-cell peak levels.[11,24,25] In our small cohort, we found statistically higher pretreatment LDH values (P<0.0001) in patients experiencing grade 4 neurotoxicity compared to those without ICANS (*Online Supplementary Figure S1A*). Also, peak IL-6 levels tended to be higher in grade ≥3 ICANS patients, although this was not statistically significant (*Online Supplementary Figure S1B* and *C*). Similarly, the patients with grade ≥3 ICANS showed a trend towards higher grades of CRS (*Online Supplementary Figure S1D*). In our prior work in the first 21 axi-cel patients of this cohort, we found that higher CAR T-cell peak values were significantly associated with favorable efficacy with a trend towards increased neurotoxicity.[21] In the here updated entire axi-cel cohort (n=35 patients), we found a similar trend with regard to the incidence of neurotoxicity (grade 0 *vs.* grades 1-4 ICANS), with significantly higher CAR T-cell peak levels in the subgroup of patients with grades 2-4 ICANS compared to grades 0-1 ICANS (P=0.0142, Figure 1A). In this small cohort of patients, the circulating axi-cel persistence was low in the single-one grade 4 ICANS patient (patient #03, Figure 1B; *Online Supplementary Figure S2*) and he recovered fully within 24 hours without ICANS-specific treatment, making alternative neurological pathologies such as a transient ischemic attack very probable. This observation might also reflect the complex and multifactorial nature of the ICANS pathogenesis, in which tumor burden, inflammation, and/or severity of CRS have also been identified as important contributing factors.[9-11]

Consistent enrichment of CAR T cells within cerebrospinal fluid after real-world CAR therapy

Ten of 48 patients in this cohort experienced grade ≥3 neurotoxicity (Table 1). The patient characteristics and outcomes are described in the *Online Supplementary Table S1*. We received aliquots of single or serial diagnostic CSF specimen that were obtained for clinical purposes from eight patients (*Online Supplementary Appendix*), including five of seven axi-cel treated patients and three of three tisa-cel-treated patients (Figure 2A). Using our recently developed and validated dPCR assays,[19-22] we first determined whether the persisting CAR T cells proliferated and/or accumulated preferentially in the central nervous system. In all patients, we observed a relative enrichment of CAR-positive T cells on a per million basis within the CSF as compared to the PBMC sample with a median 25.8-fold peak increase (range, 1.5-124.7) in the CSF (Figure 2B). Even though the absolute numbers of leukocytes and CAR T cells were higher in the peripheral blood, we observed a significantly higher (P=0.0073) proportion of CAR T cells among leukocytes within the CSF *versus* the peripheral blood (*Online Supplementary Table S2*; Figure 2C). In order to determine whether CAR T cells are detectable within the CSF in the absence of severe ICANS, we also examined the CAR T-cell kinetics in a subset of eight patients with grades 0-2 ICANS undergoing

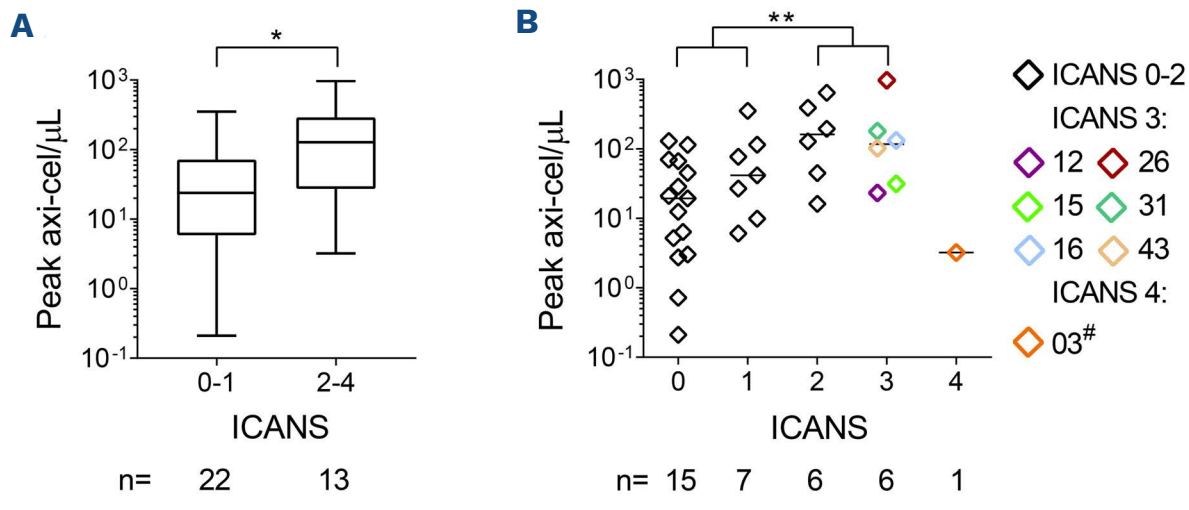

Figure 1. Chimeric antigen receptor T-cell peak levels in correlation with immune effector cell-associated neurotoxicity syndrome. 35 consecutive relapsed and/or refractory (r/r) B-cell lymphoma patients were treated with axi-cel in the non-trial setting and examined for neurotoxicity. Samples of peripheral blood mononuclear cells (PBMC) were obtained after the chimeric antigen receptor (CAR) T-cell infusion and analyzed by digital polymerase chain reaction for the presence of CAR T cells. (A) Boxplots showing the axi-cel peak levels in correlation with immune effector cell-associated neurotoxicity syndrome (ICANS). Boxplots show median (line), 25th and 75th percentiles (upper and lower box borders), and range (whiskers). (B) Individual axi-cel peak values in correlation with ICANS. The color-coded symbols identify patients with ≥grade 3 ICANS. #: Patient #03 recovered fully within 24 hours without ICANS-specific treatment, making alternative neurological pathologies very probable. Statistical significance: *P≤0.05; **P≤0.01.

Table 1. Incidence and treatment of cytokine release syndrome and neurotoxicity after real-world CD19-CAR T-cell therapy.

	CD19-CAR total		axi-cel		tisa-cel	
	N	%	N	%	N	%
Patients	48	100	35	100	13	100
Indication						
DLBCL*	44	91.7	32	91.4	12	92.3
PMBCL	3	6.3	3	8.6	0	0.0
B-ALL	1	2.1	0	0.0	1	7.7
CNS Involvement	5	10.4	4	11.4	1	7.7
CRS						
No CRS	7	14.6	5	14.3	2	15.4
CRS 1	11	22.9	7	20.0	4	30.8
CRS 2	23	47.9	18	51.4	5	38.5
CRS 3	6	12.5	5	14.3	1	7.7
CRS 4	1	2.1	0	0.0	1	7.7
CRS, death	1	2.1	0	0.0	1	7.7
ICANS						
No ICANS	23	47.9	15	42.9	8	61.5
ICANS 1	9	18.8	7	20.0	2	15.4
ICANS 2	6	12.5	6	17.1	0	0.0
ICANS 3	6	12.5	6	17.1	0	0.0
ICANS 4	4	8.3	1	2.9	3	23.1
ICANS, death	1	2.1	0	0.0	1	7.7
Treatment						
Dex/Tocilizumab	30	62.5	22	62.9	8	61.5
Anakinra	9	18.8	4	11.4	5	38.5
IT-chemotherapy	4	8.3	3	8.6	1	7.7

Axi-cel: axicabtagene ciloleucel; B-ALL: B-cell acute lymphatic leukemia; CAR T cell: chimeric antigen receptor T cell; CNS: central nervous system; CRS: cytokine release syndrome; Dex: dexamethasone; DLBCL: diffuse large B-cell lymphoma; ICANS: immune effector cell-associated neurotoxicity syndrome; IT-chemotherapy: intrathecal chemotherapy consisting of dexamethasone, cytarabine, and methotrexate; PMBCL: primary mediastinal B-cell lymphoma; tisa-cel: tisagenlecleucel. *One axi-cel treated patient was diagnosed with a progressive follicular lymphoma/DLBCL.

strictly medically indicated lumbar punctures (LP) for neurological symptoms (*Online Supplementary Appendix*; *Online Supplementary Table S3*; *Online Supplementary Figure S3A*). In line with the approval by the local ethic committee, we were able to use aliquots of those CSF samples for the molecular monitoring (*Online Supplementary Appendix*). An increased frequency of CAR T cells within the CSF compared to the peripheral blood (median, 6.2-fold; range, 0.3-106.8) was also detectable in patients with low grade ICANS and in one patient without ICANS with a diagnostic LP in the context of a grade 2 CRS (*Online Supplementary Figure S3B*; *Online Supplementary Table S3*). Also, the proportion of CAR-T cells among leukocytes was significantly higher in the CSF compared to the peripheral blood (*Online Supplementary Table S2*; *Online Supplementary Figure 3C*; P=0.0016). These results indicate that CAR T cells can be enriched in the CSF regardless of the severity of ICANS. We did not find any significant association of the CAR T-cell enrichment in CSF with the duration or severity of ICANS, responses to ICANS-directed therapies, or active or history of cerebral lymphoma (*Online Supplementary Figure S4*).

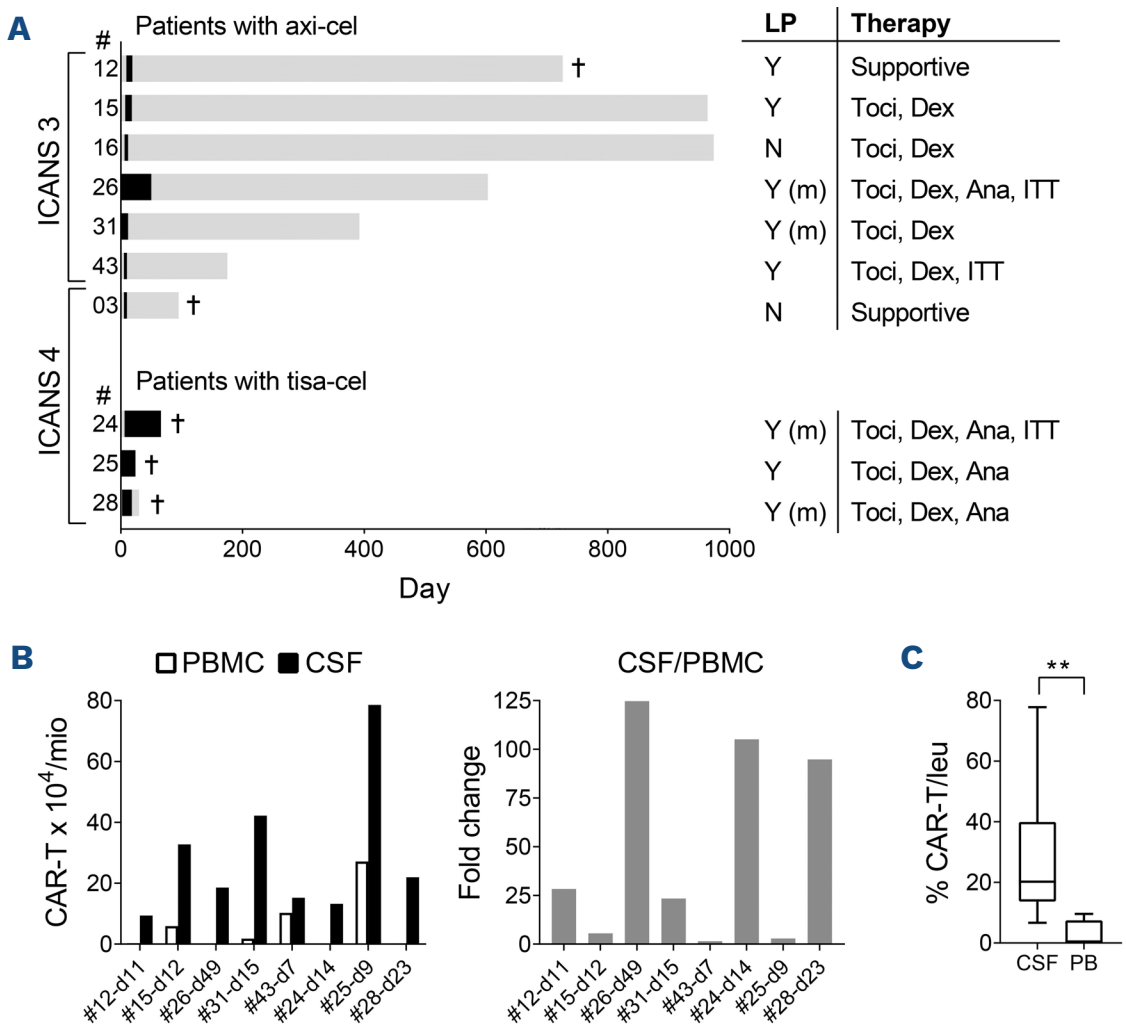

Figure 2. Chimeric antigen receptor T-cell enrichment within the cerebrospinal fluid in real-world patients with severe neurotoxicity. (A) Schematic overview of axi-cel or tisa-cel treated patients with grade ≥3 immune effector cell-associated neurotoxicity syndrome (ICANS). Days with ICANS (grades 1-4) are highlighted in black. Ana: anakinra; Dex: dexamethasone; LP: lumbar puncture; m: multiple; N: no; Toci: tocilizumab; ITT: intrathecal therapy consisting of Dex, cytarabine, and methotrexate; Y: yes; †: deceased. Updated on May 31st, 2022. (B and C) Detection of chimeric antigen receptor (CAR) T cells in peripheral blood mononuclear cells (PBMC) and diagnostic cerebrospinal fluid (CSF) specimen in patients with grade ≥3 ICANS. Aliquots of diagnostic CSF specimen and PBMC were obtained at indicated days and examined by digital polymerase chain reaction (dPCR) for the presence of CAR T cells. (B) Left panel: frequency of CAR-positive cells per million mononuclear cells (MNC) in corresponding samples of PBMC (□) and CSF (■). Right panel: Fold change (CSF *vs.* PBMC). Shown are the results on the day (d) of the maximal change if multiple samples were obtained. (C) Proportion of absolute CAR T-cell numbers among leukocytes (leu) within the CSF *vs.* the peripheral blood (PB). Statistical significance: $^{**}P \leq 0.01$.

Sustained CAR T-cell persistence despite glucocorticoid-containing therapies

Recent reports demonstrated rather detrimental effects of intense-steroid treatment with poorer outcome after CAR T-cell therapy as well as the failure to ablate CAR T cells in the peripheral blood in patients with CAR-toxicities.[26-28] However to date, data on CAR T-cell kinetics within CSF, especially in the context of glucocorticoid-containing therapies, are missing. The majority of the ICANS patients received systemic steroid-containing ICANS-directed treatment and seven of them underwent serial LP (Figure 2A; *Online Supplementary Figure S3*; *Online Supplementary Tables S1* and *S3*) for diagnostic or therapeutic purposes solely based on strong medical indication (*Online Supplementary Appendix*). Left-over material provided an opportunity to examine CAR T-cell frequencies in serial PBMC samples and diagnostic CSF specimens and to address the impact of glucocorticoid-containing treatment on the T-cell kinetics. As previously implicated,[26,27] corticosteroids did not prevent the engraftment in the peripheral blood or CSF. We observed a median 14.3-fold increase of CAR T-cell numbers per μL despite Dex treatment in serial PBMC specimen (range, 0.9-96,698) as well as in diagnostic CSF samples (median 2.7-fold; range, 0.9-35.8) after transfer. The steroid-treatment did not cause ablation of CAR T cells, but the gene-modified T cells remained detectable at only slightly reduced levels within the peripheral blood (Figure 3A and D). We also found a sustained enrichment of the frequency of CAR T cells within the CSF as compared to the PBMC sample, as indicated by higher frequencies early during steroid-treatment, with further increase to up to 95-fold levels (Figure 3B and E). As before, although the absolute CAR T-cell numbers per μL were generally higher in blood compared to CSF (Figure 3A and D), reflecting overall higher total leukocyte counts, we observed that the proportion of axi-cel or tisa-cel T cells among the leukocytes remained far higher over time within CSF compared to the peripheral blood (Figure 3C and F).

Development of treatment-refractory neurotoxicity despite intense immune effector cell-associated neurotoxicity syndrome-directed immuno-chemotherapies in a small subset of patients

Continued clinical follow-up showed that the ICANS-directed therapies mediated a resolution of the neurotoxicity in the majority of our patients (Figure 2A). However, two patients (#24 and #26) of our cohort experienced prolonged and severe courses of disease despite intense ICANS-directed treatment, including prolonged corticosteroids, anakinra, and repeated IT administration of chemotherapeutic regimen consisting of Dex, cytarabine, and methotrexate (*Online Supplementary Table S1*).[15] Even though all efforts were made to restrict the number of LP, both patients repeatedly underwent this procedure for different medical reasons including diagnostic purposes, drug administration, and/or other therapeutic interventions (for example to release pressure). In order to facilitate the procedures, patient #24 received an implanted Rickham reservoir on day 19 after the CAR T-cell infusion. Analysis of the CAR T- cell kinetics in serial PBMC and CSF samples in patient #26 showed an initial decline of detectable CAR T cells after the IT chemotherapy (Figure 4A and B), but the CAR T cells continued to persist at up to 125-fold increased frequencies per million cells analyzed in CSF *versus* PBMC throughout the follow-up (Figure 4B). This also translated in a far higher proportion of CAR T cells among leukocytes in the CSF *versus* the PB (Figure 4C). Similarly, in patient #24, there was a prolonged CAR T-cell persistence despite the intense treatment (Figure 4E). We observed a 2-fold enrichment of the absolute frequency of CAR-positive cells within CSF at the start of the ICANS-directed treatment, which further increased to up to 105-fold enriched levels in CSF *versus* PBMC by day 14 (Figure 4F). This was also accompanied by an increased proportion of absolute CAR T cells among leukocytes within CSF *versus* PB (Figure 4G). Continued ICANS-directed treatment including an IT chemotherapy mediated some decline of the detectable CAR T cells, but they remained present at >20-fold enriched frequencies over time compared to peripheral blood (Figure 4F). In this non-trial setting, we also examined selected inflammatory

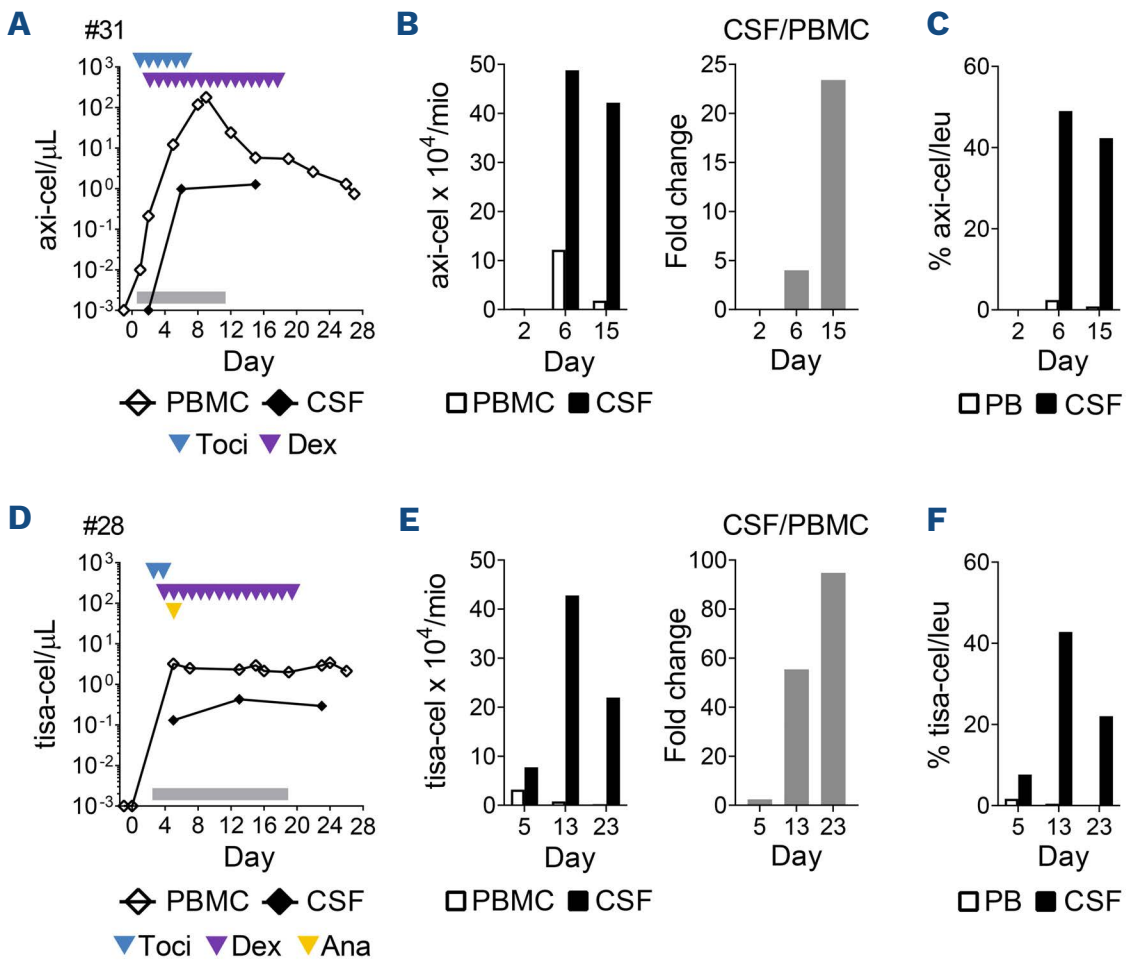

Figure 3. Sustained chimeric antigen receptor T-cell persistence and enrichment despite glucocorticoid containing immune effector cell-associated neurotoxicity syndrome-directed therapy. Chimeric antigen receptor (CAR) T-cell kinetics in serial samples of peripheral blood mononuclear cells (PBMC) and cerebrospinal fluid (CSF) in patients with grade ≥3 neurotoxicity receiving immune effector cell-associated neurotoxicity syndrome (ICANS)-directed therapies. Aliquots of PBMC and CSF were obtained at indicated days and examined by digital polymerase chain reaction (dPCR) for the presence of CAR T cells. (A and D) Shown are absolute numbers of circulating CAR T cells in PBMC (◇) and CSF (◆). The arrow heads indicate the type and times of the cytokine release syndrome (CRS) or ICANS-directed treatments: blue: tocilizumab (Toci); purple: dexamethasone (Dex); yellow: anakinra (Ana). The grey horizontal bars show the duration of ICANS. (B and E) Left panels: absolute frequencies of CAR-positive cells per million mononuclear cells (MNC) in PBMC (□) and CSF (■) on the respective day. Right panels: fold change (CSF *vs.* PBMC). (C and F) Proportion of absolute CAR T-cell numbers among leukocytes (leu) within the CSF *vs.* the peripheral blood (PB) on the indicated days after the CAR T-cell infusion.

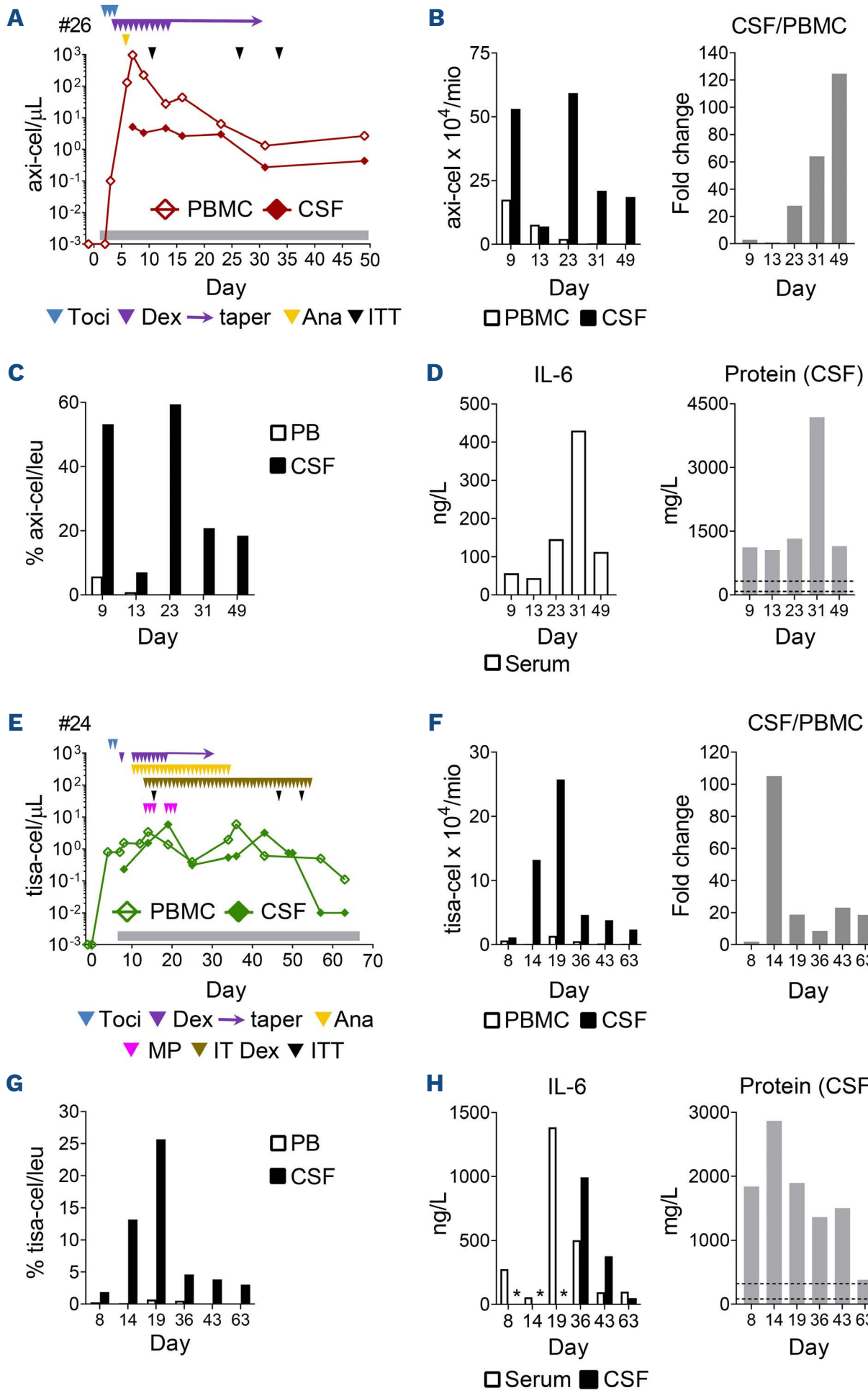

Figure 4. Sustained chimeric antigen receptor T-cell enrichment within the cerebrospinal fluid in 2 patients with prolonged treatment-refractory severe immune effector cell-associated neurotoxicity syndrome. (A and E) Chimeric antigen receptor (CAR) T-cell kinetics. Serial samples of peripheral blood and diagnostic cerebrospinal fluid (CSF) specimen were examined by digital polymerase chain reaction for the presence of CAR T cells. (A) Absolute numbers of circulating CAR T cells in peripheral blood mononuclear cells (PBMC) (◇) and CSF (◆) in (A) patient #26 undergoing axi-cel therapy and (E) patient #24 receiving tisa-cel. The arrow heads indicate type and times of the cytokine release syndrome (CRS)- or immune effector cell-associated neurotoxicity syndrome (ICANS)-directed treatments: blue: tocilizumab (Toci); purple: dexamethasone (Dex); yellow: anakinra (Ana); magenta: methyl-prednisone (MP); brown: IT-Dex; black: intrathecal therapy (ITT) with Dex, cytarabine, and methotrexate. The grey horizontal bars show the duration of ICANS. (B and F) Left panels: Absolute frequency of CAR-positive cells per million mononuclear cells (MNC) in PBMC (□) and CSF (■) on indicated days in (B) patient #26 or (F) patient #24. Right panels: fold change (CSF *vs*. PBMC). (C and G) Proportion of absolute CAR T-cell numbers among leukocytes in CSF *vs.* PB in (C) patient #26 and (G) patient #24. (D and H) Analysis of inflammatory marker (IL-6, protein) in (D) patient #26 and (H) patient #24.

marker such as IL-6 and/or total protein, respectively, and detected in both patients elevated levels in CSF compared to the serum sample (Figure 4D and H). However, high levels of inflammatory marker in CSF were also detected in patients with lower grade ICANS (*Online Supplementary Table S5*). Whereas the ICANS symptoms gradually resolved over time in patient #26, patient #24 experienced a prolonged treatment-resistant grade 4 ICANS until her death due to progression of lymphoma (Figure 2A, *Online Supplementary Table S1*). Thus, even very intense systemic and/or local ICANS-directed treatment may fail to mediate timely resolution of neurotoxicity in a subset of patients, which coincides with a continued persistence of possibly pathogenic T cells in the CSF.

Treatment-refractory immune effector cell-associated neurotoxicity syndrome concurs with profound clonal T-cell receptor-β skewing

The sustained T-cell persistence in CSF despite prolonged intense therapies in patients #24 and #26 was surprising, but could reflect the selected outgrowth of single treatment-refractory T-cell clones. We used a previously established TRB-NGS assay[23] to examine the clonal composition of T cells in the CSF of the aforementioned two patients. Our longitudinal analysis of changes in the T-cell receptor (TCR) repertoire revealed a diverse distribution in CSF and blood at the onset of ICANS, with an increasingly skewed repertoire in CSF *versus* blood during the ICANS-directed treatment. In fact, only 19 hyperexpanded clones accounted for 88% of the TRB repertoire within the CSF sample of patient #26 (Figure 5A), 49 days after the initial diagnosis of ICANS and 48 days after the initiation of intense ICANS-directed treatment (Figure 2A and 4A). Similarly, patient #24 showed a high diversity of the T-cell repertoire within the CSF at the onset of ICANS, but a profound progressive TRB skewing compared to the corresponding samples of PBMC over time, especially at later time points. In this patient, only 15 hyperexpanded clones occupied 94% of the clonal space within the CSF by day 63 (Figure 5C; *Online Supplementary Table S6*), after 55 days of ICANS-directed treatment (Figure 2A and 4E), whereas the corresponding PBMC sample showed a high level of diversity. This was also reflected by corresponding changes in the inverse Simpson index (Figure 5B and D),

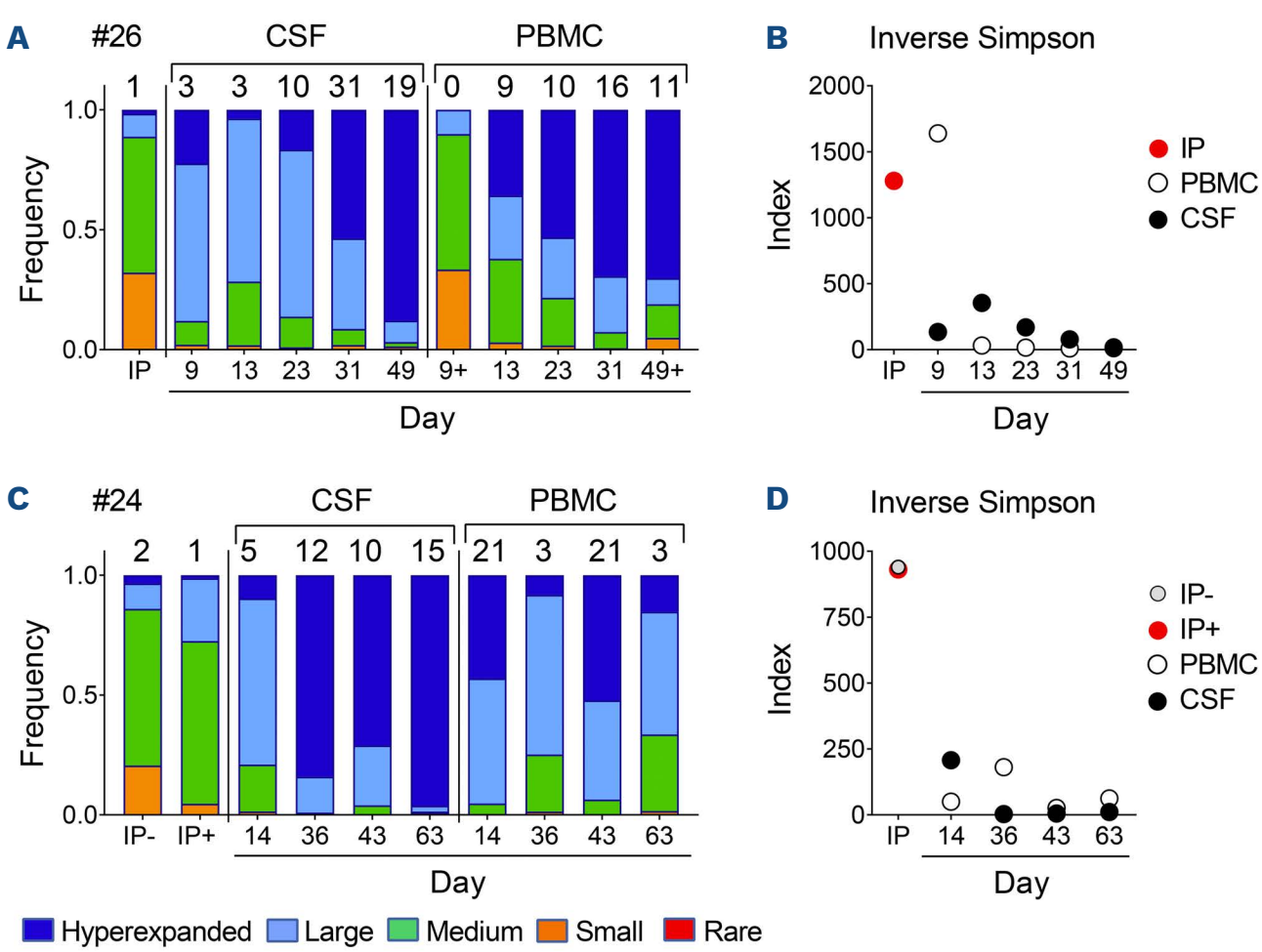

Figure 5. T-cell immune-profiling by T-cell receptor-β next-generation sequencing reveals clonal T-cell repertoire skewing within the cerebrospinal fluid in 2 patients with prolonged neurotoxicity despite intense immune effector cell-associated neurotoxicity syndrome-directed therapies. (A and C) Analysis of the clonal space distribution in (A) patient #26 undergoing axi-cel therapy and (C) patient #24 receiving tisa-cel. Genomic DNA was extracted from aliquots of the infusion product (IP), cerebrospinal fluid (CSF), or peripheral blood mononuclear cells (PBMC), either unsorted or sorted in a chimeric antigen receptor (CAR)-positive (+) or non-CAR (-) subset and examined by T-cell receptor-β next-generation sequencing (TRB-NGS). The panels demonstrate the clonal space distribution on the indicated days. Shown is the frequency of rare ($0 \leq X \leq 1^{-05}$; red bars), small ($1^{-05} \leq X \leq 1^{-04}$; orange bars), medium ($1^{-04} \leq X \leq 1^{-03}$; green bars), large ($1^{-03} \leq X \leq 0.01$; blue bars), and hyperexpanded clones ($0.01 \leq X \leq 1$; dark blue bars). The inset values above the bars represent the number of hyperexpanded clones. (B and D) Corresponding repertoire metrics of the TRB-NGS analysis shown as inverse Simpson index.

which is a measure for diversity. The total number of recovered cells for both patients are shown in *Online Supplementary Table S4*. Importantly, we did not observe similar changes in the clonal space distribution within CSF or peripheral blood in patients with low grade or no ICANS, respectively (*Online Supplementary Figure S5* and *S6*).

We next examined the fine-specificity of the hyperexpanded clones and their longitudinal evolution over time. In patient #24, we detected a total of 33 unique hyperexpanded clones within the CSF between days 14-63; not all of them persisted throughout all time points, though (*Online Supplementary Figure S7*). When we analyzed the infused T-cell product, eight of those clones that we detected in the CSF could be directly traced to the CAR-positive subset and 11 to the non-CAR T-cell fraction, with some being present in both subsets (Figure 6A and B). Interestingly, as few as five unique T-cell clones were shared at all time points within the CSF (Figure 7A) and detectable at increasing frequencies in the CSF *versus* the corresponding peripheral blood sample and/or the IP (Figure 7A) during the intense ICANS-directed treatment. Similarly, only single refractory clones were detectable within the CSF and/or blood in patient #26 (Figure 7B). Collectively, the fine-specific longitudinal analysis of the TCR repertoire within the CSF *versus* the peripheral blood suggested both, the robust proliferation or accumulation (up to 1,200-fold) of unique T-cell clones within the CSF, as well as the progressive outgrowth of a few treatment-refractory clones within the CSF over time.

Discussion

Adoptive CAR T-cell therapy targeting CD19 has recently emerged as a novel treatment modality for r/r B-cell tumors and demonstrated an impressive potential to induce durable responses of advanced disease.[1-8] However, CAR T-cell therapy can be accompanied by deleterious side effects, including neurological toxicities, which may limit the successful outcome of the approach in a subset of patients.[9-11] CAR T cells belong to a unique new entity of 'living drugs', which exert unpredictable and patient-specific *in vivo* kinetics. Efforts have been made to assess the circulating CAR T-cell engraftment in the peripheral blood in the non-trial setting.[19-22,29] However, to date still very little is known about the specific CAR T-cell kinetics within the CSF in these patients. Here, we set out to examine the migration and proliferation of CD19-CAR T cells within the CSF of patients experiencing ICANS after real-world axi-cel or tisa-cel therapy. We demonstrated a consistent enrichment of the frequency of CAR-T cells within the CSF in our patients at ICANS onset and further accumulation during ICANS-directed therapies in comparison with peripheral blood. In a small subset of patients with treatment-refractory ICANS, we found a profound progressive skewing of the TCR repertoire with an outgrowth of very few resistant clones. Notably, these clones were derived from the CAR-positive and non-CAR subset of the IP and proliferated and/or accumulated profoundly *in vivo*.

Prior published results of CD19-CAR therapies demonstrated manageable toxicities with a considerable subset of ~40-67% of patients experiencing neurotoxicities.[9-11] The observed incidence and outcome of ICANS in our non-trial cohort was in the expected range, and in line with prior research we detected a trend towards higher tumor burden and CAR T peak levels in more severe ICANS patients.[11,25] Thus, the identification of prophylactic strategies to prevent the development of ICANS, including thorough clinical and immune monitoring as well as early intervention, continues to have a high priority to further improve patient outcomes.

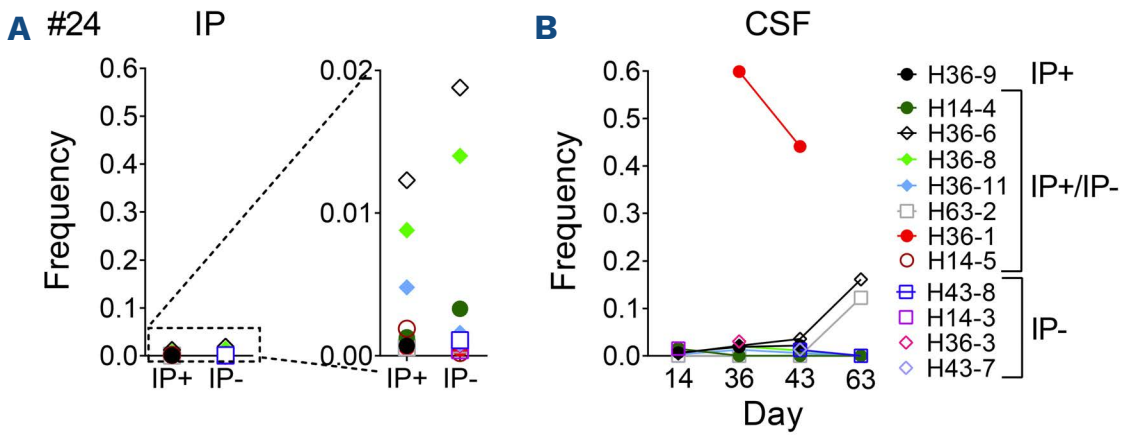

Figure 6. Hyperexpanded T-cell clones can be traced back to the infused T-cell product and proliferate strongly *in vivo*. Analysis of the T-cell receptor (TCR) repertoire in patient #24 experiencing a treatment-resistant grade 4 neurotoxicity. Genomic DNA was extracted from (A) the chimeric antigen receptor (CAR)-positive (infusion product positive [IP+]) and non-CAR (IP-) subset of the infused T-cell product and (B) serial diagnostic cerebrospinal fluid (CSF) samples, and then examined by T-cell receptor-β next-generation sequencing (TRB-NGS). Shown are the frequencies of 12 hyper-expanded clones that were both detectable in the CSF (B) between days 14-63 and could also be traced back to the CAR-positive (IP+) and/or non-CAR (IP-) subset of the infused T-cell product (A).

An important finding of our work is the consistent CAR T-cell increase within the CSF in our ICANS patients with a progressive enrichment despite steroid-containing ICANS-directed therapies. Glucocorticoids have been thought to serve as lymphotoxic drugs, but prior studies indicated that steroids fail to restrict the persistence of transferred T cells, including CAR T-cells, in the peripheral blood.[26,27,30] Additionally, recently published work by Strati *et al*. demonstrated detrimental effects of corticosteroids after CAR T-cell therapy and showed that higher cumulative doses of corticosteroids, as well as a prolonged or early use after CAR T-cell infusion were associated with significantly shorter patient survival and poor outcome.[28] In line with these data, we observed in our patients with severe ICANS that corticosteroids may not mediate ablation of CAR T cells and/or resolution of neurotoxicity. Importantly, we extend this work and show for the first time the effects of steroid-based ICANS-directed therapies on the CAR T-cell kinetics within the CSF. However, a limitation of this work in the non-trial setting is, in part due to the inherent ethical concerns, the lack of a cohort of ICANS patients not treated with corticosteroids that could serve as formal control group. Thus, systematic studies in appropriate *in vitro* or *in vivo* model systems are urgently needed.[31-33] The consistently more profound accumulation of CAR T cells in the CSF *versus* peripheral blood highlights the importance of the development of effective tools to continue to monitor and control the cell fate after adoptive transfer.

Our extensive analysis of the available samples in selected patients showed that the prolonged severe ICANS symptoms coincided with the outgrowth of several treatment-refractory T-cell clones. To the best of our knowledge, this is the first demonstration of a progressive skewing of the TCR repertoire and longitudinal fine-specific tracking of individual T-cell clones in therapy-resistant severe ICANS. The observation that the clonal diversity within the CSF is more restricted compared to the peripheral blood suggest a role of a CSF-specific environment during severe ICANS, as suggested in prior research.[10,11,13,24] A prior study of Shah *et al.*, examined the phenotype of the white blood cells within the CSF in a patient with a steroid-refractory CAR T-cell associated neurotoxicity syndrome.[15] The flow cytometric analysis of a single time point (day 13) in that patient revealed, unexpectedly, the predominant presence of a non-CAR CD4$^+$ T-cell population within the CSF. In line with these results, we found that the dominating treatment-refractory clones were at least in part derived from the CAR-negative T-cell fraction. The observation

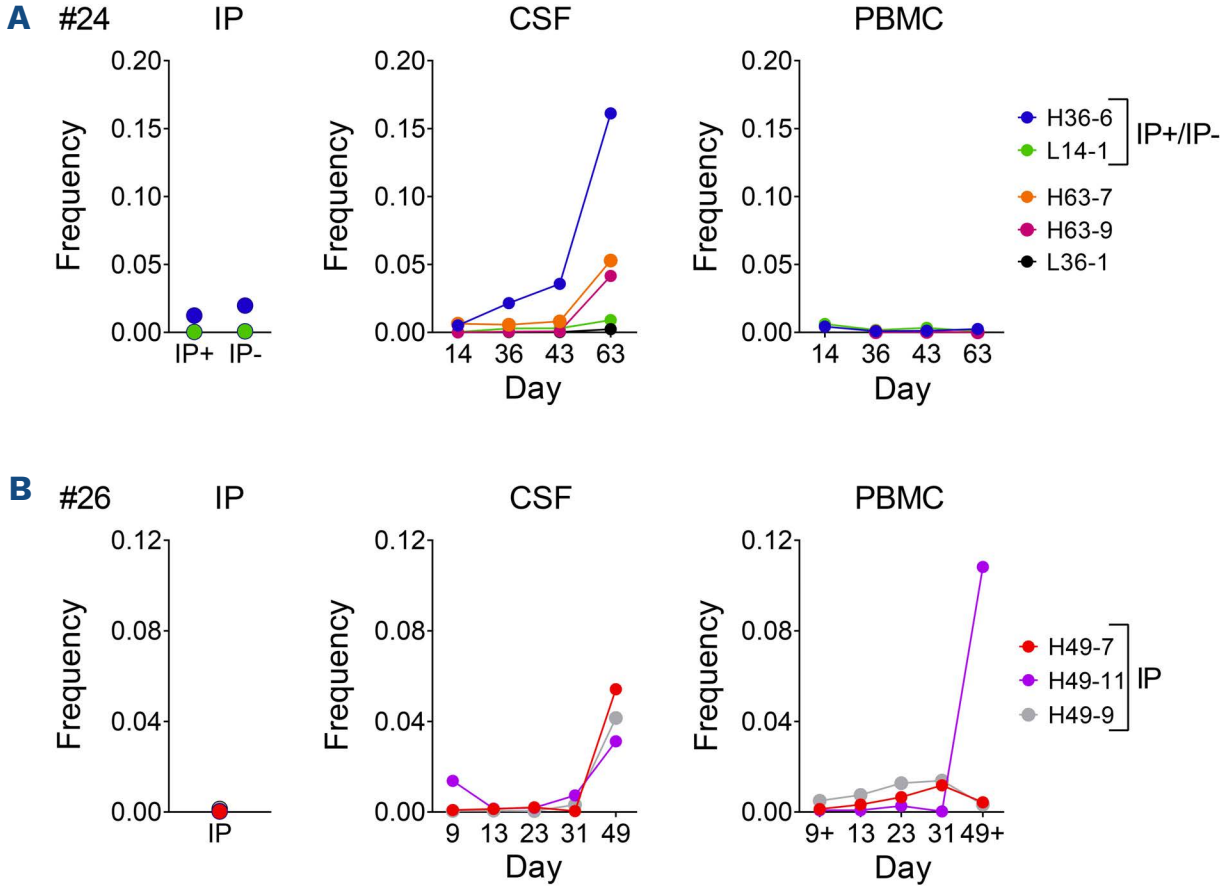

Figure 7. Detection of few persisting T-cell clones despite intense immuno-chemotherapies of severe immune effector cell-associated neurotoxicity syndrome. Longitudinal analysis of the T-cell receptor repertoire in 2 patients with prolonged treatment-resistant neurotoxicity after the CD19-chimeric antigen receptor (CD19-CAR) T-cell infusion. Genomic DNA was extracted from the infused T-cell product (left panels), and samples of cerebrospinal fluid (CSF) (middle panels), or peripheral blood mononuclear cells (PBMC) (right panels) and examined by T-cell receptor-β next-generation sequencing (TRB-NGS). (A) Patient #24: 5 unique T-cell clones were shared at all time points and increased in frequency in CSF, but not PBMC. Two of them were also detectable in the CAR-positive (IP+) and non-CAR (IP-) subset of the infused T-cell product subset. (D) Patient #26: detection of 3 unique T-cell clones throughout all time points with a pattern of increased frequency in CSF. All 3 clones were also detectable in the infusion product (IP).

that both CAR-positive and non-CAR T cells may contribute to the pathology suggests that current 'built-in' safety tools and/or suicide-switch strategies, i.e., utilizing the Herpes Simplex Virus type 1 thymidine kinase gene, inducible Caspase-9, or constitutive expression of the truncated epidermal growth factor receptor (EGFR)[34-39] may be insufficient in this setting.[9-11] Also, it is important to keep in mind that the mere presence of increased T-cell levels in the CSF is formally not sufficient to conclude that these cells themselves are directly mediating neurotoxicity rather than serving as biomarkers of increased expansion. Consequently, we cannot exclude the possibility that primarily inflammatory cytokines rather than T cells mediated the observed severe neurotoxicity.

A limitation of our work includes the relatively small number of diagnostic CSF specimens of inherently very small volumes and/or paucity of available cell number as well as the small number of patients. Our findings however emphasize the need for further systematic in-depth studies, using next-generation single cell RNA sequencing, proteomics, and/or epigenetic analysis,[40,41] to reveal the precise transcriptional signature underlying the treatment-refractory state of individual T-cell clones. Possible mechanisms may include an acquired steroid-resistance due to altered JAK-STAT-pathway signaling,[42,43] upregulation of the glucocorticoid-induced TNF receptor family-related protein (GITR) expression,[44] and/or a previously observed chemoresistance of certain central memory/memory stem T-cell subsets.[45,46] Novel approaches, including small-molecule inhibitors interfering with transcriptional pathways, i.e., Janus kinase- (ruxolitinib) and/or tyrosine kinase (dasatinib) inhibitors, have recently been considered to manage T-cell resistance and/or CAR T-cell mediated toxicities and may provide novel tools to overcome this problem.[9,47-50] Collectively, our results show that a subset of infused CAR T cells and non-CAR T cells can resist currently used ICANS-directed regimens and highlight the need to interrogate the biology of treatment-refractory T cells in severe ICANS and the rationale identification of suitable tools to control their survival, all of which will provide insights in and will facilitate the further design of ICANS-directed therapies.

Disclosures

The dPCR assays were made available as Bio-Rad "Expert Design Assays" (axi-cel: dEXD45718942 and tisa-cel/universal: dEXD88164642) based on an agreement between UMC Hamburg-Eppendorf and Bio-Rad. In accordance with the German law on employee inventions, BF, AB, and SCB received compensation payments. BF has performed consultancy work for Celgene/BMS. FAA has performed consultancy work for Celgene/BMS and is an advisory board member of Kite/Gilead, Celgene/BMS, Novartis and Janssen. NK has received honoraria from Celgene/BMS, Kite/Gilead, Novartis and Janssen. Note that Bio Rad was not involved in the study design, collection, analysis or interpretation of the data, or in the writing of this paper. None of the mentioned sources supported the work described within this manuscript. All other authors have no conflicts of interest to disclose.

Contributions

Patient treatment and oversight undertaken by FAA, NK, CW, DJ, NG, ML, DW, CF, GT, AA and MG. Conceptualization of the study by FAA, BF and SCB. Methodology by SCB, AB, NA, JD and BF. Data collection, analysis, and visualization by SCB, AB, NA, JD, SZ, FAA and BF. Data interpretation by FAA, BF, SCB and NK. Resources and/or research funding acquisition by NK, BF and SCB. The initial version of the manuscript was drafted by SCB, FAA, and BF. All authors read and approved the final version of the manuscrip. Supervision by NK, FAA, BF.

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Data-sharing statement

The data that support the findings of this study and/or protocols are available from the corresponding authors upon reasonable request. Primers and probes for the digital PCR assays are available as Expert Design assays from Bio-Rad Laboratories (axi-cel: dEXD45718942; "universal"/tisa-cel: dEXD88164642).

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