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Therapeutic options are limited for R/R central nervous system lymphomas (CNSL), including primary CNSL (PCNSL) and secondary CNSL (SCNSL)[1-3]. Chimeric antigen receptor (CAR)-T cells has been one of the most promising novel cancer therapies. Given that almost all CNSL express the CD19 antigen and that T cells are known to pass the blood brain barrier, there is a strong biological rationale for treating them with CAR-T cells but they have been excluded from most clinical trials. Recent data have shown that CAR-T cells could be efficient in R/R PCNSL and we recently reported an overall response rate (ORR) of 67% in 9 patients[4–7] However, the dynamics of CAR-T cells and their trafficking and persistence in cerebrospinal fluid (CSF) have been rarely described. Identification of biomarkers associated with increased disease control could enhance understanding of the biological basis for efficiency and enables more effective treatment interventions. We investigated the clinical outcomes and the CAR-T cell expansion and phenotype in the peripheral blood and CSF within 21 patients with $CD19^{+}$-R/R CNSL.

Patients with isolated R/R $CD19^{+}$-PCNSL or SCNSL and treated with tisagenlecleucel (n=19) or axicabtagene ciloleucel (n = 2) from January 2020 to January 2022 at Pitié-Salpêtrière Hospital (France) were retrospectively selected. The disease response was assessed at D28 by brain MRI and then every 2 months. Clinical results of 8 PCNSL patients were previously reported with shorter follow-up and without any biological data[4]. All patients gave written informed consent, the study was performed in accordance with the Declaration of Helsinki and was approved by national (CNIL 913611) and local (CPP Ile-De-France PP 13-022) ethics committees. Fresh blood samples were collected for real-time quantitative polymerase chain reaction (RT-qPCR) every 3 days the first 2 weeks, every week the following 2 weeks and then monthly. PMBC were collected and used for mass and flow cytometry at the peak of expansion. For detection of integrated CAR-expressing vectors, DNA was extracted from blood using a QIAamp DNA Blood Mini Kit. RT-qPCR was performed on 100 ng of

extracted DNA using the 2X TaqMan Universal PCR Master Mix. The specific primers and TaqMan probes detected the CD28-CD3ζ (Yescarta) and the 41BB- CD3ζ (Kymriah) junctions of the CAR-T transgene (**Supplementary Fig.1**). For CAR-T immune profiling by mass cytometry, thawed PBMCs were stained with the CD19 CAR Detection Reagent, Biotin (Miltenyi), washed, and stained with an anti-biotin-$^{106\text{-}116}$Cd antibody for 20 min as previously described[8]. They were then incubated with the MDIPA panel (Fluidigm), plus 5 antibodies (anti-PD-1-^{175}Lu, anti-TIGIT-^{209}Bi, anti-TIM-3-^{169}Tm, anti-CD69-^{162}Dy and anti-CXCR4-^{165}Ho). Samples were acquired on a Helios machine and analyzed with Maxpar Pathsetter, FlowJo, and OMIQ. For CAR-T functionality assessment by flow cytometry,1x10^{6} thawed PBMC were stimulated 5 hours with a CD19^{+} B lymphocyte cell line immortalized by Epstein-Barr Virus (Raji cell line), then incubated with biotin-labelled CD19 for 30 minutes and stained with Live/Dead, anti-biotin-PE, CD107a-APC-R700, CD3-APC780, CD8-BV605, CCR5-BV650, CCR6-BV700, CD69-PE-CF594, interleukin (IL) 17-BV421, interferon-γ (IFN-γ)-FITC, tumor necrosis factor-α (TNF-α)-PE-Cy7, and IL22-AF647. Fresh CSF samples were collected every 2 weeks the first month and then monthly, and tested after incubation with biotin-labelled CD19 for 30 minutes, with anti-CD3-APC-H7, CD4-PercPCy5.5, CD8-APC-Alexa700 and anti-biotin-PE. IL-6 levels were measured by Cytometric Bead Array technique in freshly thawed CSF samples, on a FACSCanto II cytometer.

Twenty-one R/R patients (13 PCNSL, 8 SCNSL) were selected, all but one patients with brain parenchymal or meningeal involvement and 9 with CSF involvement (**Supplementary Table 1**). The median age was 67 years and the median number of prior therapies 3 (range, 2–5), including ASCT in 16 patients. At the time of CAR-T cell infusion, 62% had progressive disease (PD), 29% partial response (PR), 5% stable disease and 5% complete response (CR). The median follow-up was 12 months (range, 1–29). At D28, ORR was observed in 67%

cases, including 29% CR and 38% PR. At month 3 (M3), ORR and CR without new treatment were observed in 9 (43%) (6/13 PCNSL, and 3/8 SCNSL) and 6 (29%) (5/13 PCNSL, and 1/8 SCNSL) patients, respectively. Among the 9 patients with response at M3, the median response duration was 19 (range, 8–29) months. On final follow-up, 8 (38%) patients had persistent response: 6 CR (4/13 PCNSL and 2/8 SCNSL) and 2 PR (1 PCNSL and 1 SCNSL), and all the 13 remaining patients died because of R/R disease. The median overall survival was 15 months and tended to be higher in PCNSL than in SCNSL (20 *vs* 12 months, p=0.63) (**Fig.1**), and the median progression free survival was 3 months. For subsequent biological analysis, patients with response ≥6 months without new systemic treatment were defined as responders (R, n=8), and the others as non-responders (NR, n=13). CRS and ICANS occurred in 16 (1 grade 3) and 7 (2 grades≥ 3) patients respectively, all having been resolutive using tociluzumab and corticosteroids for 14 and 5 patients.

We first assessed the expansion of CAR-T cells in the CSF for sixteen patients. All tested patients demonstrated CSF positivity for CAR-T cells regardless of clinical outcome with an initial phase of rapid expansion followed by a slow decrease with long-term persistence (**Fig.2).** On D30, the median number of CAR-T cells in the CSF was 0.17/mm^3, reflecting 19% (range, 4–45) of $CD3^+$ T cells, and the median CD4/CD8 CAR-T cells ratio was 4.2.. The later CSF analysis assessed 17 months after the infusion in one R patient still demonstrated the presence of CAR-T cells (41% of CD3). We demonstrated, focusing on the first month, that the expansion peak in the CSF was significantly higher in R than in NR patients (0.50/mm^3 versus 0.19/mm^3 (p=0.01)) (**Fig.2**). Finally, a transient increase in IL-6 dosage was detectable during the first month for 76% cases regardless of their outcomes.

We next addressed the phenotypic characterization of blood CAR-T cells at the expansion peak with mass cytometry for 20 patients**.** We first demonstrated that R patients had a higher frequency of CD8 T cells with a terminal effector phenotype (CD8 TE, mean=24% vs 11% of

cells; p< 0.05) and a lower frequency of regulatory T cells (Tregs, 0.5% vs 1.8%, p< 0.05) compared to NR. We next performed a data-driven non-supervised analysis using the Phenograph clustering algorithm which identified 68 distinct cells subtypes (**Fig.3.A**). Among them, PG-4 which presented a phenotype similar to T cells CD8 TE, confirming our previous observation, and PG-34 corresponding to $CD4^+$ CAR T cells expressing high levels of IL7R and IL3 were particularly over-represented in the R group (**Fig.3.B**). Conversely, 3 clusters were less present among the responders: PG-31, phenotypically comparable to TIGIT-expressing Tregs, which refines the above findings; PG-8, $CD4^+$ T cells positive for IL7R; and PG-13 which fits the description of NK cells. We further applied Phenograph specifically to CAR-T cells and we confirmed the over-representation of CD4+ $IL7R^+$ CAR T cells (PG-CAR13) among the responders (**Fig.3.C and D**). In addition, PD1 showed a weaker expression among responders, and this was particularly striking regarding the CAR T cells (**Fig.3.E**). This reduction of PD1 was paralleled by a CAR T cells-specific reduction of CXCR4 expression, especially among $CD4^+$ CARs.

Finally, we analyzed the CAR-T cells functionality and the expression of chemokine receptors at the peak of expansion by using thawed PBMC (n=16) after 5 hours of stimulation with tumor B- cells in order to replicate the *in vivo* interaction with the CAR-T cell target. We showed that the IFN-γ expression by $CD4^+$ CAR-T cells was higher in R than in NR patients (p=0.03), and that the expression of CD107a and IFN-γ by $CD8^+$ CAR-T cells tended to be higher in R patients (p=0.08) (**Supplementary Fig.2**). In addition, the expression of CCR5 and CCR6 on $CD8^+$ CAR-T cells was higher (54% and 47%, respectively) than that on the $CD8^+$ non-CAR-T cells (6% and 4%, respectively) (p<0.0001) after the stimulation with tumor B cells. This up-regulation on CAR-T cells seemed to be the result of the specific co-stimulation since their expression on the $CD8^+$ CAR-T cells cultured without target B cells (negative control) was significantly lower (22% for CCR5 and 14% for CCR6) (p=0.0096).

We first reported persistent response in 38% cases with highly R/R disease, and it is very encouraging in view of the very poor prognosis of CNSL. CNS CAR-T cell trafficking was reported in all of our patients, confirming their ability to migrate into the CNS and persist there despite the low level of target antigen in the blood. Of note, the R patients demonstrated higher CAR-T cell peaks in the CSF, reinforcing the recently published data[6] but these results should be interpreted with caution due to far apart time points. We next suggested that a strong cytotoxic TE $CD8^+$ T cell response, and a diminished suppressive mechanism mediated by Tregs may be crucial for the efficacy of CAR T cells reinforcing that non-CAR-T cells are also critical for the responses[9]. We further demonstrated that a subset of $CD4^+IL3R^+ILR7^+$ CAR- T cells are associated with a better clinical response. Indeed, IL-7 prolongs the survival time of tumor-specific T cells and the effector pool generation, and CAR-T cells engineered for IL7-R constitutive activation had a higher anti-tumor activity and persistence in preclinical models[10–14]. On the other hand, the expression of PD-1 and CXCR4 paralleled with a poorer outcome, probably reflecting an impaired capacity to migrate to the target tissue and then to kill the tumor cells. Our results strongly support that engineered CAR-T cells might increase the level of response. Finally, we showed for the first time that the production of IFN-γ was associated with clinical outcomes, suggesting that the peripheral immunological invigoration of CAR-T reflects the activity at the tumor site. In conclusion, the use of CAR-T cells therapy in CNSL patients answers an unmet medical need, and we suggest that the CSF expansion as well as the functionality and phenotype of CAR-T cells are implicated in the clinical outcome, paving the way for the development of novel CAR-T cells with higher anti-tumor activity.

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FIGURE LEGENDS

Figure 1. Overall survival and progression free survival. A. In all the patients. **B.** In PCNSL patients (n=13). **C.** In SCNSL patients (n=8). PFS, progression free survival; OS, overall survival.

Figure 2. CSF CAR-T cell expansion. A. Individual profiles of CAR-T cells expansion (% of CD3) in CSF for each patient. Red color denotes R patients, circle plots denote the patients with PCNSL and triangle plots the patients with SCNSL.**B.** Number of CAR-T cells (/mm3) in CSF on the best expansion peak during the first month. **C.** IL-6 dosage (pg/mL) in CSF. The timing scale has not been respected for the first 30 days in order to better see all the dots. Red color denotes R patients, circle plots denote the patients with PNCSL and triangle plots the patients with SCNSL R: responder patients, NR: non responder patients. *Mann-Whitney test*

Figure 3. Immune profiling of PBMCs in CNS lymphoma patients treated with CAR-T cells. A. t-SNE representation of single-cell CyTOF data for all patients (n = 20). , depicting 68 cell clusters after Phenograph unsupervised clustering based on the expression of each of the 35 markers. Numbers within colored boxes correspond to the respective cluster number. **B.** Abundances of selected clusters identified in (B) and comparison of their frequencies between responders (red) and non-responders (black) patients. **C.** t-SNE representation depicting specific 34 CAR-T cell clusters after Phenograph unsupervised clustering based on the expression of each of the 35 markers. Numbers within colored boxes correspond to the respective cluster number. **D.** Abundances of selected clusters identified in (C) and comparison of their frequencies between responders (red) and non-responders (black) patients. **E.** Overlayed histograms comparing expression of PD-1 (left) or CXCR4 (right) of gated total CAR-T cells, and specific CD8+ or CD4+ CARs between responder (red) and non-responder (black) patients. R: responder patients, NR: non responder patients.