Clonal expansion of CAR T cells harboring lentivector integration in the *PLAAT4* gene following anti-CD19 CAR T-cell therapy in a context of cutaneous T-cell lymphoma

Chimeric antigen receptor (CAR) T-cell therapies have emerged as groundbreaking treatments for high-grade hematological malignancies, extending progression-free and overall survival.[1] However, because CAR T-cell production relies on viral vectors that integrate into host cell DNA, this raises a specific safety concern that CAR integration might disrupt gene expression associated with cell proliferation, ultimately contributing to T-cell malignancies.[2] Two mechanisms have been associated with retroviral-induced malignancies: either vector insertion leading to the inactivation of a tumor suppressor gene,[3] or activation of nearby proto-oncogenes through its transcriptional enhancers.[4]

We recently reported, in a large cohort study, a case of T-cell lymphoma in which a minor CAR T-cell population, dominated by a CAR clone integrated into the phospholipase A and acyltransferase 4 (*PLAAT4*) gene, expanded.[5] This work details the characteristics of this clone and its evolution during the course of the T-cell lymphoma.

We performed the following evaluations. First, we retrospectively evaluated clinical outcomes of all patients with a history of tisagenlecleucel (tisa-cel) treatment at Pitié-Salpêtrière Hospital (Paris, France). We extracted all consecutive blood CAR T-cell levels from the medical records of the complete-responders subpopulation, as their levels were correlated with vector and clinical response,[6,7] to determine whether CAR T-cell blood levels could have warned us about the incident T-cell lymphoma. Then, integration site analyses were performed using a modified integration site loop amplification (ISLA)[8,9] to determine the exact locations of CAR transgene integration sites. Clone quantifications were performed using integration-specific primers for each CAR T-cell clone, along with a lentiviral-specific primer (1.U5) and a unique probe targeting the 3' end of the lentiviral transgene. Primer specificity was assessed using the patient's pre-CAR T-cell genomic DNA. Quantification was performed in duplicate and normalized to the RPP30 gene on a Qx200 digital droplet polymerase chain reaction (ddPCR) platform (Bio-Rad, Hercules, USA). T-cell receptor γ (*TCR*-γ) clonality was assessed as reported[10] on an Illumina™ MiSeq platform. Clonotype assignment was performed using Vidjil.[11] TRGV/TRGJ annotation and CDR3 characterization were performed using IMGT V-QUEST.[12] Sixty-one relevant genes were sequenced on an Illumina™ MiSeq platform from fresh skin biopsy samples using the Sure Select panel (Agilent, Santa Clara, California): *ARID1A, ATM, B2M, BCL2, BCOR, BIRC3* (exons 7 to 10), *BRAF* (exon15), *BTK, CARD11* (exons 4 to 10), *CCL22* (exons 2 and 3), *CCND3* (exon 5), *CCND1* (exon 1), *CD28, CD37, CD79A* (exons 4 and 5), *CD79B, CREBBP, CXCR4, DNMT3A, EGR2, EP300, EZH2* (exons 16 to 17), *FBXW7, ID3, GNA13, IDH2* (exon 4), *IGLL5, JAK1* (exons 14 to 25), *JAK3, KLF2, KMT2D, KRAS, MYC, MYD88* (exons 3 to 5), *NFKBIE, NOTCH1* (exon 34 to 3'UTR), *NOTCH2* (exon 34 and 3'UTR), *PIM1, PLCG1, PLCG2, POT1, PRDM1* (exons 1 and 2), PTPN1 (exons 2 to 6), *PTPRD, RHOA, RPS15, SAMHD1, SF3B1* (exons 12 to 18), *SMARCA4, SPI1* (exon 5), *STAT3* (exons 19 to 21), *STAT5B* (exons 14 to 17), *STAT6* (exon 12 to 18), *TBL1XR1, TCF3, TET2, TNFAIP3, TNFRSF14* (exons 2 and 4), and *TP53*, *XPO1* (exon 15). Variant detection threshold was set at 2%. CREBBP exon31 was amplified using reported primer sets[13] and Sanger sequenced with the same primers. Statistical analyses and figures were performed using R or GraphPad Prism v9.0. This study was conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from the patient.

A 70-year-old woman received tisa-cel as fourth-line therapy for a stage IV Ann Arbor diffuse large B-cell lymphoma with central nervous system involvement. Complete remission was achieved 1 month post infusion.

As reported,[5] T-cell lymphoma occurred 18 months after infusion, initially presenting as a skin nodule on the left shoulder which prompted an outpatient skin biopsy revealing a dense dermal infiltrate of small T lymphocytes. Three years post-infusion, following a community-acquired pneumonia and a femoral neck fracture, the patient was diagnosed with cutaneous $CD30^+$ T-cell non-Hodgkin lymphoma (NHL) in the setting of numerous hypermetabolic centimetric skin nodules, without lymph node involvement (Figure 1). At this time point, her CAR T cells levels in blood were comparable to the other tisa-cel complete responders (*Online Supplementary Figure S1*). Biopsy was remarkable for atypical $CD3^+$, $CD30^+$, $CD20^-$, $CD4^-$, $CD8^-$, $CD2^-$, $CD5^-$, $CD7^-$, $CD25^-$, granzyme B^-, $ALK1^-$, $MUM1^+$ cells, with a Ki-67 proliferation index of 80%. *TCR*-γ sequencing identified two predominant productive clonal rearrangements in the biopsy: *TRGV10-JP1* and *TRGV3-JP2* accounting for 58% and 20% of the analyzed sequences, respectively (*Online Supplementary Figure S2*). The two lymphoma clones were also found in blood, representing 2% and 0.4% of the reads, respectively. Importantly, T-cell clonality showed a polyclonal pattern before CAR T-cell infusion, without detection of the two lymphoma clones nor the main CAR T-cell clone (*Online Supplementary Figure S3*). Targeted sequencing of the biopsy uncovered an undescribed missense mutation in exon 31 of the *CREBBP* tumor suppressor gene (c.5381C>T, p.(Ser1794Phe)), with a

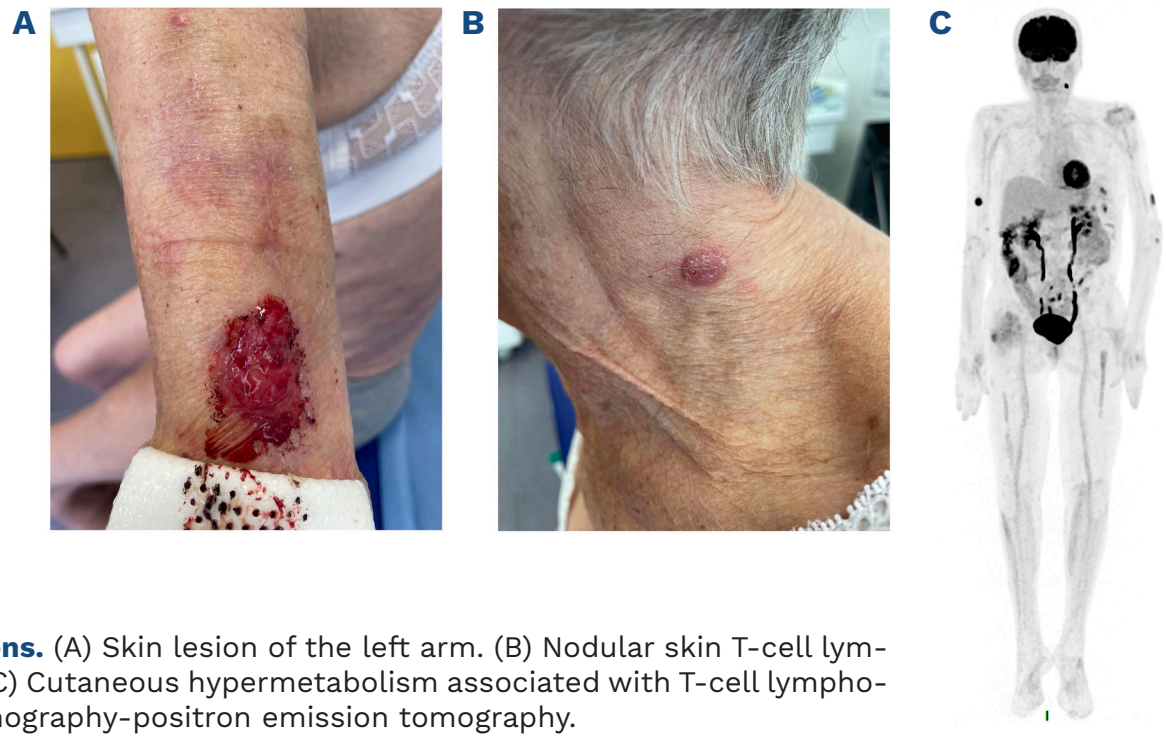


Figure 1. Clinical lesions. (A) Skin lesion of the left arm. (B) Nodular skin T-cell lymphoma on the neck. (C) Cutaneous hypermetabolism associated with T-cell lymphoma on computed tomography-positron emission tomography.

variant allele fraction of 37%.

The patient was treated with photochemotherapy for the T-cell lymphoma, with no clinical response, followed by brentuximab-vedotin, which resulted in a partial response after three cycles. Upon relapse, low-dose gemcitabine (75 mg/m², day 1-15) was initiated due to grade 3 neutropenia and grade 2 thrombopenia. Clinical course was further complicated by bronchiolitis, a second femoral neck fracture and recurrant falls. The patient died of septic shock due to a urinary tract infection 4 years post CAR T-cell infusion, in complete response for the T-cell NHL.

Due to the T-cell nature of the lymphoma, blood and biopsy samples were simultaneously analyzed for CAR T-cell quantification. At 3 years post-infusion, CAR T cells were 28-fold more enriched in the skin biopsy than in peripheral blood (5.1% *vs*. 0.18% of total cells).

As reported,[5] *PLAAT4* was the predominant site of *CAR* transgene integration in the biopsy. Further investigation retrieved additional sites, including *SETX, MEIOB*, and *ZNF512*. Among all CAR-positive clones, *PLAAT4* remained the most frequent, representing 54.1% of all *CAR* integration events, while *SETX, MEIOB*, and *ZNF512* accounted for 23.0%, 10.8%, and 6.8%, respectively. In a second cutaneous biopsy collected during disease relapse after treatment with brentuximab vedotin, the frequency of the *PLAAT4* clone increased to 80% of all CAR T cells. Notably, none of the three minor

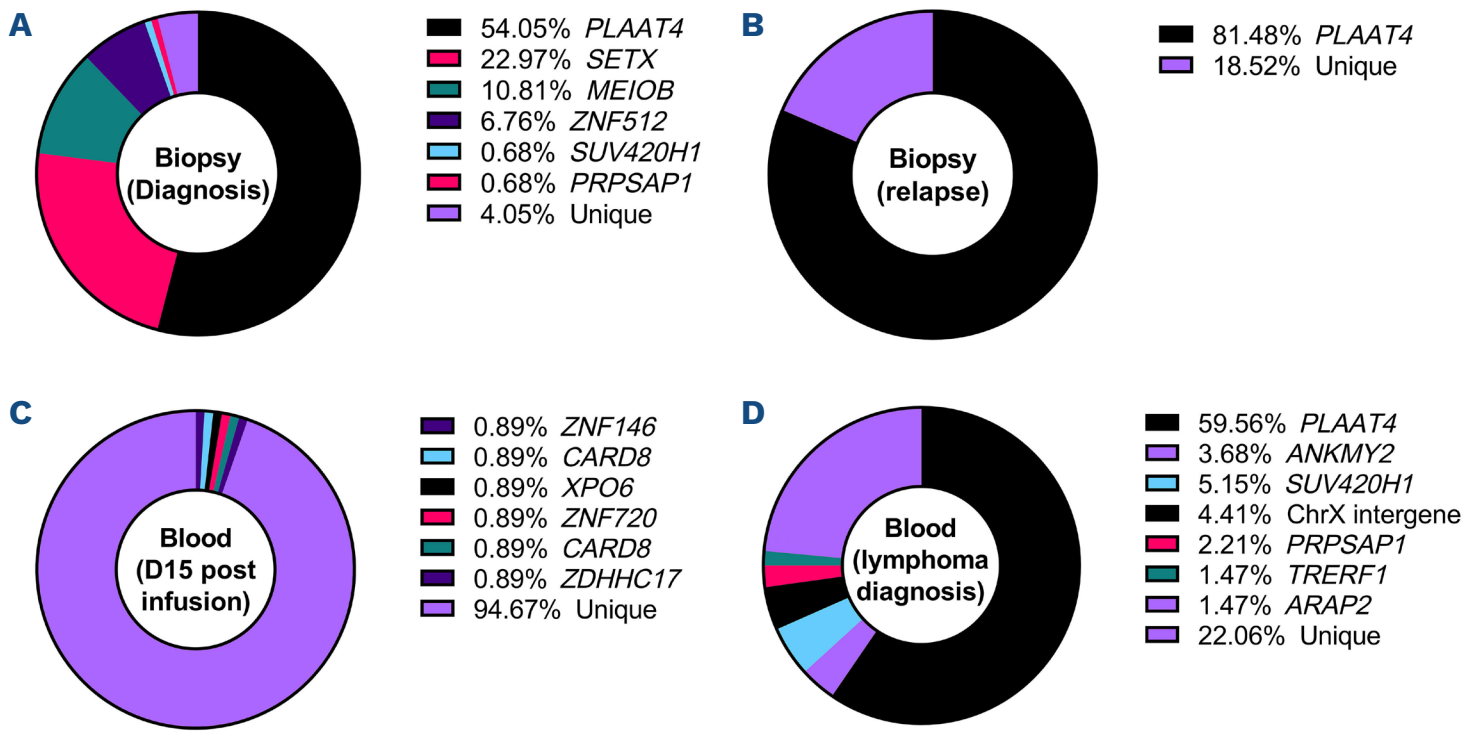


Figure 2. Clonality based on integration site analysis. Chimeric antigen receptor (CAR) T transgene integration sites from biopsy samples collected at (A) T-cell lymphoma diagnosis and (B) relapse of primary cutaneous CD30⁺ T-cell lymphoproliferative disorder, and (C) in blood samples at 15 days (D15) post CAR T-cell infusion and (D) at the time of T-cell lymphoma diagnosis. Analyses were performed using the integration site loop amplification (ISLA) assay. Clones are labeled according to their host genes. ChrX: X chromosome.

clones identified in the initial biopsy were detected using the same assay (Figure 2).

Integration site analyses were performed on whole blood samples collected at peak expansion post-infusion (day 15 post-infusion) and at the time of T-cell lymphoma diagnosis (3 years post-infusion). At peak expansion, the CAR T-cell population was polyclonal, without detection of the *PLAAT4* clone. At the time of lymphoma diagnosis, the *PLAAT4* clone was predominant, accounting for 60% of all CAR T cells, alongside six minor clones, each comprising less than 5% of the total CAR T-cell population (Figure 2).

To investigate the longitudinal dynamics of CAR T-cell clones, peripheral blood samples collected at 18 time points following CAR T-cell infusion were analyzed using integration-specific ddPCR (Figure 3). The *PLAAT4* clone became detectable around 200 days post-infusion, followed by a rapid 8-fold expansion approximately 3 years post-infusion, coinciding with the diagnosis of T-cell malignancy. A transient decrease was measured during brentuximab vedotin treatment, associated with a transient clinical response. A second expansion peak emerged during disease relapse under brentuximab vedotin treatment. Apart from the dominant clone, no clone consistently represented >5% of the total CAR T-cell pool. The *MEIOB* and *SETX* CAR clones were never detected in blood.

CAR T cells were sorted from a blood sample collected at the time of disease relapse. None of the major lymphoma-associated *TCR*-γ clonal rearrangements were detected in the sorted CAR T-cell population (*Online Supplementary Figure S4*). In addition, the *CREBBP* missense mutation identified in the lymphoma biopsy was not detected in these cells.

Finally, to assess lymphocyte survival, during the T-cell lymphoma relapse, the patient's peripheral blood mononuclear cells (PBMC) were isolated from whole blood and plated in RPMI-1640 glutamax (Gibco) supplemented with 10% fetal bovine serum to evaluate lymphocyte survival. Lymphocyte mortality was expected to reach 90% at 1 month and >99% at 2 months. CAR T cells persisted for 2 months, accounting for 2-4% of total cells. The *PLAAT4* clone rose from two-thirds to 100% of the CAR T-cell population between 1 and 2 months of culture, suggesting a survival advantage, though no cell expansion was observed and we were unable to demonstrate that this clone was immortalized.

We report a case of T-cell lymphoma associated with a minor CAR T-cell population. While transformation of CAR T cells into a tumor was unlikely, integration of the CAR transgene into the *PLAAT4* gene might have represented a genetic event contributing to the development or progression of the T-cell malignancy, as supported by the longitudinal analysis of clonal CAR populations.

This case contrasts with previous reports of CAR T cells involvement in lymphomas. To date, all T-cell lymphomas occurring after CAR T-cell infusions have presented rather unequivocal findings: they were either monoclonal or oligoclonal CAR T-cell lymphomas, or CAR T cells were absent from the biopsy samples.[14] In contrast, this lymphoma is characterized by the uncommon presence of two major non-CAR clones alongside a minor CAR T-cell population dominated by the *PLAAT4* clone.

We hypothesized that integration into the *PLAAT4* tumor suppressor gene might have contributed to the lymphoma, as this gene is frequently dysregulated in malignancies.[5] This longitudinal analysis, notably the delayed emergence and subsequent selection of the *PLAAT4* clone, also argues in favor of its potential involvement, as expansion of a major clonal CAR T-cell population is a highly uncommon event, reported in fewer than 2,200 patient-years of follow-up.[15] In contrast, mono- or oligoclonal CAR T-cell populations have

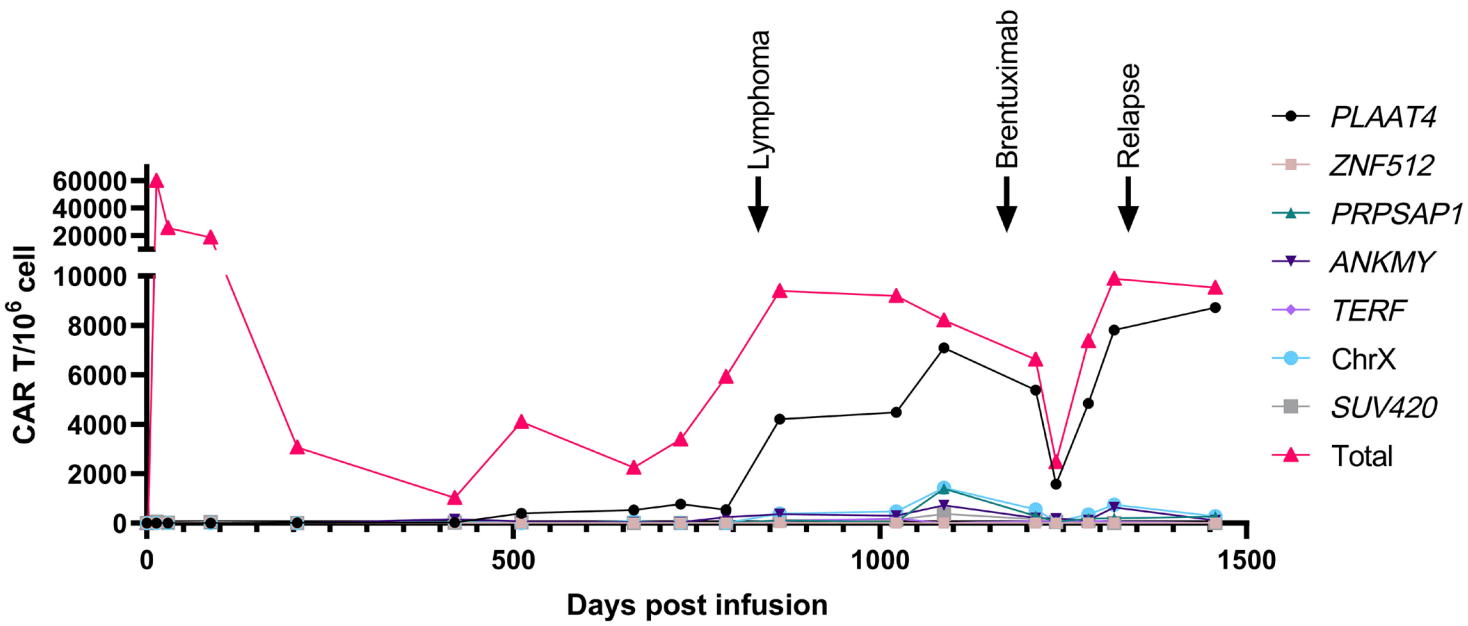


Figure 3. Longitudinal evolution of the total CAR population and CAR clones in blood post-CAR T infusion using digital droplet polymerase chain reaction. The pink line represents the total chimeric antigen receptor (CAR) T-cell population, while the other lines represent each clonal population. The *PLAAT4* clone was not detected in blood before 200 days post-infusion. Arrows mark the time of T-cell malignancy diagnosis, initiation of brentuximab vedotin treatment and disease relapse. Quantification was normalized to the housekeeping gene *RPP30*.

been consistently linked to malignancies,[14] except for the *TET2* clonal expansion case.[16] However, despite our comprehensive analysis of this case, we provide only indirect but convergent arguments regarding a potential link between the *PLAAT4* clone and the second primary malignancy. Also, few reports have emphasized that the integration site is only one of multiple contributing factors, and that CAR T-cell populations can undergo successive waves of expansion, driven primarily by T-cell phenotype[17] or, in some cases, by their integration sites.[18] Consequently, we still cannot formally exclude the possibility that a non-malignant CAR clonal population evolved alongside the two lymphoma clones.

Despite this rare event, our findings do not challenge the overall favorable safety profile of lentiviral CAR T-cell therapies. Rather, they highlight the importance of long-term molecular monitoring to better understand the biological determinants of CAR T-cell persistence and clonal dynamics.

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https://doi.org/10.3324/haematol.2026.300851

Received: March 9, 2026.
Accepted: June 3, 2026.
Early view: June 11, 2026.

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Disclosures

No conflicts of interest to disclose.

Contributions

Data curation, conceptualization, methodology, formal analysis, writing - original draft by VG, VC, SB, ET, and SC. Data curation, formal analysis, writing - review and editing by CB, AG, JAD, RL, FC, MLG, CP, and AGM. Writing - review and editing by RD and RH.

Funding

This work was supported in part by the ANRS-MIE (Agence Nationale de Recherches sur le SIDA et les hépatites virales-Maladies Infectieuses Emergentes - Medical and Pharmacological ANRS-MIE Network) and the Groupe Pasteur Mutualité. Study sponsors had no part on study design, data collection, data analyses, data interpretation, manuscript writing nor decision to submit for publication.

Data-sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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