229 LARGE-SCALE OMICS PROFILING REVEALS TYPE-2 FUNCTIONALITY SUSTAINING 8-YEAR LEUKEMIA REMISSION FOLLOWING CAR T CELL THERAPY

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Background Despite a high response rate in chimeric antigen receptor (CAR) T therapy for acute lymphocytic leukemia (ALL), ~50% of patients relapse within the first year,[1] representing an urgent question to address in the next stage of cellular immunotherapy. The pioneering clinical trials conducted at Upenn/CHOP provide a unique opportunity to examine the molecular determinants of of ultra-long CAR T persistence.

Methods We performed single-cell multi-omics profiling of ~700k pre-infusion CD19-targeted 4-BB CAR T cells from 82 pediatric ALL patients and 6 healthy donors. Patient demographics were collected between Sep-2012 to July-2022. To uncover the hallmarks of CAR T longevity, we correlated the single-cell atlas with the duration of B-cell aplasia (BCA), a widely used pharmacodynamic measurement of CAR T persistence,[2] and classified all the patients into 5 groups based on their BCA duration (table 1). To remove potential confounding variables between trial design, 42 patients from NCT01626495 were analyzed as the Discovery Cohort, while the other 40 patients from NCT02906371 were used as Validation Cohort.

Results Our analysis of CD19-specific stimulated CAR T cells from the Discovery Cohort revealed a prominent role of type-1 function, which was highly represented but had no discernible correlation with long-term persistence. Unexpectedly, we identified that elevated type-2 functionality was significantly associated with patients maintaining a median BCA duration of 8.4 years (BCA-L group) (figure 1A,B), and this signature was robustly presented in our Validation Cohort (figure 1C, D). Higher type-2 cytokine level of long-term persistent CAR T cells was independently validated using both flow cytometry and multiplexed secretomic assay (figure 1E,F). Through ligand-receptor interaction analysis, type-2 cytokines were found to regulate a cluster of Tim-3+ terminal effector cells showing overactivation of cytotoxicity, impaired immune function, elevated exhaustion signature and diminished proliferative potentiality, and CAR T cells from BCA-L patients showed reduced dysfunctional hallmarks (figure 2A-G). To examine the role of type-2 function in vivo, we performed serial proteomic profiling of post-infusion sera from patients, confirming a higher level of circulating type-2 cytokines in 5-year or 8-year relapse-free responders (figure 3A-C). In a leukemic mouse model, type-2 high CAR T cell products demonstrated superior expansion and antitumor activity particularly upon leukemia rechallenge, which could be associated with their decreased exhaustion and increased memory phenotype. (figure 4A-G).

Conclusions Our findings provide key insights into the mediators of CAR T longevity and suggest a potential therapeutic strategy to maintain long-term remission by enhancing type-2 functionality in CAR T cells.

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Trial Registration The current study is a secondary investigation using patient samples collected from existing clinical trials (ClinicalTrials.gov number, NCT01626495 and NCT02906371) for which the University of Pennsylvania Institutional Board provided insight.

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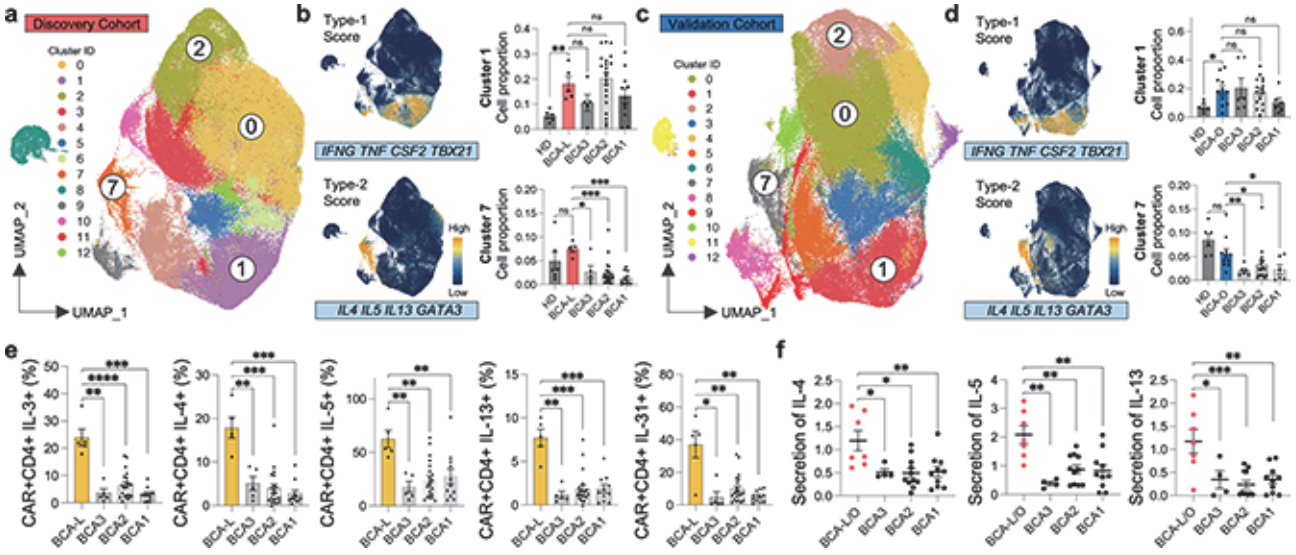
2. Greenbaum U, Mahadeo KM, Kebriaei P, Shpall EJ, Saini NY. Chimeric Antigen Receptor T-Cells in B-Acute Lymphoblastic Leukemia: State of the Art and Future Directions. *Front Oncol*. 2020;**10**:1594.

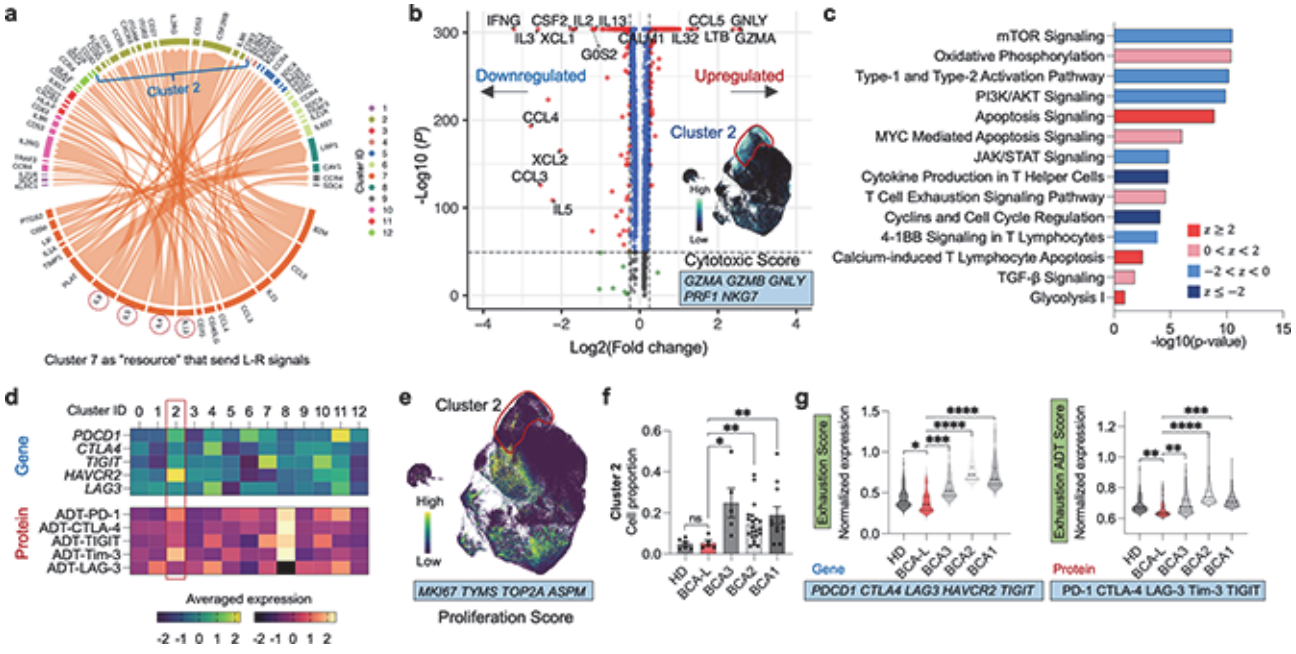
Ethics Approval The study obtained ethics approval from the University of Pennsylvania Institutional Board. Written informed consent for participation was obtained from patients or their guardians according to the Declaration of Helsinki. All laboratory operations were under principles of International Conference on Harmonization Guidelines for Good Clinical Practice with established Standard Operating Procedures and protocols for sample receipt, processing, freezing, and analysis. All ethical regulations were strictly followed. Healthy donor primary T lymphocytes were provided by the University of Pennsylvania Human Immunology Core. Samples are de-identified for compliance with HIPAA rules.

Experimental procedures in mouse studies were approved by the Swiss authorities (Canton of Vaud, animal protocol ID 3533) and performed in accordance with the guidelines from the CPG of EPFL.

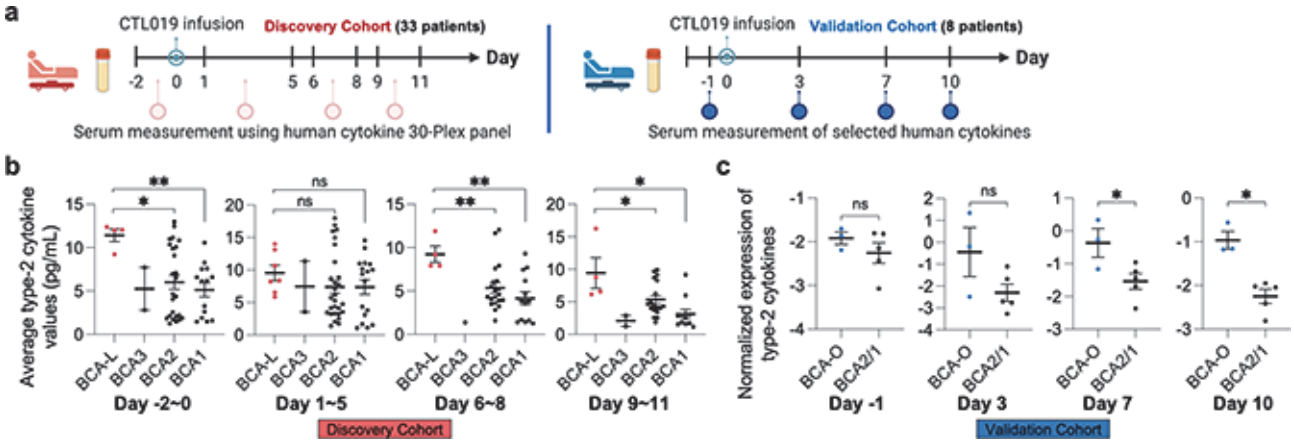
Consent The abstract doesn't contain sensitive or identifiable information.



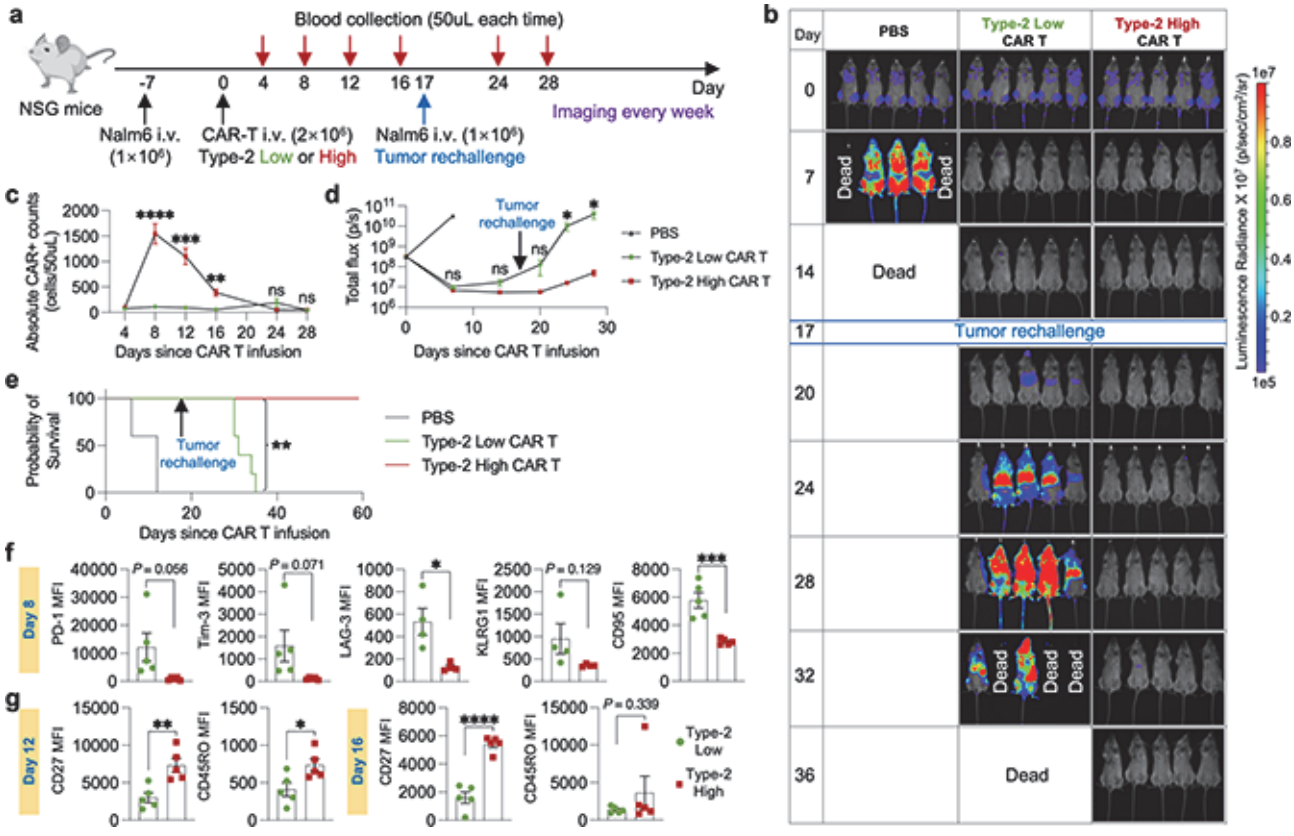
Abstract 229 Figure 1 Type-2 function CAR T cell cluster is associated with 8-year ultra-long remission. (A) UMAP clustering of CDI 9-specific stimulated CAR T cells from the Discovery Cohort patients and healthy donors. (B) Expression distribution of Type-1 (enriched in Cluster 1) and Type-2 (enriched in Cluster 7) Score on the UMAP in Fig. la, and the cell proportion comparison in Cluster 1 and Cluster 7. (C) UMAP clustering of CD19-specific stimulated CAR T cells from the Validation Cohort patients and healthy donors. (D) Expression distribution of Type-1 (enriched in Cluster 1) and Type-2 (enriched in Cluster 7) Score on the UMAP in Fig. 1c and the cell proportion comparison in Cluster 1 and Cluster 7. (E) The frequency comparison of type-2 cytokine+ population in, CD4+ CAR+ cells between persistence groups. Independent functionality assessment was performed by intracellular flow cytometry. (F) The secretion level comparison of type-2 cytokines between persistence groups. Multiplexed secretomic assay was performed for 32 patients in the cohort. The secretion level denotes to the frequency of cells secreting specific cytokine multiplied by the average signal intensity of this cytokine.



Abstract 229 Figure 2 Type-2 subpopulation regulates Tim-3+ dysfunctional effector CAR T cells through ligand-receptor (L-R) interactions. (A) Identification of all L-R interactions originating from type-2 high Cluster 7 cells in Fig. 1a, which mainly interact with Cluster 2 cells through type-2 communications. (B) Differentially expressed genes (DEGs) of Cluster 2 compared to all other clusters, and the expression distribution of Cytotoxic Score. Cytolytic effector genes were highly upregulated in this cluster, whereas key immuno-functional genes, such as *IFNG, CSF2, IL2, XCL1/2, CCL3/4*, were downregulated. (C) Corresponding signaling pathways regulated by the DEGs identified in Cluster 2 (z>0, activated/upregulated; z<0, inhibited/downregulated; z≥2 or z≤-2 can be considered significant). Central pathways involved in T cell activation and function, including mTOR, P13K/AKT, and JAK/STAT signaling were downregulated in Cluster 2 cells, whereas multiple dysfunctional pathways were upregulated. (D) Heatmap showing the average expression level of exhaustion related genes or ADT proteins of all the single cells in each identified cluster. The expression level of Tim-3 and its encoding gene *HAVCR2* is observed to be high in Cluster 2. (E) Expression distribution of Proliferation Score on the UMAP in Fig. 1a. An absent expression of this module is observed in Cluster 2. (F) Comparison of cell proportion in Cluster 2 between persistence groups. A significantly lower ratio of BCA-L compared to short-term groups is found. (G) Single-cell expression comparisons of Exhaustion Score and Exhaustion ADT Score in Cluster 2 cells between persistence groups.



Abstract 229 Figure 3 Higher type-2 cytokines are detectable in post-infusion sera from the patients showing long-term remission. (A) Schematic of the serial proteomic profiling to measure serum proteins in 33 patients from the Discovery Cohort and 8 patients from the Validation Cohort. Timepoints are relative to the day of first infusion of CTL019 (CD19-targeted CAR T) products (Day 0). (B) Comparison of average type-2 cytokine level at multiple timepoints between persistence groups in the Discovery Cohort. Each dot represents one measurement of one patient in each BCA group within the indicated time period, and the value is the averaged expression of IL-4, IL-5, and IL-13. (C) Comparison of average type-2 cytokine level at multiple timepoints between persistence groups in the Validation Cohort. The value represents the average normalized expression of IL-4, IL-5, and IL-13.



Abstract 229 Figure 4 Type-2 high CAR T cells exhibit superior expansion and antitumor activity in vivo in leukemic mouse models. (A) Schematic of in vivo leukemia model treated with type-2 low or type-2 high CAR T cells. NSG mice were intravenously (i.v.) injected with 1×10^6 Nalm6 cells. Seven days later, mice were randomly assigned to three groups and were infused i.v. with 2×10^6 CAR T cells or PBS (control). The survival mice were rechallenged with 1×10^6 Nalm6 cells 17 days post the CAR T infusion. We performed single cell analysis of CAR T cells from 6 healthy donors and identified ND463 as type-2 high and ND585 as type-2 low based on the proportion of their type-2 cells. No significant difference in type-1 signature was found between the two donors. (B) Tumor burden measured by bioluminescence at indicated days since CAR T cell infusion. (C) CAR T cell expansion in the peripheral blood of Nalm6—bearing mice measured at different timepoints after infusion. (D) Tumor burden (total flux) quantified by photons/s in mice since CAR T treatment. (E) Kaplan—Meyer curves showing mouse survival. (F) Flow cytometry analysis of exhaustion markers of peripheral CAR+ cells at day 8 after CAR T infusion. (G) Flow cytometry analysis of memory markers of peripheral CAR+ cells at day 12 and day 16 after CAR T infusion.

Abstract 229 Table 1 Patient demographics and clinical documentation. Patients are divided into 5 persistence groups based on their durations of B-cell aplasia (BCA). The Discovery Cohort includes 42 patients from clinical trial NCT01626495, and the Validation Cohort consists of 40 patients from clinical trial NCT02906371.

Demographics	BCA-L (n=5)	BCA-O (n=11)	BCA3 (n=11)	BCA2 (n=38)	BCA1 (n=17)
Cohort group, No.					
Discovery Cohort	5	N/A	5	21	11
Validation Cohort	N/A	11	6	17	6
Age in years	14.5±1.4	13.3±2.0	13.3±1.9	9.7±1.0	10.4±1.2
Median±SEM (Range)	(9.3-16.2)	(6.0-29.1)	(5.0-24.5)	(1.7-27.2)	(3.3-21.5)
BCA observed	Yes	Yes	Yes	Yes	Partial (11/17)
BCA duration in months	101±5.0	61±1.8	18±2.9	4±0.4	1±0.2
Median±SEM (Range)	(82-106)	(48-72)	(12-43)	(3-11)	(0-2)
Relapse observed	No	No	Yes	Yes	Yes
Relapse type, No. (%)					
CD19-postive	N/A	N/A	6 (54.5)	13 (34.2)	4 (23.5)
CD19-negative			4 (36.4)	19 (50.0)	5 (29.4)
Time to relapse in months	N/A	N/A	18.5±3.3	8.5±2.2	2.1±1.1
Median±SEM (Range)			(12.0-45.6)	(2.5-60.2)	(1.0-9.3)
Follow up months	101±5.0	61±1.4	51±5.7	46±4.3	4±7.0
Median±SEM (Range)	(82-106)	(54-72)	(17-78)	(4-91)	(1-78)

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