

Single-cell multiomics reveals regulatory mechanisms of CAR T-cell persistence and dysfunction in multiple myeloma

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Key Points

- Impaired effector-to-memory transition increases terminally differentiated CAR T cells and reduces in vivo persistence.
- IL-10-producing CAR T-cell clone expansion, likely via CAR and TCR coactivation, may underlie therapeutic failure in a partial responder.

Understanding the mechanisms that drive chimeric antigen receptor (CAR) T-cell function and persistence in multiple myeloma (MM) remains a critical challenge for improving therapeutic outcomes. In this study, we applied single-cell multiomics and gene regulatory network analysis to characterize the transcriptional dynamics and clonal evolution of B-cell maturation antigen-targeted CAR T-cells in longitudinally collected bone marrow (BM) and peripheral blood (PB) samples from patients with MM. Our results revealed that CAR T cells infiltrating BM exhibited a more activated and exhausted phenotype than their PB counterparts, with key transcriptional regulators driving these changes. Dysregulation in the effector-to-memory transition led to increased presence of terminally differentiated CAR T-cells, correlating with poor persistence. Additionally, we identified a hyperexpanded CAR T-cell clone in the BM of a patient in partial response, marked by elevated interleukin-10 (IL-10) expression. Functional analyses demonstrated that stimulation of endogenous T-cell receptor (TCR) enhanced IL-10 production, potentially contributing to impaired CAR T-cell proliferation and persistence. These findings uncover regulatory mechanisms influencing CAR T-cell dynamics, offering new insights into improving CAR T-cell persistence and therapeutic efficacy in MM, and highlight potential molecular targets for optimizing CAR T-cell therapy in patients with MM.

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The single-cell RNA sequencing and single-cell T-cell receptor sequencing data generated in this study have been deposited in the Gene Expression Omnibus database (accession number GSE290061).

All data needed to evaluate the conclusions in the paper are present in the paper and/or the supplemental Materials.

The full-text version of this article contains a data supplement.

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Introduction

Chimeric antigen receptor (CAR) T-cell therapies have become a promising therapeutic option for patients with B-cell hematological malignancies, including acute lymphoblastic leukemia, diffuse large B-cell lymphoma, and multiple myeloma (MM).¹ In MM, CAR T-cell therapies targeting B-cell maturation antigen (BCMA) have produced unprecedented rates of remission and increased life expectancy for patients with relapsed/refractory (R/R) disease.²⁻⁵ Nevertheless, almost every patient eventually relapses, with a median progression-free survival of 11 to 36 months, depending on the product.²⁻⁵ This lack of long-term response, one of the main challenges in CAR T-cell therapies for MM, depends on multiple factors, including CAR construct design, the quality and composition of the CAR T-cell product, CAR T-cell expansion and persistence capability, the development of resistance mechanisms, tumor burden, and the tumor microenvironment.⁶⁻¹⁴ All these data clearly indicate the need to better understand the underlying cellular and molecular features of CAR T-cell therapy leading to different patient outcomes.

The advancement of omic technologies, particularly those with single-cell resolution, has allowed gaining insights into the heterogeneity of CAR T-cell products as well as the identification of molecular factors driving CAR T-cell function *in vivo*. Moreover, the analysis of gene regulatory network (GRN) has demonstrated to be a powerful tool to unravel regulatory dynamics of CAR T-cells.^{15,16} However, most studies have been conducted analyzing CD19 CAR T-cells isolated from the infusion product or circulating in the blood from patients with acute lymphoblastic leukemia and diffuse large B-cell lymphoma.¹⁷⁻²² Even though there is increasing literature on the transcriptional features driving CAR T-cell dynamics in MM,²³⁻²⁵ there is still incomplete knowledge on the molecular factors important for long-term CAR T-cell functionality. Moreover, we lack a complete understanding of the differences between CAR T-cells infiltrating the bone marrow (BM) and their counterparts circulating in peripheral blood (PB), a critical aspect in MM due to the nature of the disease. Thus, there is a need to study the dynamics and evolution of CAR T-cells after infusion in both compartments to increase our knowledge and identify key factors responsible of long-lasting CAR T-cell responses in MM.

In this study, we interrogated longitudinal CAR T-cell samples from patients presenting different clinical outcomes enrolled in the CARTBCMA-HCB-01 trial (NCT04309981),^{26,27} a phase 1 to 2, single-arm, multicenter study assessing ARI0002h in R/R MM previously treated with a proteasome inhibitor, an immunomodulatory drug, and an anti-CD38 antibody. ARI0002h is a humanized BCMA-targeted CAR with a CD8 α hinge and transmembrane region, a 4-1BB costimulatory domain, and a CD3 ζ signaling domain.²⁸ Using single-cell RNA and T-cell receptor (TCR) sequencing coupled with SimiC,¹⁵ a machine learning algorithm for GRN inference, we characterized ~50 000 CAR T-cells from 11 samples, including infusion products (IPs) and CAR T-cells isolated from BM and PB at 1 and 3 months after infusion. We observed distinct regulatory networks in BM vs PB CAR T cells, identifying specific regulons driving the activated and exhausted BM phenotypes. Moreover, we define regulatory dysfunctions in the effector-to-memory axis promoting the lack of CAR T-cell persistence. Finally, we identify a hyperexpanded BM CAR T-cell

clone in a patient with partial response (PR) expressing interleukin-10 (IL-10) and terminal differentiation-associated genes, suggesting a potential mechanism for CAR T-cell failure in this patient.

Methods

Patient samples, clinical data, and CAR T-cell isolation

PB and BM samples were collected at the indicated time points from patients with MM enrolled in the academic clinical trial CARTBCMA-HCB-01 (NCT04309981).^{26,27} All participants provided written informed consent. Clinical response was defined according to the MM response criteria²⁹ as follows: stringent complete response (sCR), very good PR, PR, and progressive disease. PB and BM samples were processed as previously described.^{16,26} Briefly, after bulk lysis for erythrocyte elimination, CAR T cells were incubated with scFv-BT (Jackson ImmunoResearch), washed with phosphate buffered saline/bovine serum albumin, and stained with streptavidin-BV421 (BD Biosciences). CAR T cells were sorted either with BD FACSAria IIu or MoFlo Astrios BC. Data were acquired on BD FACSCanto II (BD Biosciences) and analyzed using the FlowJo software version 10 (Tree Star). Samples were prioritized for clinical monitoring (minimal residual disease) and flow cytometry. Single-cell RNA sequencing (scRNA-seq) was performed on the subset of patients ($n = 3$) for whom sufficient excess material was available.

scRNA-seq and single-cell V(D)J sequencing data

Fluorescence-activated cell sorter-sorted CAR T-cells from BM and PB samples of patients with MM were subjected to scRNA-seq and single-cell TCR α/β sequencing (scTCR-seq) using the Chromium Single Cell 5' Reagent Kit and Single Cell V(D)J Immune Profiling Solution (10X Genomics). Gene expression and variable-diversity-joining (VDJ) libraries were sequenced at a minimum depth of 30 000 and 5000 reads per cell, respectively; aligned to the human reference (GRCh38); and processed using Cell Ranger (v6.0.1) and Seurat (v3.1.5). Cell doublets (predicted with Scrublet) and cells with <250 genes, >30 000 unique molecular identifier (UMI) counts, >10% of mitochondrial RNA, and/or no detectable expression of CD3 or CAR were computationally removed. A total of 47 856 cells passed the quality control, yielding an average of 2257 genes per cell. scRNA-seq count matrices were merged and log normalized, and batch correction across patients was performed with Harmony using default parameters. Dimensionality reduction was performed using t-distributed stochastic neighbor embedding (t-SNE) and unsupervised clustering analysis with the resolution set to 0.8. Clusters were named using canonical marker genes as reference. TCR analysis was performed using scRepertoire.

Cell lines, retroviral transduction, and murine CAR T-cell generation

The Platinum Ecotropic (ATCC) cell line was cultured in Dulbecco's Modified Eagle's Medium, and MM5080 cell line³⁰ was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. All culture media were supplemented with 10% fetal calf serum, antibiotics, and 2mM glutamine. All cells were cultured at 37°C in a humidified atmosphere with 6.5% CO₂. Retroviral vector coding the murine CAR construct targeting mouse BCMA was generated as previously described.³¹ Briefly, Platinum Ecotropic cells were

transfected with the retroviral plasmid and 2 pCL-Ecoplasmid DNA using Lipofectamine 2000 (Invitrogen). Supernatants were collected at 48 and 72 hours after transfection. CD8⁺ cells were magnetically separated from the spleens and lymph nodes of CD45.1 OT-1 mice with the CD8a⁺ T Cell Isolation Kit (Miltenyi), following the manufacturer's instructions. Purified mouse CD8⁺ T cells were activated with CD3/CD28 dynabeads in complete RPMI medium containing 100 International Units (IU)/mL IL-2. After 24 hours, activated T cells were transduced with retroviral supernatants and spun at 2000g at 32°C for 90 minutes. Infection was repeated 1 day later. After infection, lymphocytes were cultured in complete RPMI medium with IL-2 until day 5, and BCMA CAR T-cells were fluorescence-activated cell sorter purified based on green fluorescent protein expression.

Analysis of proliferating and cytokine quantification

Proliferation was assessed by [methyl-3 H]-thymidine incorporation. Cells were centrifuged (500 relative centrifugal force [rcf], 5 minutes) supernatants were removed, and 0.5-μCi [methyl-3 H] thymidine was added per well. After 6- to 8-hour incubation, cells were harvested (Filtermate 96 harvester; Packard Instrument), and radioactivity was measured with a scintillation counter (TopCount; Packard Instrument). IL-10 and interferon gamma (IFN-γ) levels were quantified via mouse enzyme-linked immunosorbent assay kits (BD OptEIA), following the manufacturer's instructions. IFN-γ-producing CAR T-cells were measured using enzyme-linked immunospot (BD Biosciences). Cells were plated with irradiated MM5080 cells at tumor-to-CAR ratios of 1:1, 0.25:1, 0.1:1, and 0:1 for 24 hours. Spot-forming cells were counted using an automated enzyme-linked immunospot reader (CTL, Aalen, Germany).

Analysis of murine CAR T-cell response to BCMA, SIINFEKL, and IL-10

OT-1 BCMA CAR T-cells (1 × 10⁶ cells per mL) were seeded in 96-well plates precoated with recombinant mouse BCMA (rBCMA; 30 ng/mL; R&D Systems). SIINFEKL (0.1 ng/mL) was added to appropriate wells in complete RPMI medium. After 24-hour incubation, supernatants were collected for cytokine analysis. For sequential CAR-TCR stimulation, cells were first incubated on rBCMA-coated plates for 48 hours, then harvested, counted, and transferred to new plates containing SIINFEKL in RPMI. After 24 hours, supernatants were collected for cytokine detection. In some experiments, CAR T-cells were cultured for 3 days in complete RPMI with or without 5 ng/mL recombinant mouse IL-10 (rIL-10; PeproTech). Then, 1 × 10⁶ cell/mL were seeded into rBCMA-coated 96-well plates containing 5 ng/mL rIL-10. After 24 hours, proliferation and IFN-γ production were assessed.

Statistical analysis

Statistical analyses were performed using R version 4.1.3 and GraphPad Prism version 10.1.0. The different tests used in this work are indicated in the figure legends.

Results

Expansion of CAR T cells after infusion is preferentially driven by CD8⁺ T cells

To deepen into the transcriptomic programs governing CAR T-cell therapies, we analyzed longitudinal samples of CAR T-cells from

patients with MM treated with BCMA CAR T-cells enrolled in the CARTBCMA-HCB-01 (NCT04309981)^{26,27} clinical trial. Specifically, CAR T cells, collected from IPs as well as paired BM and PB samples at 1 and 3 months after infusion from 3 patients with R/R MM (Figure 1A-B), were profiled using scRNA-seq and scTCR-seq. After quality control and filtering, a total of 47 855 CAR T-cells (expressing CD3 and CAR; supplemental Figure 1A) were included in the analysis (median, 3847 cells per sample; range, 384-10 282; supplemental Table 1). Unsupervised clustering of integrated samples yielded 12 clusters (Figure 1C), composed of CAR T cells from all 3 patients and were defined according to the expression of canonical markers¹⁶ (Figure 1D; supplemental Figure 1B). We observed remarkable differences between IP and postinfusion samples, as reported previously.²⁰ Although IP included a diversity of CAR T-cell populations with proliferating cells, activated and memory CD4⁺ and CD8⁺, and small population of CD8⁺GNLY⁺ effector cells, postinfusion CAR T-cells were mainly composed of nonproliferating CD8⁺ CAR T-cells with effector and effector-memory phenotypes (Figure 1C-E; supplemental Figure 1A). This preferential expansion of CD8⁺ subset, representing almost 86% of cells after infusion, was also observed in most of the patients enrolled in the clinical trial and would be in accordance with the requirement of cytotoxic effector cells during the early phases of CAR T-cell therapy (supplemental Figure 1C). Differential expression analysis showed that IPs over-expressed genes linked to proliferation (*MKI67*, *TUBB*, and *TUBA*), activation (*HLA* class II and *CD25*), and memory (*CD62L* and *CCR7*), consistent with the observed CAR T-cell populations (Figure 1F; supplemental Table 2). In contrast, postinfusion CAR T-cells showed higher expression of cytotoxicity-associated genes (*NKG7*, *GZMK*, *GZMH*, and *GNLY*) and exhaustion markers (*NR4A2*^{32,33} and *PRDM1*³⁴), indicating a more terminally differentiated CD8⁺ CAR T-cell profile (Figure 1F; supplemental Table 2). To further understand postinfusion CAR T-cell function, we conducted a GRN analysis using SimiC.¹⁵ We identified 63 regulons with increased activity in postinfusion samples (supplemental Figure 1D; supplemental Table 3) that were linked to T-cell signaling (*JUN* and *FOS*³⁵) and terminal differentiation/exhaustion (*CREM*³⁶ and *ZEB2*³⁷), aligning with the transcriptomic profile of infused CAR T-cells (Figure 1G; supplemental Figure 1E). Notably, the *ZEB2* regulon correlated with effector genes (*GZMB*, *NKG7*, *CCL5*, and *GZMM*), highlighting its role in cytotoxic phenotype acquisition (Figure 1H). GRN analysis also revealed increased activity of several zinc finger factors (*ZNF800*, *ZNF292*, and *ZNF394*) associated with cell differentiation,³⁸ cell cycle suppression,³⁹ and AP1/c-JUN inhibition,⁴⁰ respectively (supplemental Figure 1F). Although their roles in T-cells remain unclear, these regulons may represent novel regulators of CAR T-cell function in vivo.

Postinfusion CD8⁺ CAR T cells exhibit an effector-to-memory phenotypic transition

Having established the global transcriptomic landscape, we next focused on the most relevant postinfusion CAR T-cell populations to see their changes over time. We identified 2 transcriptionally distinct populations within the effector subset (Figure 2A): CD8⁺ terminally differentiated effector (CD8^{TE}) cells, with increased expression of activation, cytotoxic, exhaustion, and terminal differentiation markers; and CD8⁺ effector (CD8^{EF}) cells, enriched in

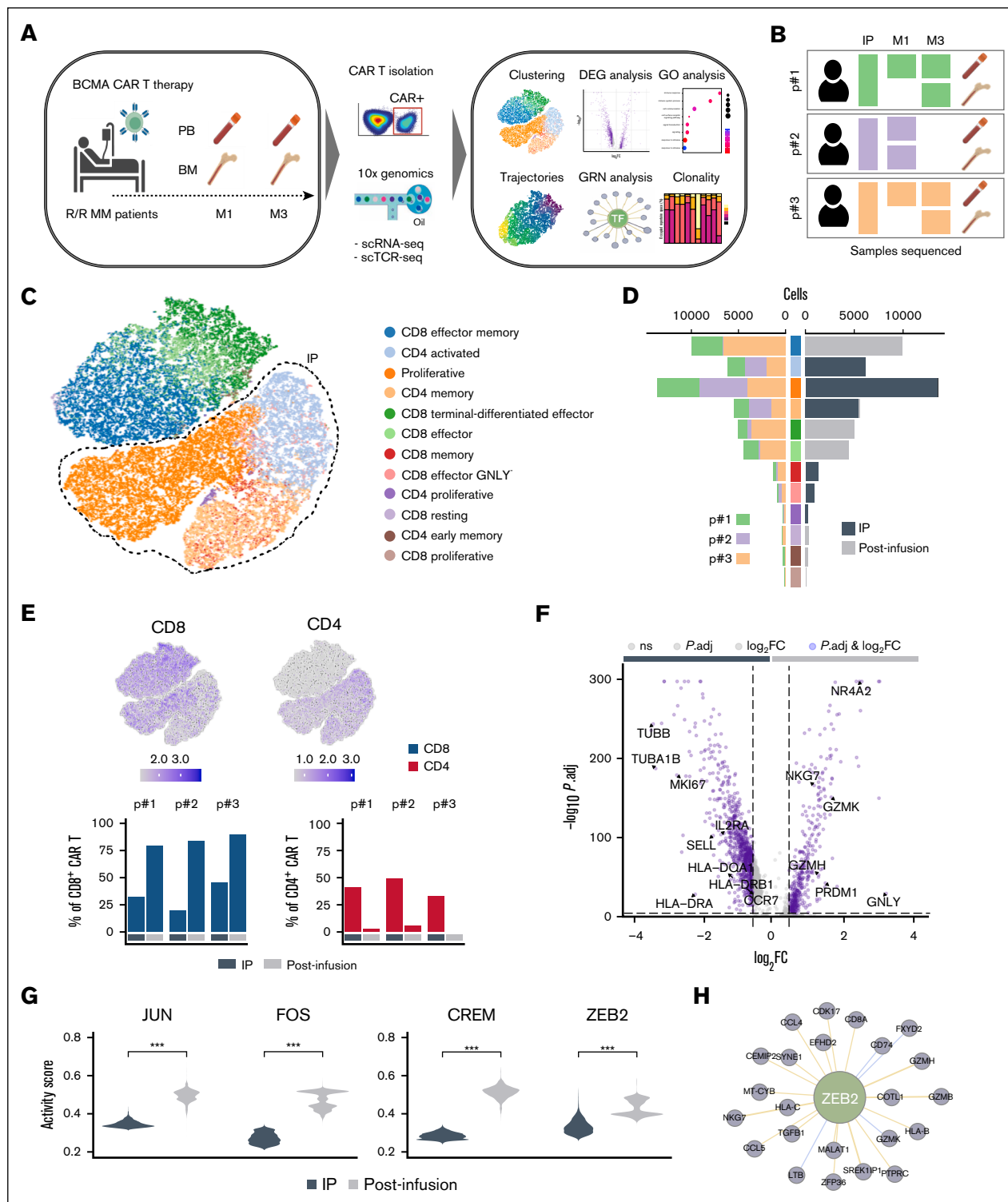


Figure 1. Preferential expansion of CD8⁺ CAR T cells with effector transcriptomic profiles after infusion. Transcriptomic analyses were performed on CAR T cells collected from patients with MM at different times after infusion (months 1 [M1] and 3 [M3]) and their paired IP. (A) Schematic representation of the followed pipeline. CAR-positive cells were FACS-isolated sorted from PB and BM samples. Then, RNA was extracted and scRNA-seq and scTCR-seq were performed. (B) Schematic representation of samples analyzed. (C) t-SNE plot of the 47 855 CAR T cells that passed quality control and filtering for subsequent analysis in the study. CAR T-cell populations were defined according to the expression of canonical markers. (D) Relative contribution of each sample to the defined CAR T-cell populations in terms of patient (left) or time point (right). (E) t-SNE plots showing the expression of CD8A and CD4, with the proportions of CD8⁺ and CD4⁺ CAR T cells in IP and postinfusion samples in each patient. (F) Differentially expressed genes (DEGs) between CD8⁺ CAR T cells in IP vs postinfusion samples (adjusted $P < .01$; $|\log \text{FoldChange}| > .25$). (G) Activity scores of regulons of interest in IP vs

translation and ribosome biogenesis pathways (Figure 2B; supplemental Figure 2A; supplemental Table 4). Moreover, CD8⁺_{TE} presented higher scores in effector-related hallmark signatures, including IFN- γ , tumor necrosis factor α (TNF- α) signaling and apoptosis (Figure 2C). These CD8⁺_{TE} and CD8⁺_{Eff} cell subpopulations, together with the CD8⁺ effector-memory cells (CD8⁺_{EM}), which showed decreased effector features compared to the CD8⁺_{Eff} subset (supplemental Figure 2B-C), constituted a postinfusion effector-to-memory axis (Figure 2A). Gene trajectory analysis performed across the defined effector-to-memory axis revealed decreasing expression of cytotoxic genes (*GZMB*, *GZMK*, *LAG3*, and *PRF1*) and transcription factors (TFs) with effector commitment (*PRDM1*, *ZEB2*) along pseudotime, with concomitant upregulation of memory-associated TFs (*TCF7*, *FOXP1*) (Figure 2D-E; supplemental Figure 2D). Mapping this axis over therapy showed that CD8⁺_{TE} cells were more abundant at month 1, whereas CD8⁺_{Eff} and CD8⁺_{EM} cells were more common by month 3 (Figure 2F), suggesting a shift from effector to memory phenotypes after infusion. Interestingly, a high-resolution reclustering of CD8⁺_{TE}, CD8⁺_{Eff}, and CD8⁺_{EM} populations revealed a bifurcating trajectory in the memory-effector axis (supplemental Figure 3A-D), with TCF7^{hi} memory cells (cluster 1) shifting toward 2 GZMB⁺ effector clusters: one with higher *PRF1* and *GZMK* expression (cluster 2) and another with expression of IFN-responding genes (cluster 3), which was found almost exclusively in patient 3 (supplemental Figure 3D-F). These results showcased interpatient heterogeneity, suggesting modulation of CAR T-cells in response to specific microenvironmental cues.

We validated the effector-to-memory transition with longitudinal flow cytometry analysis of samples from the CARTBCMA-HCB-01 cohort.²⁶ Results showed significant expansion of naïve/stem cell memory CAR T-cell populations at month 3, with central memory cells trending upward and effector memory populations declining (supplemental Figure 2E), indicating a shift toward a less differentiated phenotype, likely due to higher renewal and proliferative capacity.⁴¹ Although not significant, GZMB⁺ and CD57⁺ CD8⁺ CAR T cells slightly decreased at month 3, suggesting reduced cytotoxicity (supplemental Figure 2F). These results align with previous data,²⁶ showing transient early increases in inflammatory and effector cytokines (IFN- γ , Granzyme B, CXCL10, CCL3, IL-6, and IL-15) around 7 to 14 days after infusion, coinciding with CAR T-cell peak, followed by a progressive decline, reflecting transitional changes in CAR T-cell subpopulations.

CAR T cells infiltrating BM acquire a more differentiated and exhausted phenotype

Because MM is characterized by the presence of malignant plasma cells in the BM, we next compared CAR T cells obtained from BM and PB. Both compartments showed enrichment in CD8⁺ CAR T cells, with minor differences between patients (Figure 3A). Differential expression analysis identified 2142 differentially expressed genes in CD8⁺ CAR T cells ($|\log FC| > .10$; false discovery rate $< .05$; supplemental Table 5). Among genes upregulated in

BM, we found markers related to activation and cytotoxicity (*TNFRSF9/4-1BB*, *NKG7*, *PRF1*, *GZMH*, and *GZMB*), terminal differentiation (*DDX5* and *PRDM1*), and exhaustion (*LAG3* and *HAVCR2/TIM-3*). In contrast, genes upregulated in PB were associated with memory (*CD7*), activation (*FOS* and *JUN*), and proliferation (*TUBA1A*), consistent with differences in scores of the growth 2/mitosis (G2M) phases of the cell cycle (Figure 3B; supplemental Figure 4A). Other exhaustion terminal differentiation markers such as *NR4A2*, *TOX*, *CTLA4*, and *KLRG1* were also higher among BM CAR T cells (supplemental Figure 4B). Analyzing differentially expressed genes between BM and PB within individual patients revealed limited overlap (Figure 3C; supplemental Table 6), but genes upregulated in BM were consistently enriched in pathways related to T-cell activation and adhesion (Figure 3D), indicating common functional features in CAR T-cells from BM. GRN analysis identified 99 regulons with differential activity, 67 more active in BM and 32 in PB (supplemental Figure 4C; supplemental Table 7). Notable BM regulons included *BHLHE40* and *ZEB2* (involved in terminal differentiation of T cells),^{37,42} *PRDM1* (T cell dysfunction³⁴), and *IRF1* (IFN and antiproliferative responses⁴³; Figure 3E). In contrast, PB samples showed higher activity of *TCF7*, *KLF2*, and *JUN* family regulons (supplemental Figure 4D), aligning with overall transcriptomic differences between compartments. Finally, we extended the analysis to a larger patient cohort,²⁷ comparing CAR T-cell immunophenotypes from 42 paired BM and PB samples 1 month after infusion. BM-derived CAR T cells showed higher expression of exhaustion markers TIM-3, TIGIT, and PD-1 in both CD8⁺ and CD4⁺ subsets (Figure 3F), supporting a more exhausted phenotype in the BM. Collectively, these findings suggest that CAR T cells encountering tumor cells in the BM acquire a cytotoxic and terminally differentiated transcriptional profile, contributing to a more advanced effector-to-memory phenotype within the BM.

Limited effector-to-memory transition restricts CAR T-cell persistence

Because our study included samples from 3 patients with MM with differing CAR T-cell persistence and responses (supplemental Table 8), we analyzed patient-specific transcriptomic programs to uncover mechanisms underlying these differences. All patients showed CAR T-cell expansion after infusion, with patient 3 exhibiting substantial higher peaks in both BM and PB^{26,27} (Figure 4A; supplemental Figure 5). Patient 3 maintained detectable BM CAR T cells up to month 23, whereas patients 1 and 2 lost BM CAR T cells around months 6 and 12, respectively. CAR T cells isolated from patient 3 exhibited less differentiated profile in the effector-to-memory axis early after infusion (CD8⁺_{Eff} in BM and CD8⁺_{EM} in PB), whereas patients 1 and 2 presented more CD8⁺_{TE} and CD8⁺_{Eff} cells (Figure 4B). Similarly, the high-resolution analysis of CD8⁺ populations showed a relatively lower presence of the cytotoxic cluster 2 in patient 3, with 25% of cells corresponding to the IFN-response cluster 3 in the BM (supplemental Figure 6A). Cluster 2 expressed higher levels of activation (*TNFRSF9/4-1BB*,

Figure 1 (continued) postinfusion samples. (H) Representation of ZEB2 regulon depicting target genes with a positive (yellow) or negative (blue) correlation. Wilcoxon test for unpaired samples with Benjamini-Hochberg (BH) procedure for *P* value correction (panel G). Postinfusion data represent an aggregation of all samples collected from PB and BM at months 1 and 3 (panels D-G). ****P* < .001, ns, *P* > .05; DEG, differential gene expression; GO, gene ontology; GRN, gene regulatory network; IP, infusion product. Figure 1A-B created, in part, with biorender.com. Ariceta, B. (2026) <https://BioRender.com/1qoc709>.

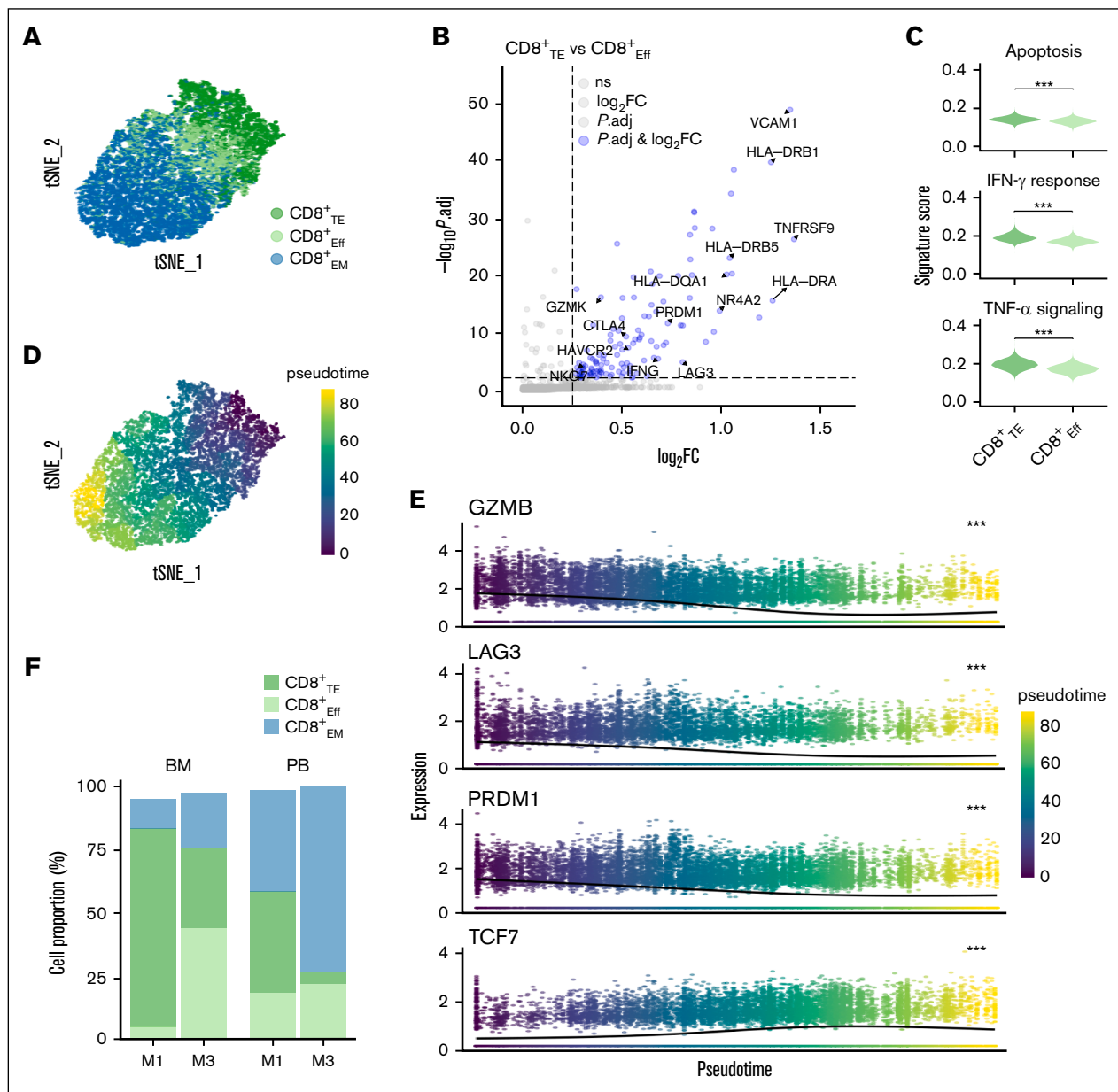


Figure 2. Postinfusion CAR T cells undergo a phenotypic transition along the effector-to-memory axis. Postinfusion CAR T cells were further characterized, focusing on the CD8⁺ cells with effector and effector-memory phenotypes. (A) t-SNE plots showing distribution of CD8⁺ TE, CD8⁺ Eff, and CD8⁺ EM populations within postinfusion CAR T cells. (B) Upregulated genes in CD8⁺ TE vs CD8⁺ Eff CAR T cell clusters (adjusted $P < .01$; $\log_2FC > 0.25$). (C) Scoring for apoptosis, IFN- γ , and TNF- α gene signatures in CD8⁺ TE and CD8⁺ Eff CAR T cells. (D) t-SNE plot showing pseudotime analysis spanning through the effector-memory axis of postinfusion CAR T cells. (E) Expression of *GZMB*, *LAG3*, *PRDM1*, and *TCF7* genes along the pseudotime. (F) Proportion of main postinfusion CAR T-cell clusters in BM and PB at months 1 and 3 after infusion. Clusters other than CD8⁺ EM, CD8⁺ Eff, and CD8⁺ TE were excluded from the visualization. Wilcoxon test for unpaired samples with BH procedure for P value correction (panel C) and Natural cubic splines regression (panel E). *** $P < .001$. ns, $P > .05$; BM, bone marrow; PB, peripheral blood.

HLA-DRB5, and *HLA-DQA2*) and exhaustion (*HAVCR2/TIM-3* and *CTLA4*) markers than cluster 3 (supplemental Figure 6B), supporting a more terminal phenotype in patients 1 and 2.

In the CARTBCMA-HCB-01 cohort ($N = 42$),²⁷ early enrichment of terminally differentiated cells (TEMRA) at month 1 negatively correlated with CAR T-cell levels at month 3 ($r_s = -0.372$; $P = .015$; Figure 4C-D), suggesting that early terminal differentiation

limits long-term persistence. Similarly, higher CD8⁺CD57⁺ percentages at month 1, associated with terminal differentiation and senescence,^{44,45} negatively correlated with month 3 CAR T-cell levels ($r_s = -0.379$; $P = .010$; supplemental Figure 7A). Transcriptomic CD8⁺ TE cells were enriched in CD57 compared with other postinfusion clusters (supplemental Figure 7B), reinforcing a terminally differentiated phenotype. Interestingly, a negative correlation was also detected between naïve/stem memory

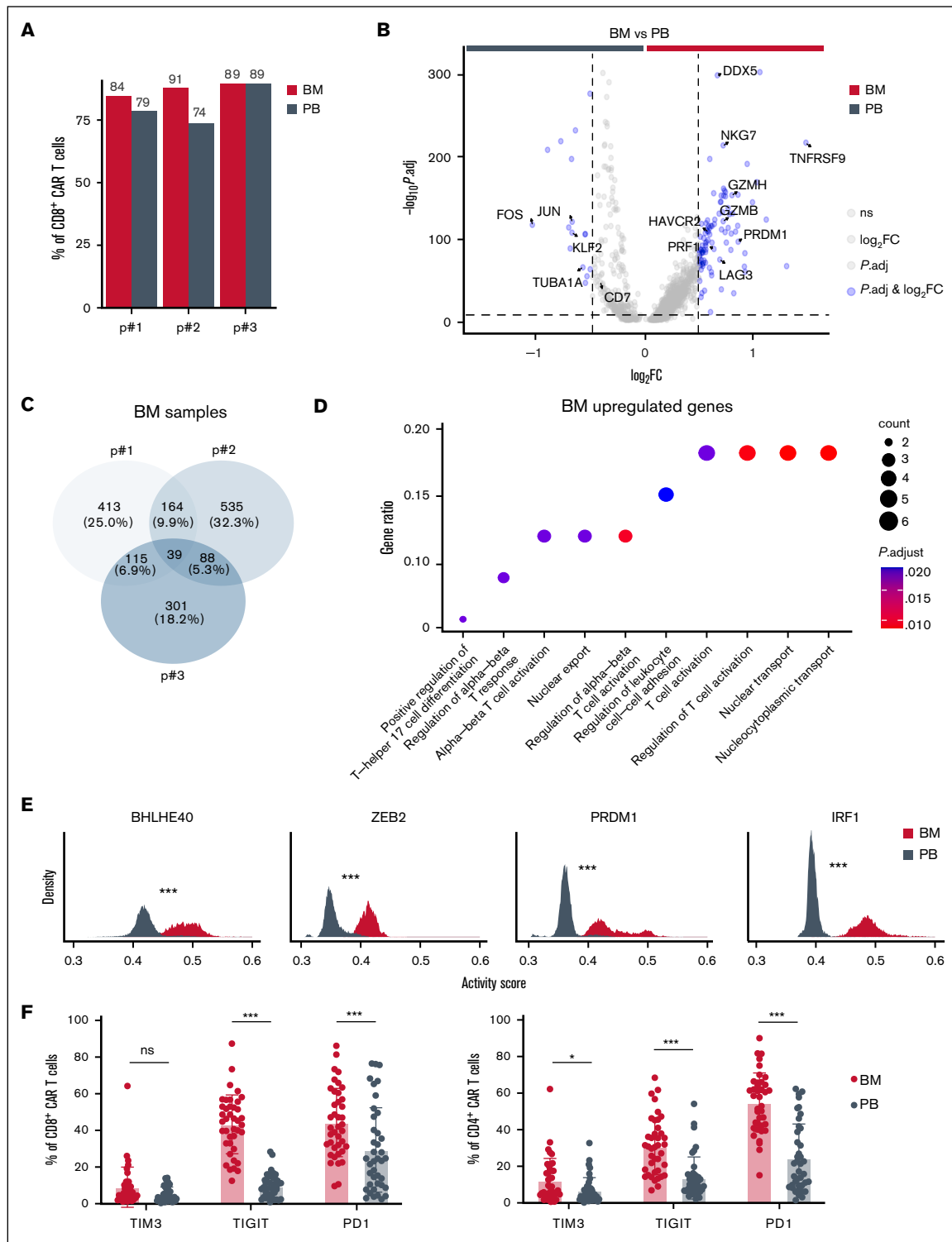


Figure 3. CAR T cells infiltrating the BM acquire a more differentiated and exhausted phenotype. Comparative analysis of paired CAR T-cell samples from bone marrow (BM) and peripheral blood (PB) was conducted. (A) Proportion of CD8⁺ CAR T cells per patient and location. (B) DEGs between paired BM and PB samples (adjusted $P < .01$; $|\log \text{FoldChange}| > .5$). (C) Intersection of DEGs among BM samples of each patient. (D) Pathway enrichment analysis of genes upregulated in the BM in all 3 patients (adjusted $P < .05$). (E) Activity scores of *BHLHE40*, *ZEB2*, *PRDM1*, and *IRF1* regulons in CAR T-cells in BM and PB. (F) Immunophenotype analysis of BM- and PB-derived CAR T-cells from the CARTBCMA-HCB-01 cohort (N = 42), 1 month after infusion. Gene ontology (GO) analysis with BH procedure for P value correction (panel D), Wilcoxon test for unpaired samples with BH correction (panel E), and Wilcoxon test for paired samples with 2-stage BH correction (panel F). *** $P < .001$. ns, $P > .05$.

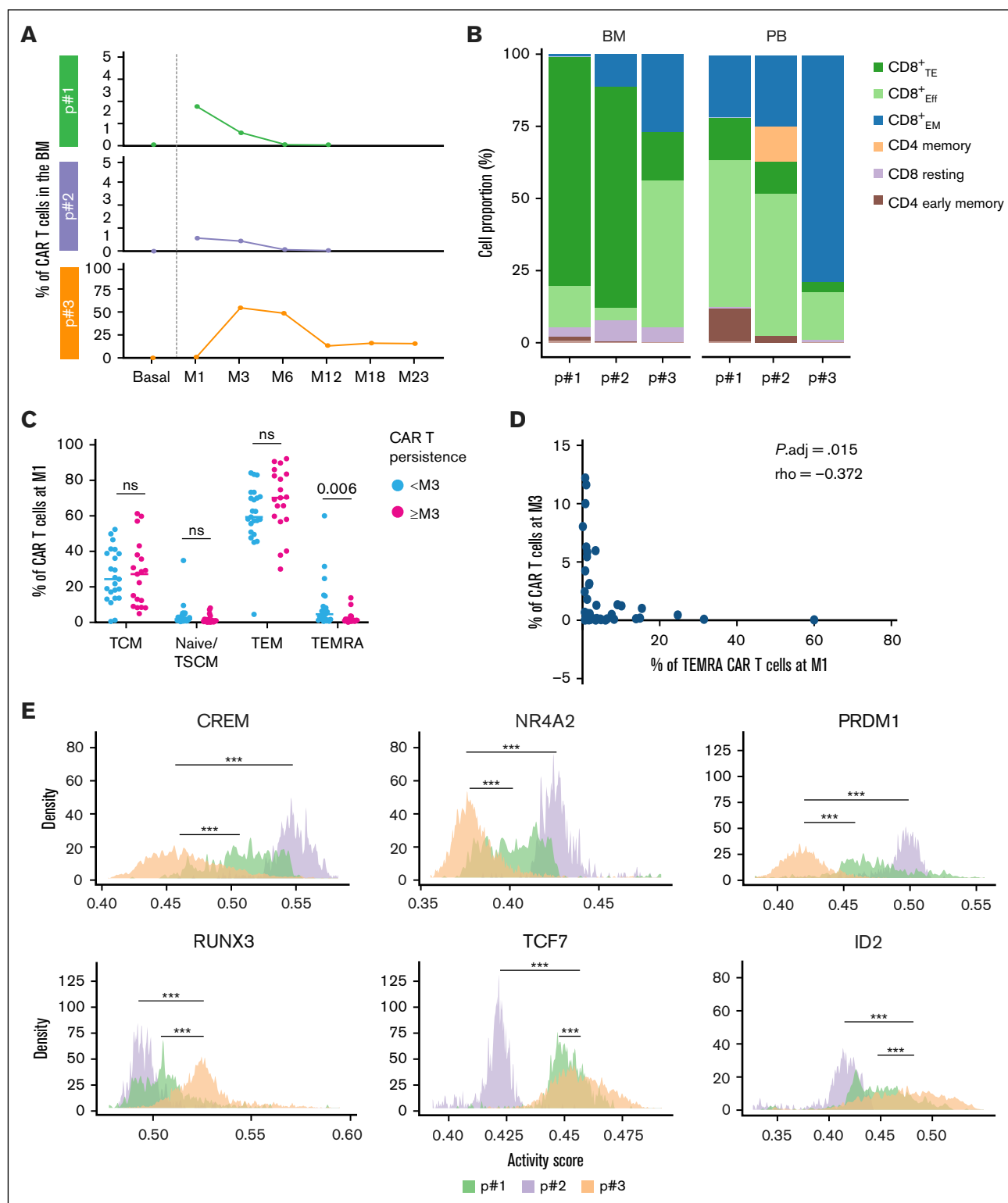


Figure 4. Gene regulatory dysfunctions in the effector-to-memory axis leads to lack of persistence. Transcriptomic features associated with long-term CAR T-cell persistence were analyzed. (A) Expansion of CAR T-cells (percentage of total cells) in the BM in each patient. Data were obtained at different times after infusion until end of treatment. (B) Composition of postinfusion CAR T cells present in the bone marrow (BM) and peripheral blood (PB) of each patient. (C) Percentage of effector and memory subsets (TEMRA, T terminal effector, CD62L⁺/CD45RA⁺; TEM, T effector memory, CD62L⁺/CD45RA⁺; TCM, T central memory, CD62L⁺/CD45RA⁺; naive/TSCM, T stem-cell memory, CD62L⁺/CD45RA⁺) present in patients of the CARTBCMA-HCB-01 cohort (N = 42) with low (<M3) and long (≥M3) CAR T-cell persistence. (D) Correlation analysis between the levels of TEMRA CAR T cells at month 1 and the presence of CAR T cells at month 3 in the BM of patients of the CARTBCMA-HCB-01 cohort (N = 42). (E) Activity scores of *CREM*, *NR4A2*, *PRDM1*, *RUNX3*, *TCF7*, and *ID2* regulons in CAR T cells from BM of each patient. Wilcoxon test for unpaired samples with 2-stage BH correction (panel C), Spearman correlation

CAR T cells at month 1 and CAR T-cell levels at month 3 (supplemental Figure 7C), which may suggest that insufficient CAR engagement and T-cell activation early after infusion can similarly impair long-term persistence.

GRN analysis of BM samples revealed shared regulatory trends across patients, indicating conserved CAR T-cell-driving GRNs with variable activity levels (supplemental Figure 7D). Patient 3 exhibited lower activity of exhaustion-associated regulons (*CREM*, *NR4A2*, and *PRDM1*) and higher activity of memory- and infiltration-related factors (*RUNX3*, *TCF7*, and *ID2*⁴⁶⁻⁴⁸; Figure 4E), consistent with sustained CAR T-cell persistence and a less differentiated phenotype. In contrast, patients 1 and 2 presented higher activity of dysfunction-related regulons. These findings suggested that differential regulon activity may shape CAR T-cell fate and persistence in vivo.

Clonality analysis identifies a hyperexpanded clone in the BM of a patient with PR

In the CARTBCMA-HCB-01 trial, 90% of patients achieved very good PR or better within 100 days after infusion, whereas 7% had PR^{26,27}. In particular, patients 1 and 3 reached sCR, with minimal residual disease negativity at month 1, whereas patient 2 had PR and progressed by month 11 (supplemental Table 8), although no significant transcriptional differences between patients in PR and sCR were observed. scTCR-seq showed similar CAR T-cell diversity across samples from the 3 patients analyzed (Figure 5A-B), with a decreased clonotype repertoire after infusion while maintaining >75% of unique clonotypes, in accordance with other previous works.¹⁸ Notably, BM sample from patient 2 presented reduced Shannon and abundance-based coverage estimator diversity indices and a reduced clonotype repertoire with only ~30% unique clonotypes, which was not found in patients 1 and 3 (Figure 5A-B; supplemental Figure 8A). Moreover, clonal space was mainly dominated (~70%) by a single clone, herein referred to as clonotype 1 (Figure 5C). This clonotype 1 mostly belonged to the CD8⁺TE cluster (Figure 5D) and was absent in paired PB and IP samples, indicating BM-specific expansion. Transcriptomic analysis showed upregulation of activation (*HLA* class II and *4-1BB*), cytotoxicity (*NKG7*, *PRF1*, and *GZMB*), and exhaustion (*LAG3*) markers (Figure 5E; supplemental Figure 8B), and GO and gene signature analysis linked this clone to T-cell activation, antigen response, apoptosis, and exhaustion (Figure 5F-G). More interestingly, IL-10, an immunosuppressive cytokine that has been shown to be produced by highly activated effector CD8⁺ cells,^{49,50} was also upregulated in clonotype 1 (Figure 5E; supplemental Figure 8B). GRN analysis identified effector- and differentiation-related TFs (*FOS*, *BHLHE40*, *PRDM1*, and *CREM*) as potential IL-10 regulators (Figure 5H). A closer look showed that IL-10⁺ cells were detected only in the BM of patient 2 and mainly in clonotype 1 (Figure 5I-J), whereas they were scarce or absent in patients 1 and 3 (supplemental Figure 8C). Moreover, analysis of publicly available BCMA CAR T-cell data sets²⁵ revealed no IL-10⁺ cells after infusion (supplemental Figure 8D), whereas in CD19 CAR T-cell data sets,^{17,18} IL-10⁺ cells appeared

only in long-term samples, being predominantly CD4⁺ or double-negative cells but not CD8⁺ cytotoxic cells (supplemental Figure 8E-H), suggesting that BM-specific expansion of IL-10⁺ CD8⁺ CAR T cells in patient 2 was a rare event.

Because CAR T-cell activation usually drives polyclonal proliferation, and clonal CAR T-cell expansions may result from hyperactivation or genetic factors such as *TET2* mutation,^{51,52} we assessed whether endogenous TCR signaling contributed to the transcriptomic profile of clonotype 1. Using a TCR-specific signature generated from publicly available data,⁵³ BM CAR T cells from patient 2 showed higher TCR activation than PB counterparts, with BM IL-10⁺ cells exhibiting higher scores than IL-10[−] cells (supplemental Figure 9A; supplemental Table 9), suggesting TCR engagement despite no reported infection at the time of sample isolation (supplemental Table 8). In OT-1 mice, independent stimulation of TCR and CAR induced dose-dependent IL-10 and IFN-γ production (supplemental Figure 9B-D), whereas combined or sequential stimulation further enhanced IL-10 production (supplemental Figure 9E), consistent with previously described cooperative signaling⁵⁴ and suggesting that CAR activation primes T cells for TCR-induced IL-10. Functionally, recombinant IL-10 inhibited CD8⁺ CAR T-cell proliferation and tended to elevate IFN-γ (supplemental Figure 9F-G), potentially driving exhaustion via immune checkpoint upregulation.⁵⁵

In summary, the CARTBCMA-HCB-01 trial achieved deep, rapid responses in most patients; however, in patient 2, disease progression was linked to a BM-restricted clonal expansion of IL-10⁺ CD8⁺ CAR T cells. This dominant clonotype exhibited strong activation, cytotoxicity, and exhaustion and was linked to IL-10 upregulation. Functional assays indicated that combined CAR and endogenous TCR signaling can enhance IL-10 secretion, potentially affecting CAR T-cell functionality. These results suggest that a rare BM-specific IL10⁺ effector CAR T-cell expansion may have compromised CAR T-cell efficacy in this patient.

Discussion

CAR T-cell therapies have transformed the treatment landscape for R/R MM, improving both overall survival and disease-free survival. However, challenges remain because relapse of the disease remains constant. In this study, the use of single-cell multiomics allowed us to uncover molecular and transcriptional mechanisms underlying CAR T-cell efficacy and persistence, which were further validated.

Results showed preferential expansion of CD8⁺ CAR T cells after infusion, with CD4⁺ cells representing <2% of postinfusion cells. Low CD4 expression may underestimate this population, but CD8⁺ cell still accounted for 86% of postinfusion cells, consistent with prior BCMA CAR T-cell studies.^{23,25} Although long-lasting CD4⁺ CAR T cells have been linked to durable remission in patients with leukemia,¹⁷ early CD8⁺ CAR T-cell expansion was observed in some of those patients,¹⁷ suggesting the importance of an initial cytotoxic CD8⁺ T-cell response for tumor control. Notably, our

Figure 4 (continued) analysis with BH correction (panel D), and Kruskal-Wallis and Dunn test with BH correction for post hoc pairwise comparisons (panel E). ns $P > .05$; *** $P < .001$. ns $P > .05$. M1 = month 1, M3 = month 3, M6 = month 6, M12 = month 12, M18 = month 18, M23 = month 23.

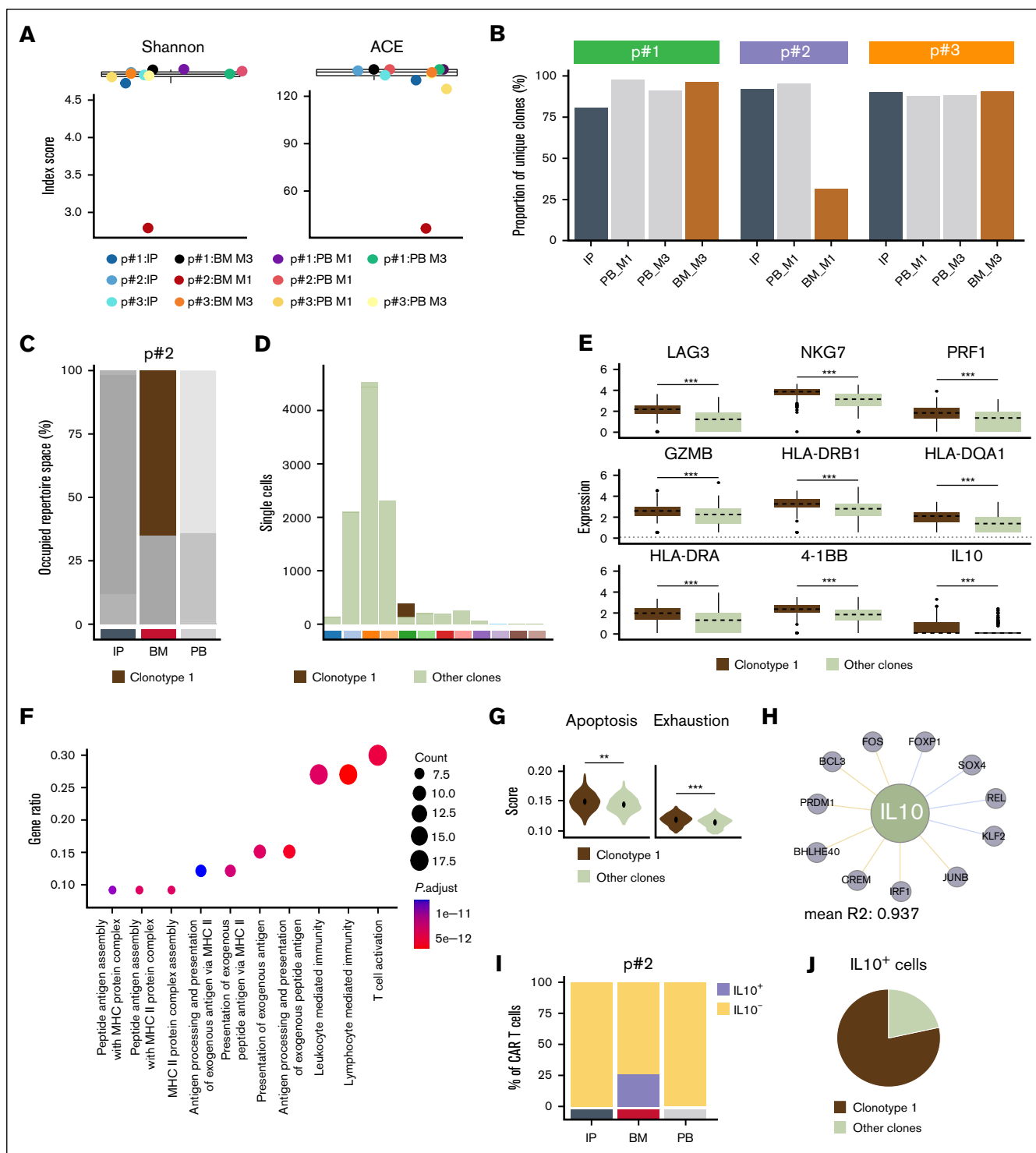


Figure 5. Clonality analysis reveals a hyperexpanded clone in the BM of a patient with PR. Clonal repertoire of CAR T cells from the different samples was evaluated by scTCR-seq. (A) Shannon and abundance-based coverage estimator (ACE) scores of each sample. (B) Percent of unique clonotypes in each sample. (C) Relative space occupied by the hyperexpanded clonotype 1 in the different sample from patient 2. (D) Distribution of the hyperexpanded clonotype 1 within the different CAR T-cell populations. (E) Differential expression of the indicated genes in clonotype 1 compared to other clones. (F) Analysis of pathway enrichment with genes upregulated in the clonotype 1 compared to other clones (adjusted $P < .05$). (G) Apoptosis and exhaustion signature scores between clonotype 1 and other clones. (H) IL-10 regulon depicting IL-10 as target and the TFs potentially regulating its activity indicating their positive (yellow) or negative (blue) correlation. (I) Percent of IL-10-expressing cells within the CAR T cells from infusion product (IP), bone marrow (BM), and peripheral blood (PB) samples of patient 2. (J) Percent of clonotype 1 cells among IL-10-expressing cells in the BM sample of patient 2. Wilcoxon test for unpaired samples with BH procedure for P value correction (panels E,G) and GO analysis with BH correction (panel F). ** $P < .01$; *** $P < .001$.

results revealed a negative correlation between early terminal differentiation and CAR T-cell persistence, underscoring the importance of memory phenotypes for long-term persistence. Effector and central memory subsets showed opposing trends to TEMRA cells, although not significant, and naïve CAR T cells at month 1 modestly negatively correlated with month 3 levels, suggesting that insufficient early CAR engagement may also impair persistence.

Paired sample analysis demonstrated that BM CAR T cells exhibited a more activated and terminally exhausted phenotype than PB cells, with upregulation of exhaustion markers and transcription factors such as *PRDM1* and *ZEB2*.^{36,51,52} Conversely, PB CAR T cells expressed higher levels of memory-associated markers (*TCF7*⁴⁷ and *JUN*^{20,35}) and regulatory networks supporting persistence. *JUN* overexpression in CAR T cells has been shown to reduce terminal differentiation in mouse tumor models,⁵⁶ consistent with our observation that PB provides a more favorable environment for maintaining functional CAR T cells. These findings suggest that direct interaction with MM cells in the BM drives terminal differentiation, whereas PB serves as a reservoir for less exhausted, longer-lasting CAR T cells, aligning with prior reports.²⁵

TCR clonality analyses revealed greater diversity in patients achieving sCR, whereas the patient with PR showed reduced CAR T-cell diversity and BM-specific hyperexpansion of an IL-10⁺ CD8⁺ terminal effector clone. This clone expressed high levels of activation, cytotoxicity, and exhaustion genes and was absent in PB and IP, indicating local BM expansion. Although clonal expansion at 1 month after infusion is common in BCMA CAR T-cell therapy,²⁵ prior studies reported broader expansion in patients with CR across PB and BM.²⁵ In contrast, we observed hyperexpansion restricted to the BM in the patient with PR, suggesting localized clonal proliferation.

Although traditionally associated with CD4⁺ T-cell immunoregulation,⁵⁷ IL-10 can be produced by effector CD8⁺ T cells during peak infection, likely to limit inflammation.⁴⁹ Several studies challenge the view of IL-10 as purely immunosuppressive,^{58,59} showing it can induce CD8⁺ tumor-specific responses and immune surveillance.⁶⁰ A recent study reported beneficial effects of IL-10-secreting CAR T cells against solid tumors,⁶¹ with increased granzyme B and IFN- γ production.⁶¹ Consistently, we observed increased IFN- γ levels during CAR T-cell and tumor cell coculture in presence of recombinant IL-10, partly due to increased number of cells producing the cytokine.

Mechanistically, IL-10 expression was driven by dual activation via CAR and endogenous TCR. In OT-1 CAR T cells, IL-10 production increased with CAR or TCR stimulation alone and further with combined or sequential stimulation, suggesting that prolonged or repeated dual activation promotes IL-10 expression. This raises the intriguing possibility that interactions between the CAR and endogenous TCR may play an underappreciated role in shaping CAR T-cell fate as has been previously described.^{54,62}

Despite the exploratory nature of the study, our findings provide actionable insights for optimizing CAR T-cell therapy in MM. First, our results show that persisting BM CAR T cells acquire a memory phenotype, whereas TEMRA fail to persist, suggesting that strategies to prevent terminal differentiation may improve persistence. Second, BM CAR T cells upregulate exhaustion markers, suggesting that CAR T-cell therapy in the context of MM may benefit from combination with immune checkpoint inhibitors targeting

TIGIT or PD-1. Finally, consistent with Rade et al,²⁵ we observed BM CAR T-cell expansion, which in this case was linked to terminal differentiation and IL-10 production. Notably, secretion by CD8⁺ CAR T cells is a rare phenomenon that has not been observed in other studies with CD19-targeted CAR T cells and appears driven by strong CAR or TCR activation. Further investigation into the role of IL-10 and TCR engagement in CAR T-cell dysfunction could pave the way for novel interventions to prevent clonal exhaustion and improve response durability in MM.

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Authorship

Contribution: M.H., J.R.R.-M., and F.P. designed the study; L.J.-U., G.S., S.C.-P., and M.E.C.-C. performed data analysis; L.J.-U., T.L., J.J.L., and J.R.R.-M. designed experimental validations; L.J.-U., S.R.-D., E.I., and T.L. performed wet laboratory experiments; P.R.-O., A.A.-P.,

E.T., and J.S.-M. provided clinical advice; A.Z., A.O.-C., M.E.-R., V.C., J.L.R., V.G.-C., M.V.M., F.S.-G., S.I., A.L.-D.d.C., A.G.-N., M.J., C.F.d.L., and B.P. provided clinical samples and/or data; P.S.M.-U. performed single-cell library preparation and sequencing; P.S.M.-U. and D.A. provided technical assistance; L.J.-U., G.S., S.C.-P., M.H., J.R.R.-M., and F.P. discussed study design and results; M.H., J.R.R.-M., and F.P. reviewed and edited the manuscript, and were responsible for research supervision, coordination, and strategy; L.J.-U. and J.R.R.-M. drafted the manuscript; and all authors reviewed and approved the final version of the manuscript.

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