

Original Article

1 α ,25(OH) $_2$ D $_3$ prevents CD19 CAR-T cell exhaustion and differentiation via VDR-dependent transcriptional reprogramming

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Abstract

CD19-directed chimeric antigen receptor T (CAR-T) cell therapy is promising for treating relapsed/refractory diffuse large B-cell lymphoma (R/R DLBCL), yet its long-term efficacy remains limited by CAR-T-cell exhaustion. Given the broad immunomodulatory activities of vitamin D, we investigate whether its active form, 1 α ,25(OH) $_2$ D $_3$, enhances CAR-T-cell functionality and improves therapeutic outcomes. We demonstrate that 1 α ,25(OH) $_2$ D $_3$ treatment significantly mitigates exhaustion and enhances the antitumor activity of CD19 CAR-T cells derived from both healthy donors and DLBCL patients, which is further validated in xenograft mouse models. Mechanistically, we show that 1 α ,25(OH) $_2$ D $_3$ upregulates the expression of the vitamin D receptor (VDR), promoting transcriptional reprogramming associated with memory-like differentiation and downregulation of exhaustion-related genes, thereby reshaping the functional heterogeneity of CAR-T cells under tumor stimulation. Our study highlights 1 α ,25(OH) $_2$ D $_3$ supplementation as a safe and accessible approach to mitigate terminal differentiation and exhaustion of CAR-T cells, offering a promising strategy to enhance the clinical efficacy of CAR-T therapy in patients with R/R DLBCL.

Key words chimeric antigen receptor T cells, diffuse large B-cell lymphoma, 1 α ,25(OH) $_2$ D $_3$, differentiation, exhaustion

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL), accounting for approximately 30%–40% of newly diagnosed cases worldwide [1,2]. DLBCL is a biologically and clinically heterogeneous disease that is often characterized by rapid progression and early relapse [3,4]. The current standard treatment for DLBCL is immunochemotherapy, which typically consists of rituximab, a monoclonal antibody

targeting CD20, combined with the CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone) [5]. While this treatment induces remission in many patients, approximately 30%–40% ultimately experience relapse or develop refractory disease, posing significant therapeutic challenges [6].

CD19-directed chimeric antigen receptor (CAR) T cell therapy has emerged as a transformative treatment for patients with relapsed or refractory (R/R) DLBCL by redirecting patient-derived T cells to

selectively recognize and eliminate CD19-positive lymphoma cells [6–8]. Multiple clinical trials have demonstrated durable responses and complete remission rates of 40%–58% in heavily pretreated patients, leading to the approval of several commercial CD19 CAR-T products for DLBCL treatment [9–12]. Despite these remarkable initial responses, the long-term efficacy of CAR-T therapy remains limited, as most relapses occur within the first year, and fewer than half of responders maintain ongoing responses after five years [13,14]. Notably, DLBCL patients who relapsed after CD19 CAR-T-cell therapy face poor outcomes, with a median overall survival (OS) shorter than 6 months [15,16]. This variability in the clinical outcomes of CD19 CAR-T treatment may be attributable to several factors, including intrinsic tumor resistance, immune evasion mechanisms within the tumor microenvironment, and, most critically, dysfunction of the infused CAR-T cells [17].

T cell exhaustion and terminal differentiation represent major forms of CAR-T cell dysfunction that lead to treatment failure [18,19]. Exhausted CAR-T cells exhibit low proliferative capacity, reduced cytokine production, and upregulated inhibitory receptor expression [18,20], whereas terminally differentiated effector T cells lose their self-renewal capacity and persist poorly *in vivo* [19]. Clinical studies have shown that a greater proportion of memory-like T cells in CAR-T products is correlated with superior clinical responses, whereas increased expression of exhaustion markers predicts early relapse [21–24]. Therefore, identifying strategies to preserve CAR-T-cell stemness and prevent premature exhaustion is critical for sustaining therapeutic efficacy [20,25].

1 α ,25-dihydroxyvitamin D $_3$ (1 α ,25(OH) $_2$ D $_3$), also known as calcitriol, is the biologically active form of vitamin D that binds to the vitamin D receptor (VDR), which acts as a transcription factor to regulate various biological processes [26,27]. In addition to its well-established role in calcium homeostasis and bone metabolism [28], 1 α ,25(OH) $_2$ D $_3$ has been shown to regulate diverse immune functions, particularly T-cell proliferation, differentiation, and cytokine production [29–31]. Notably, epidemiologic studies have linked low serum levels of 25-hydroxyvitamin D (25-OHD), a metabolic precursor of 1 α ,25(OH) $_2$ D $_3$, to increased cancer risk and poor outcomes in lymphoma patients [32,33]. More importantly, recent studies have suggested that DLBCL patients with low vitamin D levels exhibit worse clinical outcomes with CD19 CAR-T-cell therapy [34], whereas 1 α ,25(OH) $_2$ D $_3$ supplementation is associated with improved outcomes in DLBCL patients receiving rituximab-based treatment [35]. However, the mechanisms underlying this association remain poorly understood. Given the immunomodulatory effects of 1 α ,25(OH) $_2$ D $_3$, we hypothesized that 1 α ,25(OH) $_2$ D $_3$ may improve CAR-T-cell function by alleviating exhaustion and preserving memory phenotypes.

This study aimed to elucidate the mechanism by which 1 α ,25(OH) $_2$ D $_3$ enhances the therapeutic efficacy of CD19 CAR-T cells in DLBCL models. We found that signaling through the 1 α ,25(OH) $_2$ D $_3$ /VDR pathway downregulates the expression of inhibitory receptors (programmed cell death protein 1 (PD-1), T-cell immunoglobulin and Mucin-domain containing-3 (TIM-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT)) on CAR-T cells and reduces the proportion of terminally differentiated/exhausted T-cell subsets, thereby contributing to the maintenance and enhancement of CAR-T-cell antitumor functionality. This effect was validated *in vivo* via a DLBCL lymphoma xenograft model. To further dissect the mechanism of action of 1 α ,25(OH) $_2$ D $_3$, we

employed an integrated approach utilizing multiple techniques: bulk and single-cell RNA sequencing (scRNA-seq) characterized the transcriptional changes in CAR-T cells following 1 α ,25(OH) $_2$ D $_3$ treatment; the VDR-specific inhibitor MeTC7 was used to disrupt 1 α ,25(OH) $_2$ D $_3$ /VDR binding, confirming the requirement for this pathway; and cleavage under targets and tagmentation (CUT&Tag) analysis profiled the epigenetic regulatory landscape of the CAR-T cells. These results strongly support a role for the 1 α ,25(OH) $_2$ D $_3$ /VDR axis in promoting CD19 CAR-T-cell cytotoxicity and reveal potential therapeutic targets for enhancing immunotherapy.

Materials and Methods

Cell lines and cell culture

The DLBCL cell lines SU-DHL-4, SU-DHL-2, and U2932, as well as the OCI-LY1 cells were purchased from ATCC (Manassas, USA). The DLBCL cell lines were maintained in RPMI-1640 medium (L210KJ; BasalMedia, Shanghai, China), and the OCI-LY1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM, L610KJ; BasalMedia). All media were supplemented with 10% fetal bovine serum (FBS, F103-01; Vazyme Biotech, Nanjing, China) and 1% penicillin-streptomycin (60162ES76; YEASEN, Shanghai, China). All the cells were incubated in a humidified incubator with 5% CO $_2$ at 37°C. All the cell lines were authenticated via short tandem repeat (STR) profiling.

CAR-T-cell generation

The CAR construct contained a CD19-specific scFv, CD28 and 4-1BB costimulatory domains, and a CD3 ζ signaling domain. The fluorescent protein mCherry was connected via the self-cleaving peptide P2A and cloned and inserted into the pHR lentiviral vector for tracking *in vitro* and *in vivo*.

Peripheral blood mononuclear cells (PBMCs) were isolated from newly diagnosed (ND) DLBCL patients ($n = 8$), R/R DLBCL patients ($n = 5$), and healthy donors ($n = 6$) via lymphocyte separation medium (1858; Serumwerk Bernburg, Bernburg, Germany), with informed consent and institutional ethical approval (IACUC number: JUMC2024-229-A). CD3 $^+$ T cells were enriched with the Human CD3 $^+$ T-cell Enrichment Kit (17951; Stemcell, Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% FBS and 100 IU/mL recombinant human IL-2 (200-02; PeproTech, Rocky Hill, USA). Lentiviral transduction (MOI = 10) was performed after 72 h of CD3/CD28 Dynabeads stimulation (11141D; Gibco, Carlsbad, USA). CAR expression was validated by flow cytometry via mCherry detection, with a median transduction efficiency of 55% (range: 38%–72%). Unless otherwise noted, all CAR-T cells were pretreated three times at 2-day intervals with 50 nM 1 α ,25(OH) $_2$ D $_3$ (Calcitriol, HY-1002; MCE, Shanghai, China). Dimethyl sulfoxide (DMSO, 0.1%; D4540; Sigma, Livonia, USA) or phosphate-buffered saline (PBS, 10110023; Gibco) was used as a control. Absolute cell counts were recorded every two days.

Flow cytometry and cell sorting

For *in vitro* analysis, the cells were harvested, washed with PBS, and stained with specific antibodies diluted in flow cytometry staining buffer at room temperature for 15 min. After staining, the cells were washed again with PBS, centrifuged to remove the supernatant, and suspended in PBS for analysis with BD Symphony A3 or BD LSRFortessa (BD Biosciences, Franklin Lakes, USA). For *in vivo* experiments, single-cell suspensions were prepared from

mouse peripheral blood and tissue. Red blood cell lysates were prepared via red blood cell lysis buffer (G2015; Servicebio, Wuhan, China), followed by antibody staining. Data analysis and visualization were performed via FlowJo v10.8.1 (BD Biosciences). The following antibodies were used: APC anti-human CD62L (304810; Biolegend, Beijing, China), Brilliant Violet 421TM anti-human CD197 (CCR7) (353208; Biolegend), Brilliant Violet 605TM anti-human CD45RO (304237; Biolegend), ApotrackerTM Green (427402; Biolegend), APC/Cyanine7 anti-human CD8 (344714; Biolegend), APC anti-human CD69 (310909; Biolegend), FITC anti-human CD27 (302806; Biolegend), PE/Cyanine7 anti-human CD45RA (304125; Biolegend), Brilliant Violet 711TM anti-human CD95 (Fas) (305644; Biolegend), APC anti-human PD-1 (621610; Biolegend), APC anti-human TIM-3 (345011; Biolegend), Brilliant Violet 421TM anti-human TIM-3 (345007; Biolegend), PE/Cyanine7 anti-human LAG-3 (369310; Biolegend), PE/Cyanine7 anti-human TIGIT (372714; Biolegend), PE/Cyanine7 anti-human CTLA4 (369614; Biolegend), FITC anti-human CD4 (300506; Biolegend), Brilliant Violet 510TM anti-human CD3 (300448; Biolegend), Brilliant Violet 738TM anti-human CD3 (300472; Biolegend), APC anti-human CD8 (344722; Biolegend), FITC anti-human CD45RA (561882, BD Biosciences), PerCP/Cyanine 5.5 anti-human CCR7 (353219; Biolegend), PE/Cyanine7 anti-human TIM-3 (353219; Biolegend), Brilliant Violet 785TM anti-human TIM-3 (345031; Biolegend).

Cell apoptosis analysis

Apoptosis was evaluated in DLBCL cell lines and CD19 CAR-T cells via an Annexin V-APC/PI Apoptosis Kit (AP107; Multi Sciences, Hangzhou, China). Following treatment with different concentrations of 1 α ,25(OH) $_2$ D $_3$, MeTC7 (HY-147337; MCE) or DMSO for 7, 24, and 48 h *in vitro*, the cells were harvested, washed twice with ice-cold PBS, and resuspended in 500 μ L of binding buffer. The cells were then stained with 5 μ L of PI and incubated for 15 min at room temperature under light-protected conditions. Quantitative apoptosis analysis was subsequently performed via flow cytometry.

Cytotoxicity assay

SU-DHL-4, OCI-LY1, SU-DHL-2, and U2932 target cells were co-cultured with CD19 CAR-T cells for 7 h. Effector-to-target (E/T) ratios were determined via flow cytometry based on the proportion of CAR-positive T cells. Cytotoxicity was assessed using both flow cytometry and a lactate dehydrogenase (LDH) release assay kit (G1780; Promega, Beijing, China).

Cytotoxicity was calculated in the form of LDH release as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental} - \text{effector spontaneous} - \text{target spontaneous}}{\text{Target maximum} - \text{target spontaneous}} \times 100$$

Killing was calculated via flow cytometry as follows:

$$\text{Killing (\%)} = \frac{\text{Experimental group death rate} - \text{Natural target cell mortality}}{100 - \text{Natural target cell mortality}} \times 100$$

Cell counting kit-8 assay

DLBCL cells were collected and seeded in a 96-well plate at a density of 1 $\times$ 10⁴ cells per well. Various concentrations of 1 α ,25(OH) $_2$ D $_3$ (0 nM, 250 nM, 750 nM, 2 μ M, 10 μ M, 50 μ M, and 150 μ M) were administered to the cells for 24 h. Cell viability was then assessed

via the Cell Counting Kit-8 (CCK-8, C005; TargetMol, Shanghai, China) according to standard protocols. The half maximal inhibitory concentration (IC₅₀) value was calculated via GraphPad Prism V.10.2.1 (GraphPad Software, Jolla, USA). The suppression rate of 1 α ,25(OH) $_2$ D $_3$ was calculated as (OD value of the experimental group–OD value of the blank)/(OD value of the control group–OD value of the blank) $\times$ 100.

Cytokine analysis

CD19 CAR-T cells were co-cultured with different DLBCL cell lines (SU-DHL-4, OCI-LY1, SU-DHL-2, and U2932) for 7 h. The concentrations of several cytokines, including IL-2, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , IL-4, IL-6, and IL-10, in the culture media were assessed using the Cytometric Bead Array kit (551809; BD Biosciences).

Western blot analysis

CD19 CAR-T cells were pretreated three times at 2-day intervals with 50 nM 1 α ,25(OH) $_2$ D $_3$. On day 6, CD19 CAR-T cells were collected. A total of 2 $\times$ 10⁶ CD4⁺ CAR-T and CD8⁺ CAR-T cells were sorted by flow cytometry and lysed in lysis buffer containing protease inhibitors. Protein concentrations were determined using a BCA Protein Assay Kit (ZJ102; Epizyme, Shanghai, China). Proteins were resolved by SDS-PAGE, transferred to PVDF membranes (ISEQ00010/IPVH00010; Immobilon-P, Shanghai, China), and subsequently blocked with 5% bovine serum albumin (BSA, 9048-46-8; Sangon Biotech, Shanghai, China). The membranes were incubated with specific antibodies at 4°C overnight, including anti-VDR (1:1000, 12250; Cell Signaling Technology, Boston, USA), anti-PI3K (1:1000, 4249; Cell Signaling Technology), anti-p-PI3K (1:1000, PC6417S; Abmart, Shanghai, China), anti-p-AKT (1:1000, 4060; Cell Signaling Technology), anti-AKT (1:1000, 9272; Cell Signaling Technology), anti-p-mTOR (1:1000, 5536; Cell Signaling Technology), and anti-mTOR (1:1000, 2983; Cell Signaling Technology) antibodies. Following incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000, M21007; Abmart) secondary antibody and anti-GAPDH (HPR conjugate, 1:1000; 8884S; Cell Signaling Technology) at room temperature (RT) for 1 h, signals were visualized via chemiluminescent detection kit (SQ201; Epizyme) on an Amersham Imager 600 (Cytiva, Beijing, China).

Animal experiment

NOD-SCID mice (6–8 weeks) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). All animal experiments were approved by the Animal Care and Use Committee of Ruijin Hospital (JUMC2024-229-A). The mice used in the experiments were sex-matched. The animals were randomly allocated to four groups.

A total of 5 $\times$ 10⁶ SU-DHL-4 cells were injected subcutaneously into the right flank. After the tumors reached a volume of 50 mm³, the mice were randomized into four groups: CD19 CAR-T (1 $\times$ 10⁷), 1 α ,25(OH) $_2$ D $_3$ -pretreated CD19 CAR-T (1 $\times$ 10⁷), 1 α ,25(OH) $_2$ D $_3$ alone (0.05 μ g/kg), or PBS. CD19 CAR-T cells were given via the tail vein on day 0. 1 α ,25(OH) $_2$ D $_3$ or PBS was injected intraperitoneally daily from day 0 to day 15. The tumor volume (TV) was calculated as TV = (width² $\times$ length)/2. Peripheral blood was collected on day 15 for flow cytometry analysis. Mice with tumors exceeding 15 mm in diameter were euthanized.

Tumor-infiltrating lymphocyte (TIL) isolation

On day 15, the tumors were excised, minced, digested and filtered into single-cell suspensions. Leukocytes were isolated via lymphocyte separation medium, stained for surface markers, and analyzed via flow cytometry.

H&E staining

Primary liver, spleen, and tumor samples from DLBCL cell-implanted mice were collected and fixed in 4% paraformaldehyde (G1101; Servicebio) overnight at 4°C. The samples were dehydrated the next day, paraffin embedded, sliced into 4- μ m slices and subjected to hematoxylin (E4009; Sigma) and eosin (H9627; Sigma) staining. After staining, the slides were examined and analyzed via a KF-PRO-120 digital pathology slide scanner (KFBIO, Ningbo, China).

Multiplex immunofluorescence microscopy

Four-micron-thick tumor sections were deparaffinized in xylene and subjected to antigen retrieval. After being blocked, the sections were incubated with antibodies, including anti-CD8 (1:4000, ab217344; Abcam, Shanghai, China), anti-PD-1 (1:500, ab214421; Abcam), and anti-TIM-3 (1:100, ab13445; Abcam), for 30 min at 37°C, followed by incubation with fluorescein-secondary antibodies, including try-488 (Bry-try488; runnerbio, Shanghai, China), try-cy3 (Bry-trycy3; runnerbio), and try-cy5 (Bry-trycy5; runnerbio). The nuclei were stained with DAPI (G1012; Servicebio). The fluorescence signals were observed and imaged with a digital slide scanner (PANNORAMIC SCAN II (Nikon DS-fi2; Nikon, Japan). Standardized image thresholding ensured comparability across groups.

Real-time polymerase chain reaction analysis

Total RNA was isolated from CAR-T cells via TRIzol reagent (AG21102; Accurate Biology, Changsha, China). Reverse transcription was performed with Evo M-MLV RT Premix (AG11706; Accurate Biology) to generate cDNA. Quantitative PCR analysis was performed using a SYBR Green Premix Pro Taq HS qPCR Tracking Kit (Rox Plus, AG11735; Accurate Biology) on a ViiATM 7 Sequence Detection System (Applied Biosystems, Shanghai, China). Relative gene expression values were normalized to the endogenous *GAPDH* control. The following primers were used: *TCF7*, 5'-CGAGAAGAGCAGGCCAAGTA-3' (forward) and 5'-GCCTCTTCTTCTCCCGTAGT-3' (reverse); *NR4A1*, 5'-AGCTTGGGTGTTGATGTTCC-3' (forward) and 5'-CGATCAGTGATGAGGACCAG-3' (reverse); *NFATC2*, 5'-TGCATCTAACCCATCGAGTG-3' (forward) and 5'-TGAGGATCATTTGCTGGCC-3' (reverse); *ZBTB32*, 5'-AACGTCTGGGCAAGGGTTC-3' (forward) and 5'-TGGTGAGATGGGCAGTTCAG-3' (reverse); *PDCD1*, 5'-ACGAGGGACAATAGGAGCCA-3' (forward) and 5'-GGCATACTCCGTCTGCTCAG-3' (reverse); *HAVCR2*, 5'-CCTTGGAATTTCCCTGCCAA-3' (forward) and 5'-CCCAGCACATAGGCACAAGT-3' (reverse); *NR4A3*, 5'-AAACCTTGACAGCCTGAACC-3' (forward) and 5'-CTGGTGTCCTTTAAGCTGC-3' (reverse); *LEF1*, 5'-GTGGACATCGGCATGATGAAG-3' (forward) and 5'-GGTCGTAGGTCTTATGGTGGG-3' (reverse); *ETV7*, 5'-AAGCAGAGTCCAAGGCAGAGAA-3' (forward) and 5'-ACCTTGGCTCTCATCTCTTCA-3' (reverse); *VDR*, 5'-CTGCTGTGGGATTACGTGTATC-3' (forward) and 5'-GTTCTTGATTTCCCCAGAGTC-3' (reverse); *c-Fos*, 5'-CACTCCAA GCGGAGACAGAC-3' (forward) and 5'-AGGTCATCAGGGATCTTG CAG-3' (reverse); *CD28*, 5'-TTTCCGGACCTTCTAAGC-3' (for-

ward) and 5'-CGGGGAGTCATGTTTCATGTAG-3' (reverse); *CD69*, 5'-AGCTCTTCACATCTGGAGAGAG-3' (forward) and 5'-CCACTATTAACACAGCCCAAGG-3' (reverse).

CUT&Tag

CD19 CAR-T cells from three healthy donors were pretreated with 50 nM 1 α ,25(OH) $_2$ D $_3$ or DMSO three times at 2-day intervals. On day 6, CD19 CAR-T cells were collected. CD8⁺ CAR-T cells were sorted via flow cytometry. CUT&Tag was conducted by Novogene (Beijing, China) via Vazyme's Hyperactive Universal Kit (TD904; Vazyme Biotech). CD8⁺ CAR-T cells bound to ConA-coated beads were permeabilized and incubated with an anti-VDR primary antibody (12250; Cell Signaling Technology). The DNA was tagged with the pA-Tn5 transposase and PCR amplified for library construction. Libraries were sequenced on an Illumina NovaSeq (150 bp PE). The CUT&Tag data reported in this article have been deposited into the NCBI Gene Expression Omnibus database. The accession number of the CUT&Tag data is GSE305373.

Bulk RNA-seq analysis

CD19 CAR-T cells from a healthy donor were prepared as described previously. Total RNA was isolated from $\geq 1 \times 10^6$ bulk cells via TRIzol reagent (AG21102; Accurate Biology) per the manufacturer's protocol. mRNA was enriched from total RNA with poly-T oligo-attached magnetic beads. Fragmentation was performed in First Strand Synthesis Reaction Buffer using divalent cations at elevated temperature. First-strand cDNA synthesis was performed with random hexamers and M-MuLV Reverse Transcriptase (RNase H⁻), followed by second-strand synthesis with DNA Polymerase I and RNase H. The remaining overhangs were blunted by exonuclease/polymerase treatment. After 3' end adenylation, hairpin loop adaptors were ligated. Libraries were size-selected (370–420 bp) via AMPure XP beads. PCR amplification was performed with Phusion High-Fidelity DNA Polymerase with Universal and Index (X) primers. The final libraries were purified (AMPure XP) and quality-checked on an Agilent Bioanalyzer 2100. Differential expression between two conditions ($n = 2$ replicates/group) was analyzed via DESeq2 (v1.20.0). Genes with adjusted *P* values (adj. *P*) ≤ 0.05 were considered differentially expressed (DEGs). The functional enrichment analyses of the DEGs included Gene Ontology (GO) Enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment and Gene Set Enrichment Analysis (GSEA). The bulk RNA-seq data reported in this article have been deposited into the NCBI Gene Expression Omnibus database. The accession number of the bulk RNA-seq data is GSE298264.

Single-cell RNA-seq analysis

CD19 CAR-T cells from a healthy donor were prepared as described previously. Then, CD19 CAR-T cells, co-cultured with SU-DHL-4 cells for 24 h at an E:T ratio of 1:1, were harvested by cell suspension preparation. Cellular suspensions were loaded on a 10 $\times$ Genomics GemCode Single-cell instrument that generates single-cell Gel Beads-In-Emulsion. Libraries were generated and sequenced from the cDNAs with Chromium Next GEM Single Cell 3' Reagent Kits v3.1. scRNA-seq raw data were aligned to GRCh38 via Cellranger (v3.1; default parameters), generating raw count matrices converted to Seurat objects (Seurat v3.2). Genes detected in fewer than three cells were removed. Cells meeting quality

thresholds (> 200 expressed genes, < 15 % mitochondrial content, > 3000 UMIs) were retained. Data normalization was performed with Seurat's default normalization. PCA employed the top 2000 variable genes, with batch correction by Harmony (v0.99.9) on PCA embeddings. Cell clustering and Uniform Manifold Approximation and Projection (UMAP) visualization were performed utilizing the first 40 Harmony components. The single-cell RNA-seq data reported in this article have been deposited into the NCBI Gene Expression Omnibus database. The accession number of the scRNA-seq data is GSE298369.

Statistical analysis

All *in vitro* experiments consisted of three or more replicates. Each replicate is presented as an individual data point. All *in vivo* studies consisted of more than five mice per group. Statistical analysis was performed via GraphPad Prism V.10.0. The data are presented as the mean $\pm$ SD. Statistical significance was set to $P < 0.05$. Student's *t* test was used for comparison between two groups, whereas one-way analysis of variance (one-way ANOVA) was used for comparison among more than two groups. Correlation analysis was performed via Pearson's correlation test.

Results

1 α ,25(OH) $_2$ D $_3$ exerts limited cytotoxicity against DLBCL cells *in vitro*

We first assessed the direct cytotoxicity of 1 α ,25(OH) $_2$ D $_3$ to DLBCL cells. To represent the two major molecular subtypes of DLBCL, activated B-cell (ABC) and germinal center B-cell (GCB) [36], we selected the SU-DHL-2 and U2932 cell lines for the ABC subtype and OCI-LY1 and SU-DHL-4 for the GCB subtype. Among them, SU-DHL-2 and SU-DHL-4 cells were more susceptible to 1 α ,25(OH) $_2$ D $_3$ treatment than OCI-LY1 and U2932 cells were (Supplementary Figure S1A). Nevertheless, the half-maximal inhibitory concentration (IC $_{50}$) values for all four cell lines exceeded the physiological range of 1 α ,25(OH) $_2$ D $_3$, suggesting that 1 α ,25(OH) $_2$ D $_3$ is weakly cytotoxic to DLBCL cells under physiological conditions [37,38]. To establish the optimal concentration of 1 α ,25(OH) $_2$ D $_3$ for use with CD19 CAR-T cells, we performed a concentration gradient experiment. The results revealed that the expression levels of inhibitory receptors (PD-1 and TIM-3) were maximally reduced by 50 nM 1 α ,25(OH) $_2$ D $_3$ (Supplementary Figure S1B,C). This finding is consistent with reports that 1 α ,25(OH) $_2$ D $_3$ at 50 nM could effectively alleviate cytotoxic T-cell exhaustion in non-small cell lung cancer (NSCLC) patients [39]. To determine whether 1 α ,25(OH) $_2$ D $_3$ directly affects DLBCL cells, we treated DLBCL cell lines with 50 nM 1 α ,25(OH) $_2$ D $_3$. No significant induction of apoptosis was observed across the tested cell lines (Supplementary Figure S1D–G), which collectively indicated that 1 α ,25(OH) $_2$ D $_3$ alone does not exert significant cytotoxic effects on DLBCL cells.

1 α ,25(OH) $_2$ D $_3$ alleviates exhaustion in healthy donor-derived CD19 CAR-T cells

Given that 1 α ,25(OH) $_2$ D $_3$ does not directly inhibit the proliferation of DLBCL cells, we next explored the effects of 1 α ,25(OH) $_2$ D $_3$ on the phenotype and function of CD19 CAR-T cells derived from healthy donors. CD19 CAR-T cells were prepared via our previously validated third-generation CAR construct [40]. Over 50% of the T cells successfully expressed the CD19 CAR construct (Figure 1A,B) and were subsequently treated with either DMSO (control) or 50 nM

1 α ,25(OH) $_2$ D $_3$ (Figure 1C). Compared with the control, continuous exposure to 50 nM 1 α ,25(OH) $_2$ D $_3$ did not significantly affect CAR-T-cell expansion (Figure 1D), apoptosis (Figure 1E), or the CD4 $^+$ /CD8 $^+$ T-cell ratio (Figure 1F). Interestingly, 1 α ,25(OH) $_2$ D $_3$ treatment markedly reduced the expression of the early activation marker CD69 on both T cells and CD19 CAR-T cells (Figure 1G), which may help mitigate tonic signaling-induced exhaustion. Notably, the expression levels of the exhaustion-related inhibitory receptors PD-1, TIM-3, and TIGIT in both cell types was significantly reduced following 1 α ,25(OH) $_2$ D $_3$ treatment (Figure 1H). Furthermore, 1 α ,25(OH) $_2$ D $_3$ -treated CAR-T cells showed increased frequencies of naïve T cells (Tn, CCR7 $^+$ CD45RA $^+$), along with decreased proportions of terminal effector (Te, CCR7 $^+$ CD45RA $^+$) and effector memory (Tem, CCR7 $^+$ CD45RA $^-$) subsets within the CD8 $^+$ population (Figure 1I). The percentage of CCR7 $^+$ memory-like cells within the CD8 $^+$ population was significantly elevated upon 1 α ,25(OH) $_2$ D $_3$ treatment (Figure 1J). In contrast, within the CD4 $^+$ population, 1 α ,25(OH) $_2$ D $_3$ treatment increased the proportion of Tn cells and decreased the number of Tem cells, whereas the frequency of Te cells mildly increased (Supplementary Figure S1H,I). These findings suggest that 1 α ,25(OH) $_2$ D $_3$ may enhance the phenotypic fitness of CD19 CAR-T cells during *in vitro* manufacturing by promoting memory-like differentiation and mitigating early exhaustion signals.

1 α ,25(OH) $_2$ D $_3$ enhances the antigen-specific immune response of CD19 CAR-T cells

To evaluate the effector function of 1 α ,25(OH) $_2$ D $_3$ -treated CD19 CAR-T cells upon antigen stimulation, we co-cultured the CD19 CAR-T cells with different types of DLBCL cell lines. As expected, 1 α ,25(OH) $_2$ D $_3$ -treated CAR-T cells consistently exhibited enhanced tumor-killing activity in an effector-to-target (E/T) ratio-dependent manner, with the most pronounced effects observed in U2932 and SU-DHL-4 cells (Figure 2A–D). Interestingly, although 1 α ,25(OH) $_2$ D $_3$ reduced CD69 expression in unstimulated T cells, CD69 expression was significantly upregulated upon tumor antigen stimulation (Figure 2E), which is consistent with its role as a short-term activation marker indicative of cytotoxic function [41]. Compared with controls, when co-cultured with germinal central B cell (GCB) type SU-DHL-4 cells, 1 α ,25(OH) $_2$ D $_3$ -treated CD19 CAR-T cells displayed significantly reduced co-expression levels of inhibitory receptors, including PD-1, TIM-3, and TIGIT (Figure 2F). Additionally, CD8 $^+$ CAR-T cells retained a reduced proportion of Te cells and a greater proportion of CCR7 $^+$ memory-like cells (Figure 2G,H), whereas this effect was less evident in CD4 $^+$ CAR-T cells and non-transduced T cells (Supplementary Figure S2A,B). Given the clinical significance of DLBCL molecular subtypes, particularly the adverse prognosis associated with activated B cell (ABC) type [42], we extended our analysis to U2932 cells (ABC type). Critically, 1 α ,25(OH) $_2$ D $_3$ -treated CD19 CAR-T cells co-cultured with U2932 cells recapitulated the effects observed in GCB-type models: reduced co-expression levels of exhaustion markers, decreased Te proportion, and increased CCR7 $^+$ memory-like cell frequency (Supplementary Figure S2C–E). Notably, similar modulatory effects were detected in CD4 $^+$ CAR-T cells and non-transduced T cells (Supplementary Figure S2F,G). Functionally, 1 α ,25(OH) $_2$ D $_3$ treatment increased the secretion of key antitumor cytokines, including IL-2, IL-6, IFN- γ , and TNF- α , from CD19 CAR-T cells (Figure 2I). In contrast, the production of IL-10 was reduced in co-cultures with OCI-LY1 and SU-DHL-2 cells, whereas the IL-4 levels remained

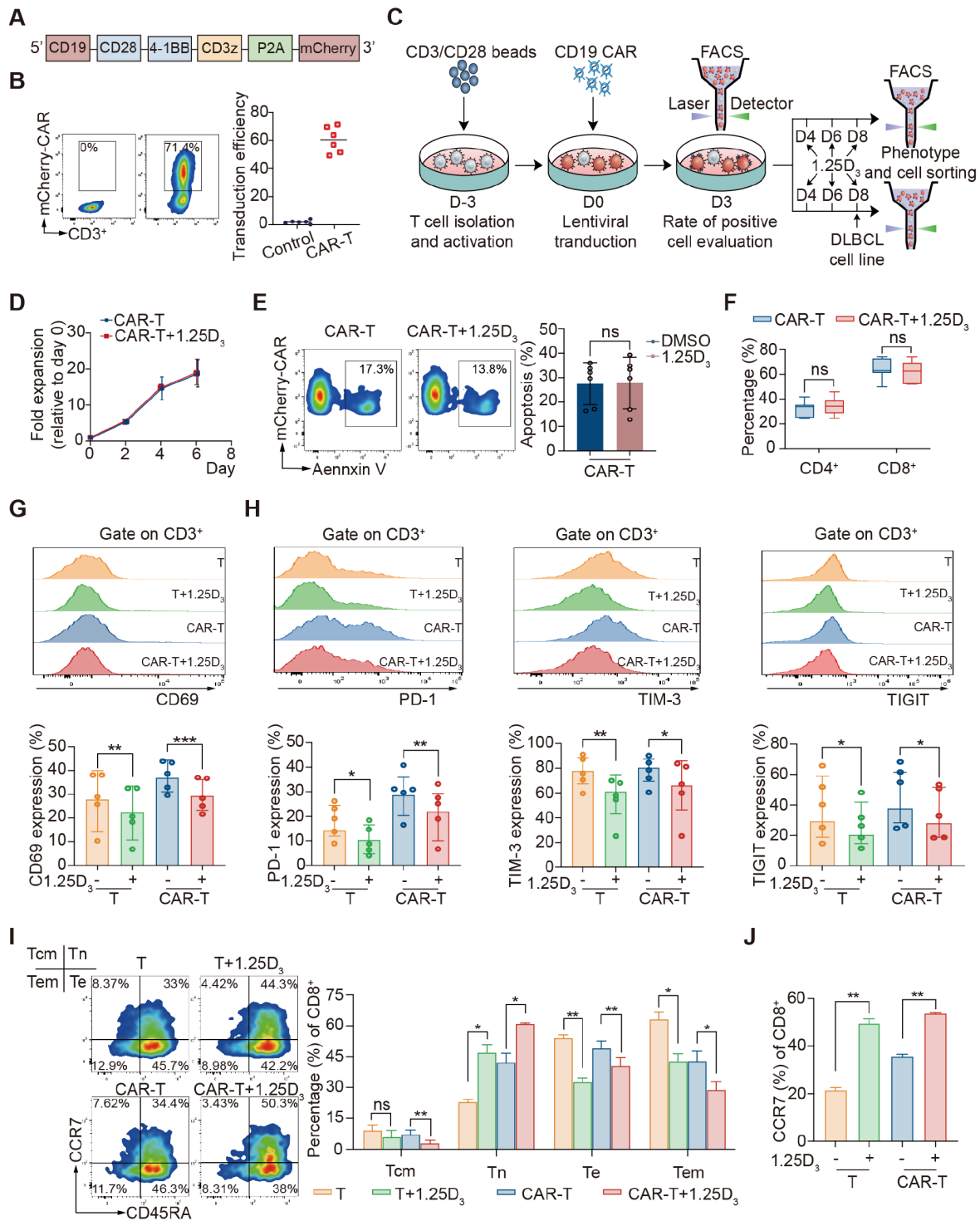


Figure 1. 1 α ,25(OH) $_2$ D $_3$ potentiates early memory differentiation and counteracts CD19 CAR-T-cell exhaustion *in vitro*. (A) Schematics of the CD19 CAR constructs. (B) Flow cytometry analysis profiles and quantification analysis of the positive rate of CAR-T cells. (C) Experimental design: T cells were isolated from PBMCs, enriched, and activated on day 3. CAR lentivirus was transduced on day 0, and the percentage of CD19 CAR-T cells was detected via flow cytometry. CD19 CAR-T cells were co-cultured with or without 1 α ,25(OH) $_2$ D $_3$ for 6 days, and CAR-T cells on day 8 were collected for flow cytometry analysis. (D) Fold expansion of control and 1 α ,25(OH) $_2$ D $_3$ -treated CD19 CAR-T cells *in vitro* ($n = 6$). (E) Flow cytometry analysis profiles and quantification of apoptosis in CD19 CAR-T cells after treatment with 1 α ,25(OH) $_2$ D $_3$ or DMSO for 24 h ($n = 6$). (F) Percentage of CD4 $^+$ and CD8 $^+$ CAR-T cells among control and 1 α ,25(OH) $_2$ D $_3$ -treated CAR-T cells ($n = 5$). (G,H) Flow cytometric analysis of the expression levels of an activation marker (CD69) (G) and inhibitory receptors (PD-1, TIM-3, and TIGIT) (H) ($n = 5$). (I) Flow cytometry analysis profiles and quantification analysis of the subpopulations of control or 1 α ,25(OH) $_2$ D $_3$ -treated CD8 $^+$ T and CD8 $^+$ CAR-T cells ($n = 3$). Tcm, central memory T; Tn, naïve T; Te, effector T; Tem, effector memory T. (J) The percentage of CCR7 expression in control or 1 α ,25(OH) $_2$ D $_3$ -treated CD8 $^+$ T and CD8 $^+$ CAR-T cells. Data are presented as the mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant).

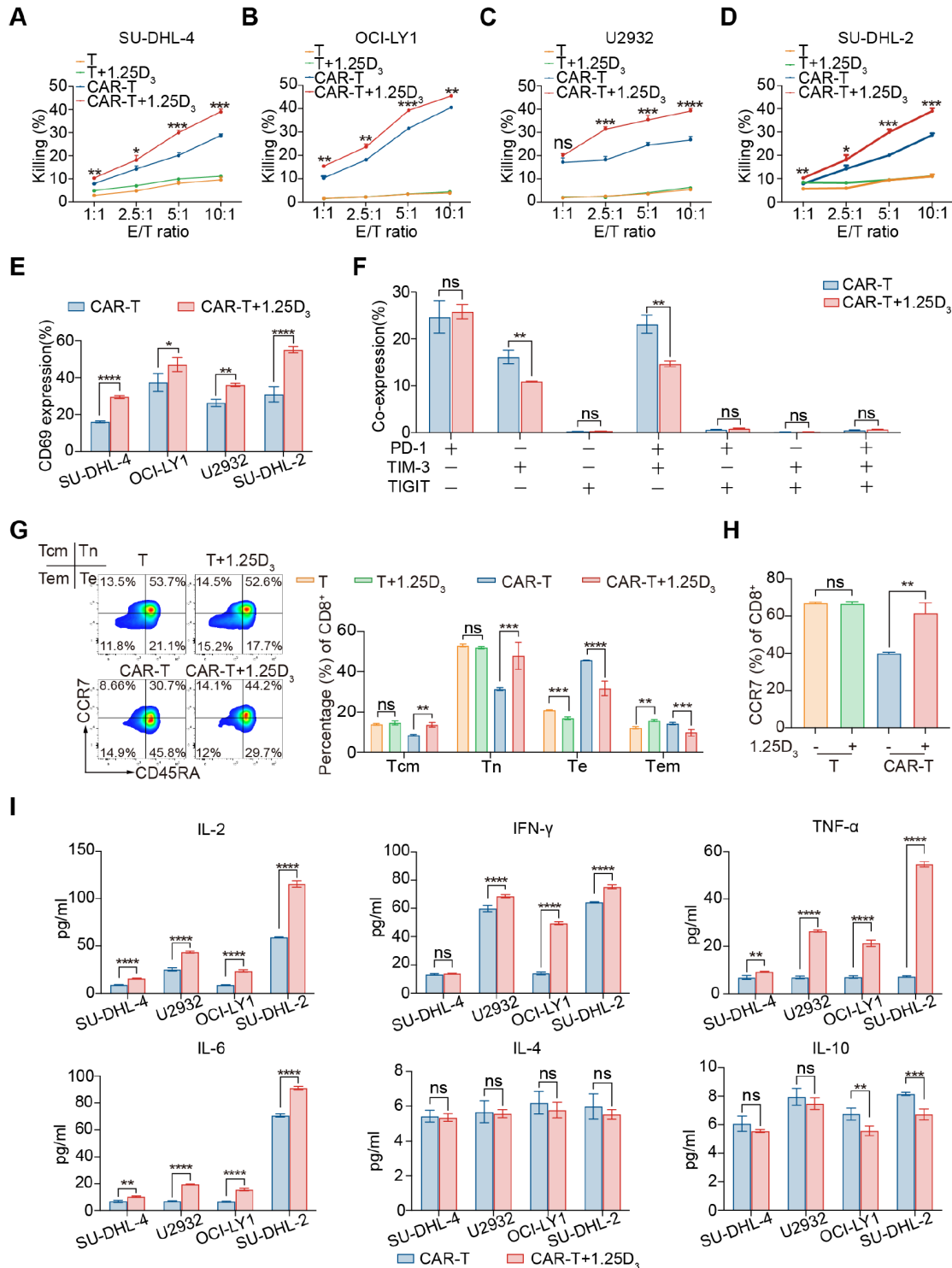


Figure 2. 1 α ,25(OH) $_2$ D $_3$ enhances the antigen-specific immune response of CD19 CAR-T cells (A–D) Anti-lymphoma activity of CD19 CAR-T cells *in vitro*. CD19 CAR-T cells were co-cultured with DLBCL cell lines for 7 h ($n = 3$). Cytotoxicity was measured by flow cytometry. (E) Flow cytometric analysis of the expression levels of an activation marker (CD69) in control and 1 α ,25(OH) $_2$ D $_3$ -treated CD19 CAR-T cells after the co-cultured with DLBCL cell lines for 7 h at an E/T ratio of 1:1 ($n = 3$). (F) Percentage of inhibitory receptor co-expression (PD-1, TIM-3, and TIGIT) in control and 1 α ,25(OH) $_2$ D $_3$ -treated CAR-T cells after the co-cultured with SU-DHL-4 cells for 24 h at an E/T ratio of 1:1 ($n = 3$). (G) Flow cytometry analysis profiles and quantification analysis of the subpopulations of control or 1 α ,25(OH) $_2$ D $_3$ -treated CD8 $^+$ T and CD8 $^+$ CAR-T cells after the co-cultured with SU-DHL-4 cells for 24 h at an E/T ratio of 1:1 ($n = 3$). (H) The percentage of CCR7 expression in control or 1 α ,25(OH) $_2$ D $_3$ -treated CD8 $^+$ T and CD8 $^+$ CAR-T cells after the co-cultured with SU-DHL-4 cells for 24 h at an E/T ratio of 1:1 ($n = 3$). (I) Secretion of IL-2, IFN- γ , TNF- α , IL-6, IL-4, and IL-10 by control and 1 α ,25(OH) $_2$ D $_3$ -treated CD19 CAR-T cells after co-culture with four DLBCL cell lines for 7 h at an E/T ratio of 1:1 ($n = 3$). Data are presented as the mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant).

largely unchanged. Taken together, these results demonstrate that 1 α ,25(OH) $_2$ D $_3$ not only preserves memory phenotypes but also enhances the antigen-specific cytotoxicity and cytokine response of CD19 CAR-T cells *in vitro*.

1 α ,25(OH) $_2$ D $_3$ reverses exhaustion in patient-derived CD19 CAR-T cells

Next, we examined the effects of 1 α ,25(OH) $_2$ D $_3$ on patient-derived CD19 CAR-T cells. Specifically, we generated CD19 CAR-T cells from newly diagnosed (ND) and R/R DLBCL patients and cultured them in the presence or absence of 1 α ,25(OH) $_2$ D $_3$ for 6 days. Consistent with findings in healthy donor cells, 1 α ,25(OH) $_2$ D $_3$ had no significant effect on apoptosis (Supplementary Figure S3A) or the CD4/CD8 ratio (Supplementary Figure S3B). Interestingly, the baseline serum 25-OHD was negatively correlated with the pre-treatment expression levels of PD-1, TIM-3, TIGIT, lymphocyte activation gene-3 (LAG-3), and CD69 on CAR-T cells (Figure 3A). In CAR-T cells derived from both ND and R/R DLBCL patients, 1 α ,25(OH) $_2$ D $_3$ treatment markedly reduced the expression levels of the early activation marker CD69 (Figure 3B) and the exhaustion markers PD-1, TIM-3, TIGIT, and LAG-3 (Figure 3C–F). Moreover, 1 α ,25(OH) $_2$ D $_3$ treatment increased the proportion of CCR7 $^+$ cells (Figure 3G) and the frequency of naïve T cells and decreased the percentage of Te subsets (Figure 3H,I). Similarly, this effect was more pronounced among CD8 $^+$ T cells than among CD4 $^+$ T cells (Supplementary Figure S3C,D). Notably, LAG-3 expression in healthy donor-derived CAR-T cells was not significantly affected by 1 α ,25(OH) $_2$ D $_3$ treatment, suggesting the strong relevance of this intervention for patients with DLBCL, possibly due to prior lymphotoxic treatments.

Next, we characterized the effects of 1 α ,25(OH) $_2$ D $_3$ treatment on CD19 CAR-T-cell functions in the presence of tumor antigen stimulation. Consistent with the *in vitro* findings, compared with the controls, the 1 α ,25(OH) $_2$ D $_3$ -treated CAR-T cells exhibited significantly enhanced tumor-killing efficacy in an E/T ratio-dependent manner (Figure 4A–D). Treatment with 1 α ,25(OH) $_2$ D $_3$ also upregulated CD69 expression on CAR-T cells when co-cultured with DLBCL cells (Figure 4E). Following co-culture of CAR-T cells with SU-DHL-4 (GCB-type), the 1 α ,25(OH) $_2$ D $_3$ -treated group presented downregulated expression of single- and double-positive inhibitory markers (Figure 4F). Additionally, treatment increased the proportions of the Tcm and Tn subsets while reducing the proportions of the Te and Tem subsets among the CD8 $^+$ but not the CD4 $^+$ T cells (Figure 4G and Supplementary Figure S4A). The frequency of CCR7 $^+$ cells was significantly elevated in the CD8 $^+$ subset but not in the CD4 $^+$ subset (Figure 4H and Supplementary Figure S4B). Extending to U2932 cells (ABC-type) revealed the conserved anti-exhaustion effects of 1 α ,25(OH) $_2$ D $_3$ -treated CD19 CAR-T cells. Phenotypic recapitulation of the GCB-type response was evidenced by significantly reduced co-expression of exhaustion markers, diminished Te cells and increased CCR7 $^+$ memory-like subset frequencies following co-culture with U2932 cells (Supplementary Figure S4C–E). Notably, analogous modulatory effects were consistently observed in both CD4 $^+$ T cells and nontransduced T cells (Supplementary Figure S4F,G), confirming the broad immunomodulatory activity of 1 α ,25(OH) $_2$ D $_3$ across T cell populations. Finally, the levels of the effector cytokines IL-2, IL-6, TNF- α , and IFN- γ were significantly increased, whereas the levels of IL-4 and IL-10 were decreased following 1 α ,25(OH) $_2$ D $_3$ treatment in the

co-culture system (Figure 4I). These findings highlight the immunomodulatory function of 1 α ,25(OH) $_2$ D $_3$ in enhancing the efficacy of CD19 CAR-T-cell therapy in DLBCL patients by reversing exhaustion and terminal differentiation.

1 α ,25(OH) $_2$ D $_3$ enhances the tumor infiltration and antitumor efficacy of CD19 CAR-T cells *in vivo*

To further evaluate whether 1 α ,25(OH) $_2$ D $_3$ enhances the antitumor efficacy of CD19 CAR-T cells *in vivo*, we established an SU-DHL-4 subcutaneous xenograft mouse model. The tumor-bearing mice were injected with CD19 CAR-T cells pre-treated with either 1 α ,25(OH) $_2$ D $_3$ or DMSO, followed by additional systemic administration of 1 α ,25(OH) $_2$ D $_3$ or PBS (Figure 5A). Compared with the control, CD19 CAR-T-cell therapy significantly inhibited tumor growth, while compared with CD19 CAR-T-cell therapy alone, treatment with 1 α ,25(OH) $_2$ D $_3$ further suppressed tumor progression (Figure 5B). No significant differences in body weight were observed across the treatment groups, suggesting a favorable safety profile (Supplementary Figure S5A).

Given that CAR-T-cell expansion is significantly correlated with the therapeutic response *in vivo* [43], we quantified the number of CAR-T cells in the peripheral blood, spleen, and tumor tissue. Compared with those not treated with 1 α ,25(OH) $_2$ D $_3$, mice receiving 1 α ,25(OH) $_2$ D $_3$ showed significantly increased CAR-T-cell infiltration in the tumor microenvironment (Figure 5C) and accumulation in the spleen (Figure 5D), whereas no significant difference was observed in the peripheral blood (Figure 5E). The CD4/CD8 ratio of CAR-T cells remained unchanged in the peripheral blood and tumor tissue (Supplementary Figure S5B). Consistent with previous findings, the expression levels of the memory marker CCR7 were significantly increased in CD8 $^+$ T cells in both the blood and tumor after 1 α ,25(OH) $_2$ D $_3$ treatment (Figure 5F). Consistent with our *in vitro* findings, CAR-T cells isolated from the peripheral blood and spleen of the 1 α ,25(OH) $_2$ D $_3$ -treated group presented markedly reduced expression levels of exhaustion-related inhibitory receptors (PD-1, TIM-3, and TIGIT), especially in the double-positive populations (Figure 5G and Supplementary Figure S5C). Furthermore, histological staining of liver and spleen tissues revealed that CAR-T-cell therapy combined with 1 α ,25(OH) $_2$ D $_3$ treatment did not significantly impact the normal morphology or histology of organs (Supplementary Figure S5D). Immunofluorescence analyses of tumor tissues confirmed significantly lower expression of PD-1 and TIM-3 in tumor-infiltrating CD8 $^+$ CAR-T cells following 1 α ,25(OH) $_2$ D $_3$ treatment (Figure 5H). These results collectively suggest that 1 α ,25(OH) $_2$ D $_3$ treatment improves the anti-DLBCL efficacy of CAR-T cells *in vivo*, potentially by attenuating T-cell exhaustion.

1 α ,25(OH) $_2$ D $_3$ inhibits CAR-T-cell exhaustion via VDR-mediated transcriptional reprogramming

To elucidate how 1 α ,25(OH) $_2$ D $_3$ treatment enhances CD19 CAR-T-cell functionality, RNA sequencing was performed on sorted CD4 $^+$ and CD8 $^+$ CAR-T cells treated with DMSO or 1 α ,25(OH) $_2$ D $_3$ in the absence of tumor stimulation. A total of 12,250 and 12,357 differentially expressed genes (DEGs) were identified in CD8 $^+$ and CD4 $^+$ CAR-T cells, respectively (Figure 6A and Supplementary Figure S6A). In the CD8 $^+$ subset, memory-associated transcription factors such as *MAPK13* and *TCF7* were upregulated [44–46], whereas exhaustion-related regulators such as *HOPX* and *PRF1*

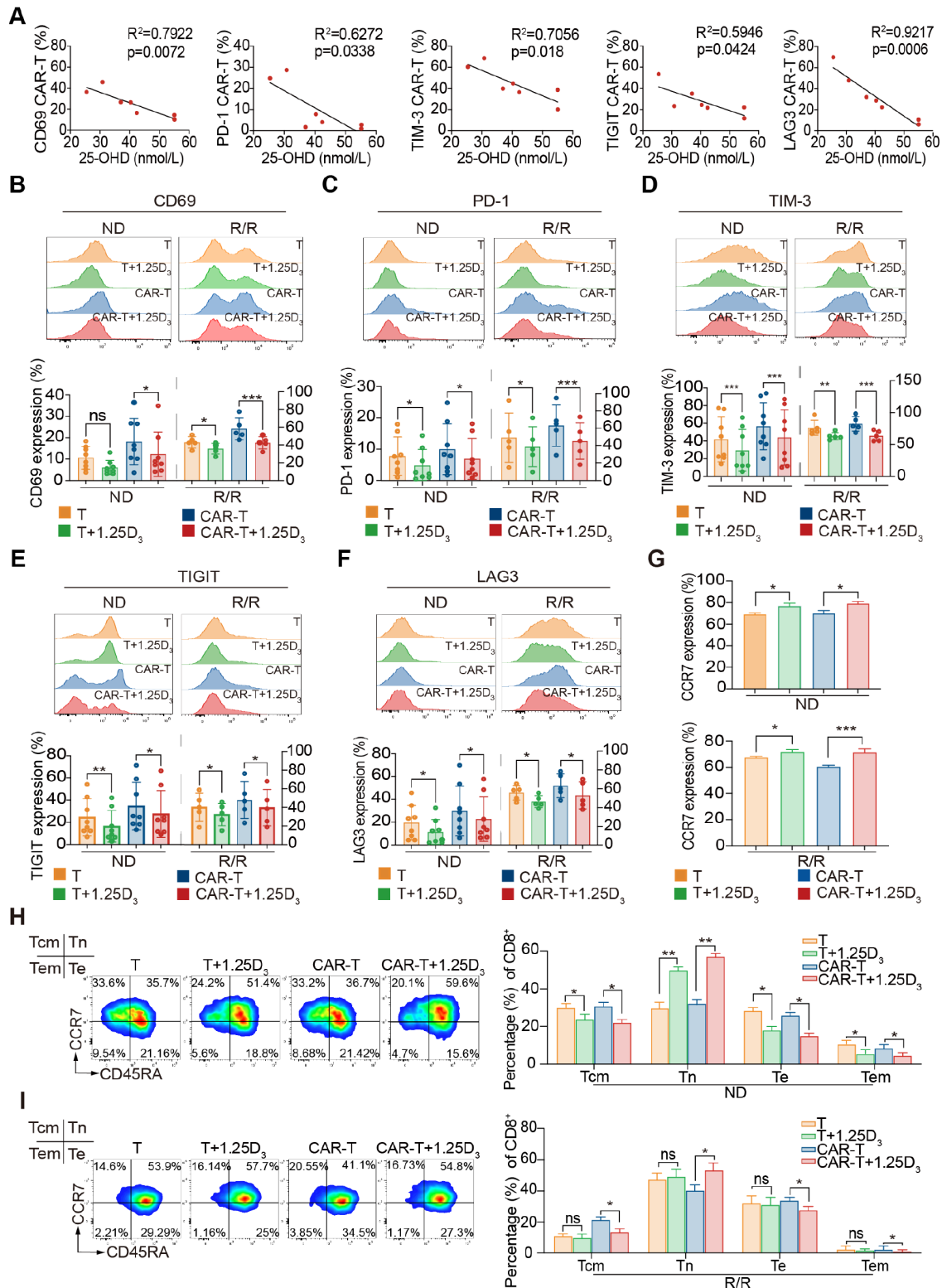


Figure 3. 1 α ,25(OH) $_2$ D $_3$ improves T cell differentiation and exhaustion of CD19 CAR-T cells from ND and R/R DLBCL patients (A) Linear regression analysis between the 25-OHD level and the expression of immune checkpoint receptors (PD-1, TIM-3, TIGIT, and LAG-3) and an activation marker (CD69) on CAR-T cells derived from the PBMCs of patients with ND DLBCL ($n=7$). (B-F) Flow cytometric analysis of the expression levels of an activation marker (CD69) (B) and inhibitory receptors (PD-1, TIM-3, TIGIT, and LAG-3) (C-F) in ND ($n=8$) and R/R ($n=5$) patients. (G) The percentage of CCR7 expression in control or 1 α ,25(OH) $_2$ D $_3$ -treated CD8 $^+$ T and CD8 $^+$ CAR-T cells from ND and R/R patients ($n=3$). (H,I) Flow cytometry analysis profiles and quantification analysis of the subpopulations of control or 1 α ,25(OH) $_2$ D $_3$ -treated CD8 $^+$ T and CD8 $^+$ CAR-T cells from ND (H) and R/R patients (I) ($n=3$). Data are presented as the mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant).

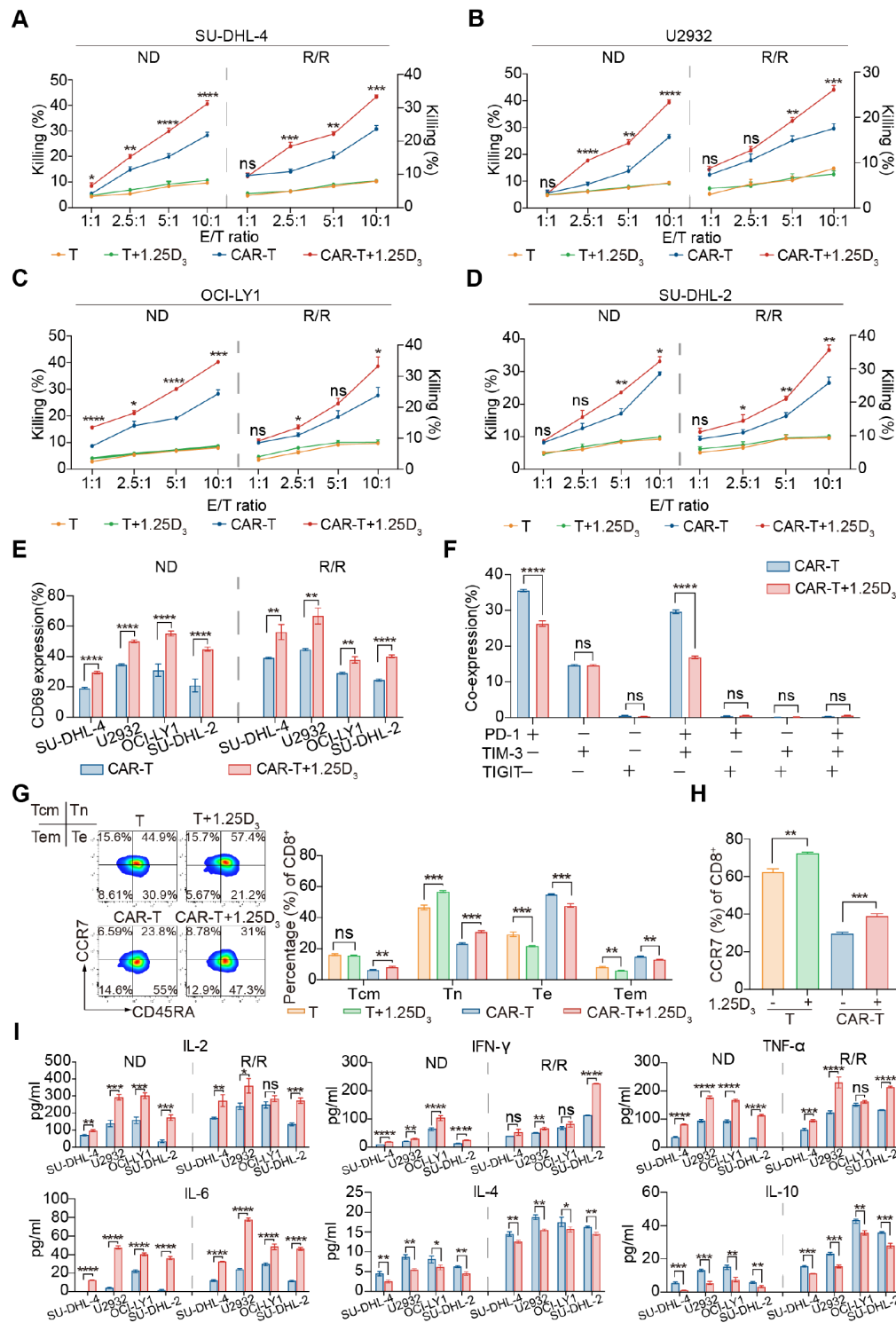


Figure 4. 1 α ,25(OH) $_2$ D $_3$ enhances the antigen-specific immune response of CD19 CAR-T cells from ND and R/R DLBCL patients (A–D) Antilymphoma activity of CAR-T cells *in vitro*. CAR-T cells (effector cells, E) were co-cultured with four DLBCL cell lines (target cells, T) for 7 h ($n = 3$). Cytotoxicity was measured by flow cytometry. (E) Flow cytometric analysis of the expression levels of an activation marker (CD69) in control and 1 α ,25(OH) $_2$ D $_3$ -treated CD19 CAR-T cells after co-culture with DLBCL cell lines for 7 h at an E/T ratio of 1:1 ($n = 3$). (F) Percentage of inhibitory receptor co-expression (PD-1, TIM-3, and TIGIT) in control and 1 α ,25(OH) $_2$ D $_3$ -treated CAR-T cells after co-culture with SU-DHL-4 cells for 24 h at an E/T ratio of 1:1 ($n = 3$). (G) Flow cytometry analysis profiles and quantification analysis of the subpopulations of control or 1 α ,25(OH) $_2$ D $_3$ -treated CD8 $^+$ T and CD8 $^+$ CAR-T cells after co-culture with SU-DHL-4 cells for 24 h at an E/T ratio of 1:1 ($n = 3$). (H) The percentage of CCR7 expression in control or 1 α ,25(OH) $_2$ D $_3$ -treated CD8 $^+$ T and CD8 $^+$ CAR-T cells after co-culture with SU-DHL-4 cells for 24 h at an E/T ratio of 1:1 ($n = 3$). (I) Secretion of IL-2, IFN- γ , TNF- α , IL-6, IL-4, and IL-10 by control and 1 α ,25(OH) $_2$ D $_3$ -treated CD19 CAR-T cells after co-culture with DLBCL cell lines for 7 h at an E/T ratio of 1:1 ($n = 3$). Data are presented as the mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant).

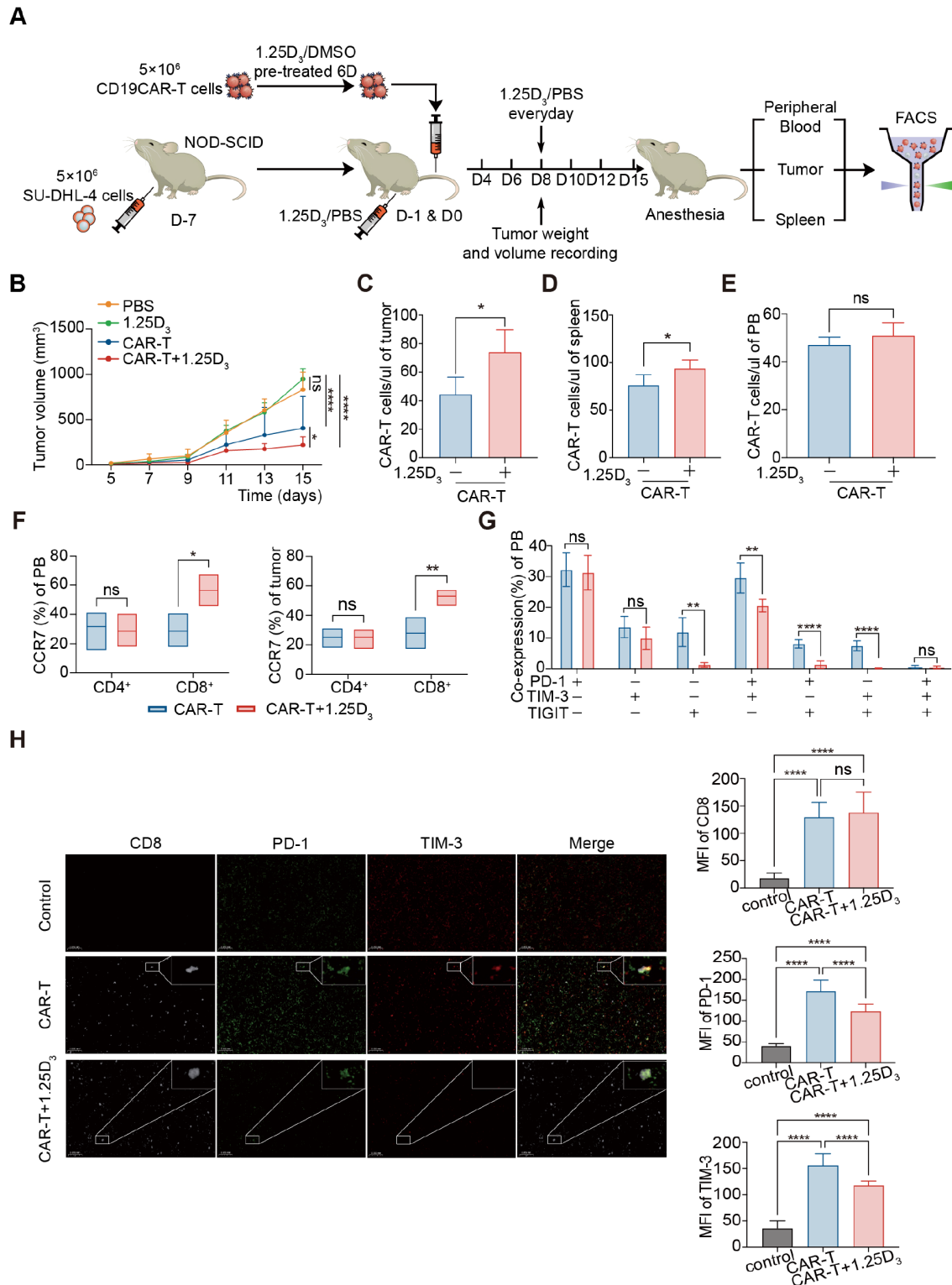


Figure 5. 1 α ,25(OH) $_2$ D $_3$ sensitizes DLBCL cells to CD19 CAR-T cells *in vivo* (A) Schematic depicting the protocol used for the animal experiments. (B) Graphs showing tumor growth, presented by TV ($n = 6$ per group). (C–E) CAR-T-cell expansion in the tumor (C), spleen (D), and peripheral blood (E) of the mice in each group was assessed, and flow cytometry was performed to calculate the number of CAR-T cells. (F) CCR7 expression levels in CD4 $^+$ or CD8 $^+$ CAR-T cells were analyzed via flow cytometry on day 15 after CAR-T-cell infusion. (G) Percentage of inhibitory receptor (PD-1, TIM-3, and TIGIT) co-expression levels in peripheral blood of CAR-T cells in the control or 1 α ,25(OH) $_2$ D $_3$ -treated groups. (H) Immunofluorescence images showing the expression levels of CD8 (white), PD-1 (green), and TIM-3 (red) in tumor. Mean fluorescence intensity (MFI) of CD8, PD-1, and TIM-3 was displayed on the right (day 15 after CAR-T cell infusion). Data are presented as the mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant).

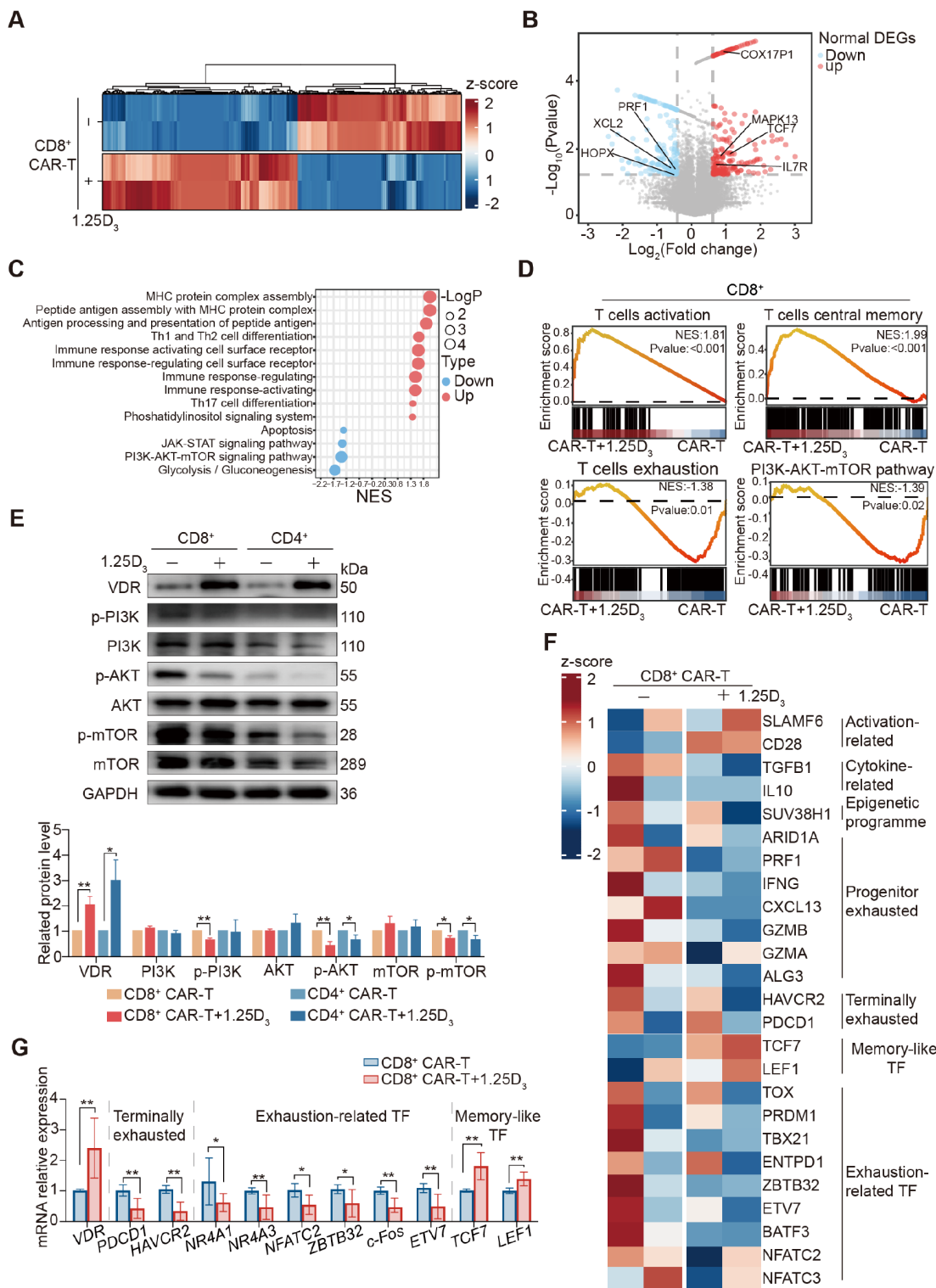


Figure 6. RNA sequencing of CD19 CAR-T cells after 1 α ,25(OH) $_2$ D $_3$ treatment (A) Heatmap showing the FPKM values of the DEGs in 1 α ,25(OH) $_2$ D $_3$ -treated CD8 $^+$ CAR-T cells compared with those in the control group. (B) Volcano plot illustrating the significant DEGs in the 1 α ,25(OH) $_2$ D $_3$ -treated group compared with the control group. Red or blue dots represent genes with upregulated or downregulated expression, characterized by a $|\log_2(\text{fold change})| > 1$ and $P < 0.05$, respectively. (C) Bubble plot of the GO and KEGG analysis of the DEGs between the 1 α ,25(OH) $_2$ D $_3$ group and the control group. (D) GSEA showing central memory, exhaustion, and the PI3K-AKT-mTOR pathway in CD8 $^+$ CAR-T cells treated with 1 α ,25(OH) $_2$ D $_3$ compared with those in the control group. (E) Protein levels of VDR, p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR determined by western blot analysis. GAPDH was used as a loading control. (F) Heatmap showing the expression profiles of selected DEGs (FDR < 0.05) between the two groups. (G) mRNA levels of VDR, terminally exhausted molecules, and exhaustion-related or memory-like-related transcription factors in the control and 1 α ,25(OH) $_2$ D $_3$ -treated groups. Data are presented as the mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$).

were significantly downregulated (Figure 6B) [47,48]. Similar patterns were also observed in the CD4 $^+$ subsets (Supplementary Figure S6B). GSEA further revealed that 1 α ,25(OH) $_2$ D $_3$ treatment enriched pathways related to T-cell activation, antigen presentation, central memory, exhaustion, cytokine signaling, and the PI3K/AKT/mTOR pathway in CD8 $^+$ subsets (Figure 6C,D). Pathways enriched in CD4 $^+$ CAR-T cells included cytokine-cytokine receptor interaction, CXCR chemokine receptor binding, T-cell activation and the JAK-STAT signaling pathway (Supplementary Figure S6C, D).

Downregulation of the PI3K/AKT/mTOR pathway has been reported to be closely associated with T-cell memory formation and terminal differentiation [49]. Furthermore, 1 α ,25(OH) $_2$ D $_3$ was reported to inhibit the PI3K/Akt/mTOR axis through a vitamin D receptor (VDR)-dependent mechanism [50]. Therefore, we examined the expression of key proteins involved in this pathway. As expected, 1 α ,25(OH) $_2$ D $_3$ treatment led to increased VDR protein expression and decreased phospho-PI3K, phospho-AKT, and phospho-mTOR protein levels without affecting total PI3K, AKT, or mTOR protein levels, further reflecting the inhibition of CAR-T-cell exhaustion after 1 α ,25(OH) $_2$ D $_3$ treatment (Figure 6E). Further transcriptomic profiling confirmed that VDR and a memory-like transcription factor, *TCF7*, were upregulated at the mRNA level, whereas genes associated with progenitor exhaustion or terminal exhaustion, including *IFNG*, *CXCL13*, *NFATC2*, *NR4A1*, *PDCD1*, and *HAVCR2*, were downregulated after 1 α ,25(OH) $_2$ D $_3$ treatment (Figure 6F). These trends were validated by qPCR, which revealed increased transcription of *VDR*, *TCF7*, and *LEF1* and decreased expression of exhaustion-related TFs, such as *PDCD1*, *HAVCR2*, *NR4A1/3*, *c-Fos*, *ZBTB32*, and *ETV7* (Figure 6G) [51–55].

The canonical action pathway of 1 α ,25(OH) $_2$ D $_3$ involves binding to its receptor (VDR), leading to nuclear translocation where VDR functions as a transcription factor [56]. Based on the hypothesis that 1 α ,25(OH) $_2$ D $_3$ modulates CD19 CAR-T-cell exhaustion and differentiation via the VDR, we utilized the VDR antagonist MeTC7 to validate these functional phenotypes [57]. We first established an optimal concentration of MeTC7 for CD19 CAR-T cells using a dose-response assay. The results demonstrated that cell viability decreased with increasing MeTC7 concentration (Supplementary Figure S7A), whereas PD-1 and TIM-3 expression levels increased along MeTC7 concentration (Supplementary Figure S7B,C). Based on these collective findings, 50 nM was determined to be the optimal MeTC7 concentration for treating CD19 CAR-T cells, balancing its effects on cell viability and inhibitory receptor expression. CD19 CAR-T cells were treated for 48 h under four conditions: (1) 50 nM 1 α ,25(OH) $_2$ D $_3$, (2) 50 nM MeTC7, (3) vehicle control (equivalent DMSO), and (4) sequential treatment with 50 nM MeTC7 (24 h) followed by 50 nM 1 α ,25(OH) $_2$ D $_3$ (24 h). After treatment, inhibitory receptor expression (PD-1, TIM-3) and CD4 $^+$ /CD8 $^+$ T or CAR-T-cell subset differentiation were analyzed via flow cytometry. The results demonstrated that MeTC7 blocked the 1 α ,25(OH) $_2$ D $_3$ -induced downregulation of inhibitory receptors (PD-1, TIM-3) (Figure 7A,B). Analysis of subsets revealed that MeTC7 abrogated the ability of 1 α ,25(OH) $_2$ D $_3$ to maintain CD8 $^+$ CD19 CAR-T cells in a naïve state and increase the CCR7 $^+$ frequency (Figure 7C,D). Similar impairment of 1 α ,25(OH) $_2$ D $_3$ -mediated differentiation was observed in the CD4 $^+$ compartment (Supplementary Figure S7D,E). Collectively, these data demonstrate that 1 α ,25(OH) $_2$ D $_3$ regulates CD19 CAR-T-cell exhaustion and differ-

entiation through VDR-dependent mechanisms.

To map the genomic binding landscape of VDR, we employed CUT&Tag, a high-resolution method for profiling protein-DNA interactions. After 1 α ,25(OH) $_2$ D $_3$ treatment, VDR binding to chromatin was markedly increased, with 35 % of the peaks localized in promoter regions (Figure 7E–G). GO and KEGG analyses revealed that these VDR target genes were enriched in pathways involving T cell activation/differentiation, Wnt signaling, JAK-STAT, NF- κ B, and metabolism (Supplementary Figure S7F,G). By overlapping the RNA-seq and VDR CUT&Tag datasets, we identified key regulatory genes directly modulated by the VDR (Figure 7H). We found that 1 α ,25(OH) $_2$ D $_3$ treatment upregulated the expression of memory-promoting genes, such as *TCF7* and *CD28*, and suppressed the expression of exhaustion-related genes, such as *NFATC2*, *NR4A3*, *CD69*, and *c-Fos* (Figure 7I–L and Supplementary Figure S7H,I). These findings collectively suggest that 1 α ,25(OH) $_2$ D $_3$ may enhance CAR-T-cell function through VDR-mediated transcriptional reprogramming, promoting a memory-like state while mitigating exhaustion.

1 α ,25(OH) $_2$ D $_3$ enhances the memory phenotype of CAR-T cells under tumor stimulation

To investigate how 1 α ,25(OH) $_2$ D $_3$ influences individual CAR-T-cell states upon tumor stimulation, we performed single-cell RNA sequencing on SU-DHL-4-stimulated CD19 CAR-T cells treated with DMSO or 1 α ,25(OH) $_2$ D $_3$. UMAP analysis identified seven cell clusters, comprising six T cell subtypes and one DLBCL population (Figure 8A and Supplementary Figure S8A–C). VDR expression was notably enriched in two T cell clusters, suggesting its transcriptional regulatory role following 1 α ,25(OH) $_2$ D $_3$ treatment in these cells (Figure 8B). Functional annotation based on exhaustion and memory gene signatures revealed a reduction in exhausted T cells and an increase in memory-like T cells in the 1 α ,25(OH) $_2$ D $_3$ group (Figure 8C). In particular, the frequency of CD8 $^+$ T cell sub-cluster 3 was significantly elevated upon 1 α ,25(OH) $_2$ D $_3$ treatment (Figure 8D and Supplementary Figure S8D), resulting in reduced exhaustion markers and features of early memory differentiation (Figure 8E). Volcano plot and KEGG analyses further revealed that the CD8 $^+$ sub-cluster 3 was enriched in pathways associated with T cell activation, antigen processing, and cytotoxicity (Figure 8F,G). CD4 $^+$ cells in the treatment group were also enriched in the JAK-STAT signaling, TNF signaling, and cytokine-cytokine receptor interaction pathways (Supplementary Figure S8E). Similar to previous findings, 1 α ,25(OH) $_2$ D $_3$ treatment upregulated the expression levels of memory-related TFs (e.g., *TCF7* and *LEF1*) and activation markers (CD28) but decreased the expression of exhaustion-related TFs (e.g., *ETV7*, *TOX* and *BATF3*) in sub-cluster 3 (Figure 8H). Notably, enhanced intercellular communication was observed between B cells and CD8 $^+$ T cell sub-cluster 3, suggesting functional interplay in the tumor microenvironment (Figure 8I). Taken together, these data demonstrate that 1 α ,25(OH) $_2$ D $_3$ promotes the expansion of memory-like CAR-T cells and attenuates exhaustion programs under tumor stimulation, thus contributing to improved antitumor efficacy.

Discussion

In this study, we demonstrated that 1 α ,25(OH) $_2$ D $_3$, the active form of vitamin D, enhances the antitumor efficacy of CD19-directed CAR-T cells against DLBCL. The results from healthy donor- and

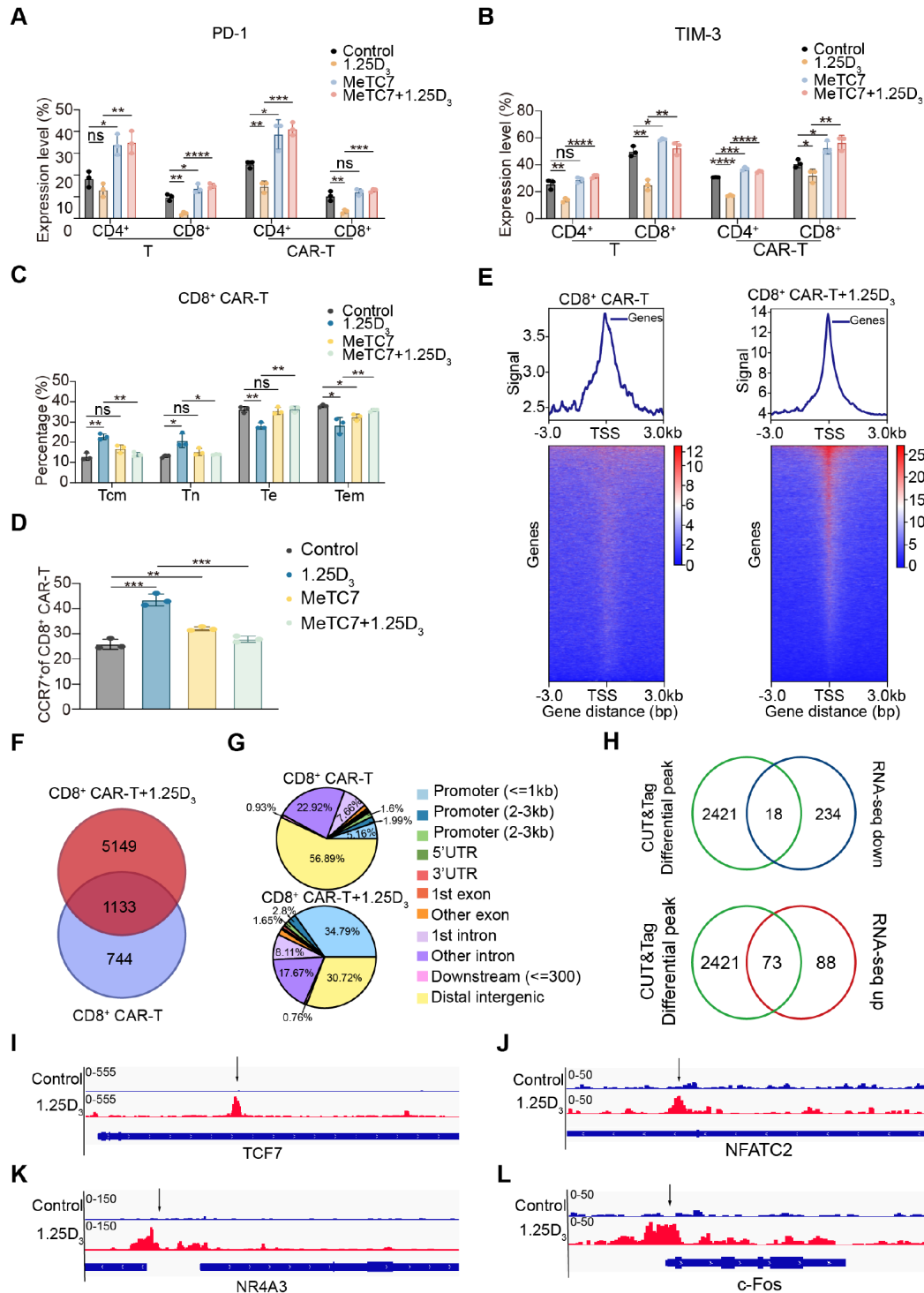


Figure 7. 1 α ,25(OH) $_2$ D $_3$ limits CD19 CAR-T-cell exhaustion and differentiation through the VDR transcription factor (A,B) Flow cytometry analysis of the expression levels of PD-1 and TIM-3 in the control (DMSO), 1 α ,25(OH) $_2$ D $_3$ alone, MeTC7 alone, and the combination of 1 α ,25(OH) $_2$ D $_3$ and MeTC7 ($n = 3$) groups. (C) Quantification of the subpopulations of CD8⁺ CAR-T cells in the control (DMSO), 1 α ,25(OH) $_2$ D $_3$ alone, MeTC7 alone, and the combination of 1 α ,25(OH) $_2$ D $_3$ and MeTC7 groups ($n = 3$). (D) The percentage of CCR7-expression CD8⁺ CAR-T cells in the control (DMSO), 1 α ,25(OH) $_2$ D $_3$ alone, MeTC7 alone, and the combination of 1 α ,25(OH) $_2$ D $_3$ and MeTC7 ($n = 3$) groups. (E) Line chart of the read distribution relative to the TSS (top), and the binding density of the VDR in CD8⁺ CAR-T cells was visualized via deepTools (bottom). (F) The number of peaks identified in the CD8⁺ CAR-T and CD8⁺ CAR-T cells in the 1 α ,25(OH) $_2$ D $_3$ groups. (G) The peak distribution in the whole genome of genes with increased VDR binding after 1 α ,25(OH) $_2$ D $_3$ treatment. (H) Venn diagram displaying the overlap between DEGs and differential peaks between CD8⁺ CAR-T cells treated with 1 α ,25(OH) $_2$ D $_3$ and those treated with DMSO. (I-L) Representative CUT&Tag sequencing tracks showing the binding of VDR to the promoter regions of *TCF7*, *NFATC2*, *NR4A3*, and *c-Fos* in DMSO- and 1 α ,25(OH) $_2$ D $_3$ -treated CD8⁺ CAR-T cells. Data are presented as the mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant).

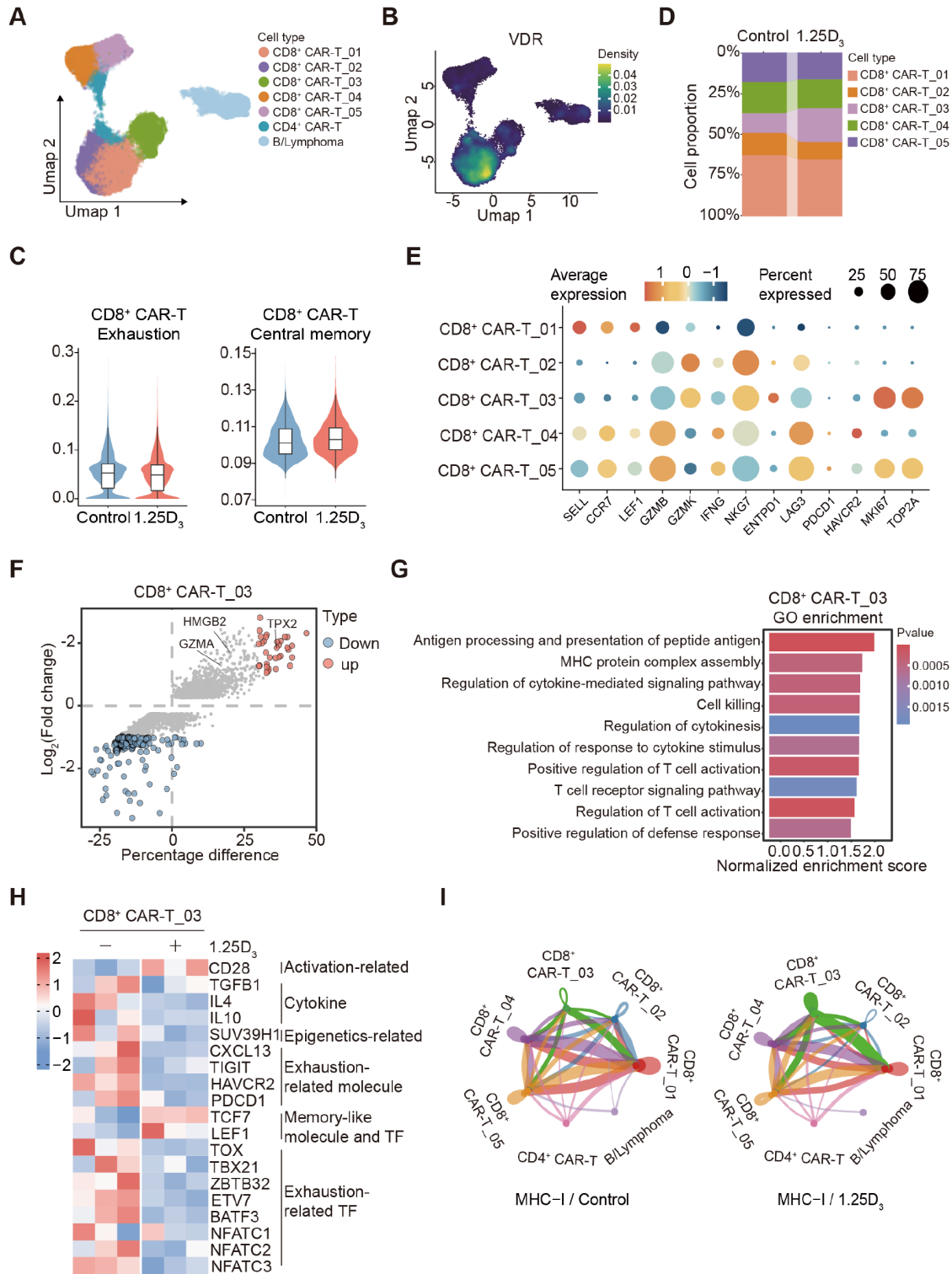


Figure 8. Single-cell transcriptome analysis of CD19 CAR-T cells after 1 α ,25(OH) $_2$ D $_3$ treatment (A) UMAP plot of the scRNA-seq datasets color coded by seven distinct cell types. (B) UMAP plot showing the expression of the VDR in seven distinct cell types. (C) Violin plots showing the exhausted and central memory-related scores for CD8⁺ CAR-T cells. (D) Proportion of subpopulations with CD8⁺ CAR-T-cell populations. (E) Dot plot for marker genes of each CD8⁺ CAR-T-cell type. The color gradient represents the average scaled expression of marker genes in each cell type, and the dot size represents the percentage of cells expressing the marker genes in each cell type. (F) Volcano plot illustrating the significant DEGs in the 1 α ,25(OH) $_2$ D $_3$ -treated CD8⁺ CAR-T_03 group vs the control group. Red or blue dots represent genes with upregulated or downregulated expression, characterized by |log $_2$ -fold change| > 1 and P < 0.05, respectively. (G) GO enrichment plot for genes upregulated in CD8⁺ CAR-T_03 cells in the 1 α ,25(OH) $_2$ D $_3$ -treated group. (H) Heatmap showing the expression profiles of selected DEGs (FDR < 0.05) among the groups. (I) The extensive cell-cell communication in the control and 1 α ,25(OH) $_2$ D $_3$ -treated groups.

patient-derived CAR-T cells, as well as those from xenograft mouse models, suggest that 1 α ,25(OH) $_2$ D $_3$ enhances CAR-T-cell expansion, persistence, and tumor infiltration while alleviating T-cell exhaustion. Mechanistically, our findings further suggest that 1 α ,25(OH) $_2$ D $_3$ may exert these effects through the upregulation of VDR, which transcriptionally reprograms CAR-T cells, promoting memory-like differentiation and downregulating exhaustion-associated genes. Specifically, our CUT&Tag and single-cell RNA sequencing analyses revealed that VDR may bind to the regulatory regions of key transcription factors, such as TCF7 and NR4A3, thereby reshaping the functional heterogeneity of CAR-T cells under tumor stimulation. Collectively, these findings provide mechanistic insight and translational potential for leveraging 1 α ,25(OH) $_2$ D $_3$ to optimize CAR-T-cell therapies in patients with R/R DLBCL, potentially enhancing long-term survival and reducing the risk of relapse by mitigating T-cell exhaustion and promoting a memory-like phenotype.

T cell exhaustion remains a major barrier for sustained CAR-T efficacy in the treatment of R/R DLBCL. To overcome this limitation, previous studies have explored various immunomodulatory interventions. For example, administration of the anti-PD-1 antibody, pembrolizumab, has been shown to reinvigorate antitumor T cell immunity [58,59]; however, the clinical use of this approach is hindered by the occurrence of immune-related adverse events (irAEs), such as autoimmune reaction. Several other studies have evaluated small-molecule drugs that improve T cell function, such as ibrutinib, lenalidomide, and dasatinib [40,60,61]. Nevertheless, the optimal dosing, treatment schedule, and safety profiles of these small molecules require further clinical verification, and their use may substantially increase the overall cost of treatment. In contrast, our study revealed that 1 α ,25(OH) $_2$ D $_3$ can effectively rescue T-cell exhaustion during CAR-T-cell therapy for DLBCL without the need for additional cytotoxic drugs or immune checkpoint blockade, suggesting a safe and cost-effective strategy to improve CAR-T-cell performance in the long-term by mitigating exhaustion and enhancing T-cell persistence.

The associations between vitamin D or 1 α ,25(OH) $_2$ D $_3$ and immunotherapy for DLBCL have been well documented. For example, vitamin D insufficiency is associated with inferior clinical outcomes in CAR-T recipients [34], and low serum concentrations of 1 α ,25(OH) $_2$ D $_3$ are independently associated with inferior event-free survival (EFS) and OS in DLBCL patients [33,62]. Moreover, vitamin D supplementation is associated with improved rituximab-based treatment in DLBCL patients with suboptimal vitamin D levels [35]. While these studies highlighted the prognostic relevance of vitamin D status in DLBCL, the underlying mechanisms remain largely unexplored. Our study provides mechanistic insight by demonstrating that 1 α ,25(OH) $_2$ D $_3$ helps reprogram CAR-T cells, especially CD8 $^+$ T cells, through the upregulation of VDR, which enhances the expression of memory-associated transcription factors (e.g., TCF7 and LEF1) while suppressing the expression of exhaustion markers (e.g., NR4A1/3, ZBTB32, NFATC2, PDCD1, HAVCR2, and ETV7), thereby alleviating CAR-T-cell exhaustion. This functional reprogramming was further validated by single-cell RNA sequencing, which revealed an increased prevalence of memory-like CAR-T cells following 1 α ,25(OH) $_2$ D $_3$ treatment under tumor antigen stimulation. Consistent with enhanced memory differentiation, we observed reduced phosphorylation of PI3K/AKT/mTOR pathway components, a signaling axis whose inhibition has

been associated with memory T-cell formation. These findings suggest that VDR-mediated transcriptional modulation may mechanistically link the clinical benefits of vitamin D supplementation to enhanced CAR-T-cell functionality.

Nevertheless, this study has several limitations. First, although our data suggest that the immunomodulatory effects of 1 α ,25(OH) $_2$ D $_3$ on CAR-T cells are mediated through VDR signaling, this conclusion is primarily based on correlative transcriptomic and epigenetic profiling. Functional validation via the use of VDR-deficient models, such as T-cell-specific VDR-knockout mice or CRISPR-mediated VDR deletion in CAR-T cells, is needed in future research to verify these signaling networks and elucidate the role of the VDR in CAR-T-cell differentiation and exhaustion. Second, while we demonstrated the therapeutic potential of 1 α ,25(OH) $_2$ D $_3$ in a subcutaneous DLBCL xenograft model, this system does not fully reflect the physiological and pathological microenvironments in DLBCL. More relevant models, such as murine-derived CAR-T-cell models or humanized mouse platforms, will be necessary to better recapitulate tumor-immune interactions and evaluate the translational applicability of this strategy. Third, given the broad immunomodulatory effects of vitamin D [63], comprehensive profiling of other immune cells, such as regulatory T cells and dendritic cells, as well as cytokine levels, is needed to understand the observed therapeutic benefits beyond those of CAR-T cells.

In summary, our study identified 1 α ,25(OH) $_2$ D $_3$ as a potent immunomodulatory agent that may enhance the efficacy of CD19-directed CAR-T-cell therapy through VDR-mediated transcriptional reprogramming. Our findings highlight the potential of combining 1 α ,25(OH) $_2$ D $_3$ with CAR-T-cell therapy as a safe and cost-effective strategy to enhance therapeutic responses, particularly in patients with R/R DLBCL. Further clinical studies are warranted to explore the translational applicability of this combinatorial approach and to optimize dosing strategies.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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