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Prior chemotherapy deteriorates T-cell quality for CAR T-cell therapy in B-cell non-Hodgkin's lymphoma

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ABSTRACT

Background Chimeric antigen receptor (CAR) T-cell therapy depends on T cells that are genetically modified to recognize and attack cancer cells. Their effectiveness thus hinges on the functionality of a patient's own T cells. Since CAR T-cell therapy is currently only approved for advanced cancers after at least one line of chemotherapy, we evaluated the potential negative effects of prior exposure to chemotherapy on T-cell functionality.

Methods We studied T cells of two B-cell non-Hodgkin's lymphoma patient cohorts, one collected before treatment (pre-therapy) and the other after one or more (median 3) lines of chemotherapy (post-therapy). Leveraging advanced multiparameter flow cytometry, single-cell RNA sequencing (scRNA-seq), whole-genome DNA methylation arrays and in vitro functionality testing of generated CAR T cells, we compared patient samples in their suitability for effective CAR T-cell therapy.

Results We discovered significant modifications in T-cell subsets and their transcriptional profiles secondary to chemotherapy exposure. Our analysis revealed a discernible shift towards phenotypically more differentiated T cells and an upregulation of markers indicative of T-cell exhaustion. Additionally, scRNA-seq and DNA methylation analyses revealed gene expression and epigenetic changes associated with diminished functionality in post-therapy T cells. Cytotoxicity assays demonstrated superior killing efficacy of CAR T cells derived from treatment-naïve patients compared with those with chemotherapy history.

Conclusions These findings corroborate that employing T cells collected prior to frontline chemotherapy could enhance the effectiveness of CAR T-cell therapy and improve patient outcomes.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The importance of chimeric antigen receptor (CAR) T-cell product composition and characteristics, such as phenotype and CD4/CD8 ratio, was previously shown to be an important indicator of response to CAR T-cell therapy. It has moreover been observed that chemotherapy-exposed patients show a reduced amount of naïve T cells compared with healthy donors. However, the possibly extensive ramifications of chemotherapy exposure on T-cell functionality in the context of CAR T cell therapy have not yet been fully elucidated.

WHAT THIS STUDY ADDS

⇒ We have, to our knowledge for the first time, performed an in-depth analysis of patients with B-cell non-Hodgkin's lymphoma (B-NHL) T cells in order to uncover the effects of chemotherapy exposure on T-cell quality for CAR T-cell therapy. Leveraging multiparameter flow cytometry, single-cell RNA sequencing, whole-genome DNA methylation screens and functional assays, we were able to uncover T-cell alterations secondary to chemotherapy not only on a phenotypical level, but also including transcriptome and epigenome changes indicating a reduced suitability for adoptive T-cell therapies. Furthermore, using in vitro cytotoxicity assays, we could show a functional advantage of using non-exposed T cells as starting material for CAR T-cell production compared with chemotherapy-exposed ones.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The results of our study highlight the negative effects previous lines of treatment have on T-cell quality in the context of CAR T-cell therapy. On one hand, this could be applied by including a T-cell preservation step before the initiation of first-line therapy in high-risk patients in order to have access to a more effective starting material for CAR T-cell generation down the line. On the other hand, our findings strongly support current studies investigating the benefits of incorporating CAR T-cell therapy into the first-line treatment algorithm for B-NHL.

BACKGROUND

Chimeric antigen receptor (CAR) T-cell therapy has revolutionized the treatment of relapsed or refractory (r/r) B-cell non-Hodgkin's lymphoma (B-NHL) by leveraging genetically modified T cells to target the B-cell antigen CD19.[1 2] While initial response rates are high, many patients show

disease progression within 1 year of therapy.[1 2] Mechanisms of resistance include antigen escape, hampered trafficking of CAR T cells to the tumor and limited T-cell functionality, including T-cell exhaustion and insufficient persistence.[3] Recent studies indicate that the therapeutic success of CAR T cells is determined by certain factors, such as a higher CD4:CD8 T-cell ratio, higher proportion of less-differentiated T cells and reduced expression of exhaustion markers.[4–6] These findings underscore the importance of T-cell condition for CAR T-cell manufacturing, which varies from patient to patient and may be influenced by the progression of the disease and previous anticancer treatments.[7]

Despite the emergence of novel immunotherapies, chemotherapies still play an indispensable role, not only in the front-line setting but also as salvage therapy in r/r B-NHL. The immuno-chemotherapy regimen R-CHOP (rituximab with cyclophosphamide, doxorubicin, vincristine and prednisolone) represents an essential backbone of lymphoma therapy, mainly as standard first-line therapy in diffuse large B-cell lymphoma (DLBCL).[8] Rituximab, a monoclonal antibody targeting the B-cell antigen CD20, induces depletion of malignant B cells, but unfortunately also harms the physiological B cells,[9] resulting in hampered antibody production and antigen presentation that can impair T-cell activation, proliferation and survival.[10 11] Moreover, while chemotherapy mainly targets rapidly dividing cancer cells, it can also stimulate immune responses by triggering the release of antigens from dying tumor cells, leading to cell activation and differentiation.[12] Thus, chemotherapeutic agents can affect T cells in their activation, polarization, migration and survival,[13–15] or even cause lymphopenia, which is associated with negative treatment outcomes after CAR T-cell therapy.[16] Collectively, these studies suggest a relevant effect of prior chemotherapy on the patient's immune system, including T cells, which serve as the basis for the manufacturing of an effective CAR T-cell product.

Here, we provide comprehensive insights into the impact of past immuno-chemotherapy on T-cell functionality for subsequent adoptive T-cell therapy. We performed a multimodal analysis of patient T cells, including multiparameter flow cytometry, single-cell RNA sequencing (scRNA-seq) and whole-genome DNA methylation profiling, as well as functional assays. Overall, our findings indicate that T cells collected prior to chemotherapy exposure retain characteristics associated with a more effective CAR T-cell product.

METHODS

Study design

We examined peripheral blood samples from two groups: (1) patients with newly diagnosed B-NHL before any treatment and (2) patients with B-NHL who had received prior chemotherapy (median of 3 lines). Cohorts include patients with DLBCL, mantle cell lymphoma and follicular lymphoma (FL). Clinical details are listed in online supplemental table 1.

Sample preparation and culture

Peripheral blood mononuclear cells were isolated from whole blood using density gradient centrifugation. T cells were isolated with the EasySep Human T Cell Isolation Kit (STEMCELL Technologies) and cryopreserved in 10% dimethyl sulphoxide (DMSO) by freezing down to −80°C in a Mr Frosty (Nalgene) freezing container. Samples were then stored in liquid nitrogen until use. The human DLBCL cell lines DB, U2932, Rck8, OciLy1 and SU-DHL-10 were propagated in Roswell Park Memorial Institute 1640 medium (RPMI-1640 medium) (Gibco), supplemented with 10% heat-inactivated fetal calf serum (Merck), 100 U/mL penicillin/streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Gibco). Primary human T cells were cultured in T-cell medium (TCM), consisting of RPMI 1640 additionally supplemented with 1× non-essential amino acid solution, 1 mM sodium pyruvate (PAN Biotech) and 54 µM β-Mercaptoethanol (Sigma Aldrich). All medium was optimized for the corresponding cell types in advance. All cells were counted using the LUNA-FL dual fluorescence cell counter (Logos Biosystems).

Manufacturing of CAR T cells

A CD19-targeting CAR was encoded by a plasmid connecting the CD19 single chain variable fragment (scFV) (derived from the FMC63 monoclonal antibody) with the hinge and transmembrane domain of CD8 and the intracellular domains of 4-1BB and CD3ζ (online supplemental figure S1A). The CAR-encoding lentivirus was generated as previously described.[17]

For transduction, T cells were activated with Dynabeads Human T-Activator CD3/CD28 (Invitrogen) at a bead:-cell ratio of 1:3 and cultured in TCM supplemented with 100 U/mL IL-2 (Miltenyi Biotech) for 3 days. The activated T cells were transduced with the CAR-encoding lentivirus at a multiplicity of infection (MOI) of 1 and 6 µg/mL polybrene (Sigma-Aldrich). Cells were expanded in TCM supplemented with 10 ng/mL interleukin (IL)-7 (Miltenyi) and 50 ng/mL IL-15 (Miltenyi) for 7 days after transduction. Subsequently, CAR T cells were purified by magnetic cell sorting using an integrated myc-tag.

Flow cytometric analysis

Cells were stained for viability using the Zombie UV Fixable Viability Kit (BioLegend). Antibody staining for CD3, CD4, CD8, PD-1, TIGIT, LAG-3, TIM-3, annexin, CD25, CCR7, CD45RA, CD69, CD62L, CD94, CD27, CD40L, IFN-γ, TNF-α, IL-2, granzyme B, CD107a and CD137 was performed for 20 min at room temperature in the dark. Data were acquired using a CytoFLEX LX flow cytometer and analyzed using the CytExpert software (V.2.4.0.28). Unsupervised clustering was performed using the XShift clustering algorithm plugin of FlowJo (V.10.6.2). For gating, we focused first on lymphocytes,

excluding doublets and dead cells. CD3+T cells were then split into either CD4+ or CD8+ T cells before gating for the shown markers.

Functional assays

A repetitive stimulation assay was carried out by co-culturing CAR T cells with the GFP$^+$ DLBCL target cell lines at a 1:1 effector:target (E:T) ratio in TCM. Every 3 days, the cells were counted and analyzed via flow cytometry. The E:T ratio was restored by the addition of fresh cancer cells for a total of three to five cycles. 24 hours after each cell seeding step, an additional sample was analyzed via flow cytometry for the expression of cytokines and activation markers on the CAR T cells.

Methylation analysis

DNA methylation was analyzed using the Infinium MethylationEPIC Kit (Illumina). Bisulfite conversion was performed using the EZ-DNA methylation Gold Kit (Zymo Research) and hybridized according to the manufacturer's instructions. The array was scanned using the HiScan platform (Illumina). Raw data were analyzed in R (V.4.3.1) using the minfi package (V.1.48.0).[18] Background correction was performed using the preprocessNoob function.[19] The following packages were used for further analysis: ggplot2,[20] ComplexHeatmap,[21] missMethyl.[22]

Single-cell sequencing analysis

Processing of the sequencing library was performed using cellranger multi (V.7.1.0) and the GRCh38 genome annotation, and analysis was run with Seurat (V.4.0.11). Cells with more than 10% mitochondrial RNA content and ones with less than 250 or more than 5,000 genes were filtered out. Seurat's reference mapping workflow was applied to assign cell type labels and embed coordinates from a peripheral blood mononuclear cells (PBMC) reference.[23] We used level 2 annotation for T cells. We used DoubletFinder (V.2.0.3) to detect and remove doublets and additionally removed cells with a cell type prediction on level 1 of less than 0.75. We used scRepertoire (V.1.1.22) to process cellranger VDJ output. Clonal diversity was assessed using the inverse Simpson score. Differential cell–cell signaling was assessed using scDiffCom (V.0.1.05). The area under the curve (AUC) scores for non-exhaustion, exhaustion, apoptosis, hypoxia, early memory, stress,[6] dysfunction[24] and persistence[25] were computed using AUCell (V.1.22.0).[26]

Statistical analysis

Variations in cell type composition were analyzed using mixed-effects binomial models from the lme4 package (V.1.1–27.1). Differential gene expression was assessed with DESeq2 (V.1.30.13), employing a pseudo-bulk method. Data description was presented using the appropriate statistical position and dispersion parameters. For functional experiments regarding univariate group comparisons, t-tests were conducted. Confirmatory comparisons between the two cohorts with respect to the development of killing efficacy over time were performed using generalized estimating equations, with the time vector of killing efficacy as the dependent variable and cohort, time, and the cohort×time interaction term as predictive variables.[27] Statistical analyses were performed with GraphPad Prism (V.9.4.1) and IBM SPSS Statistics (V.29.0.0.0).

Data sharing statement

The scRNA-seq and methylation data sets presented in this study are deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus, accession number GSE275947 and GSE274821, respectively.[28 29] This study does not report original code.

RESULTS

Prior chemotherapy exposure alters T-cell composition of patients with B-NHL

We compared T cells of patients with B-NHL collected before initiation of first-line therapy (pre-therapy) and after a median of 3 lines of treatment (post-therapy), in order to assess the impact of administered chemotherapeutics on T-cell functionality for an effective CAR T-cell therapy (figure 1A, online supplemental table S1).

Using multiparameter flow cytometry, we revealed profound differences in CD4$^+$ and CD8$^+$ T-cell compositions. Post-therapy patients showed a reduced CD4/CD8 ratio (figure 1B) and exhibited a shift towards differentiated T cells. This was reflected in a reduced percentage of naïve T cells (CCR7$^+$CD45RA$^+$) and an increase in effector memory (EM) T cells (CCR7$^-$CD45RA$^-$), while the levels of central memory and effector T cells were comparable between groups (figure 1C,D). After dimensionality reduction and unsupervised clustering, t-distributed stochastic neighbor embedding (t-SNE) and XShift analysis were performed on a flow cytometry panel of T-cell exhaustion markers. We observed distinct T-cell exhaustion profiles between pre-therapy and post-therapy samples (figure 1E, online supplemental figure S1B). Cluster three mainly comprises pre-therapy CD4$^+$ T cells lacking exhaustion marker (programmed cell death protein 1 (PD-1), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT)) expression. In contrast, cluster nine is enriched in post-therapy CD8$^+$ T cells expressing the highest levels of the assessed exhaustion markers. We observed only marginal differences in the analyzed markers when subclustering our patient data based on time since therapy, number of lines of therapy, certain types of therapy, tumor burden or entity (data not shown).

Single-cell RNA sequencing uncovers altered gene expression in post-therapy T cells

To determine alterations in gene expression secondary to chemotherapy, we performed scRNA-seq of T cells

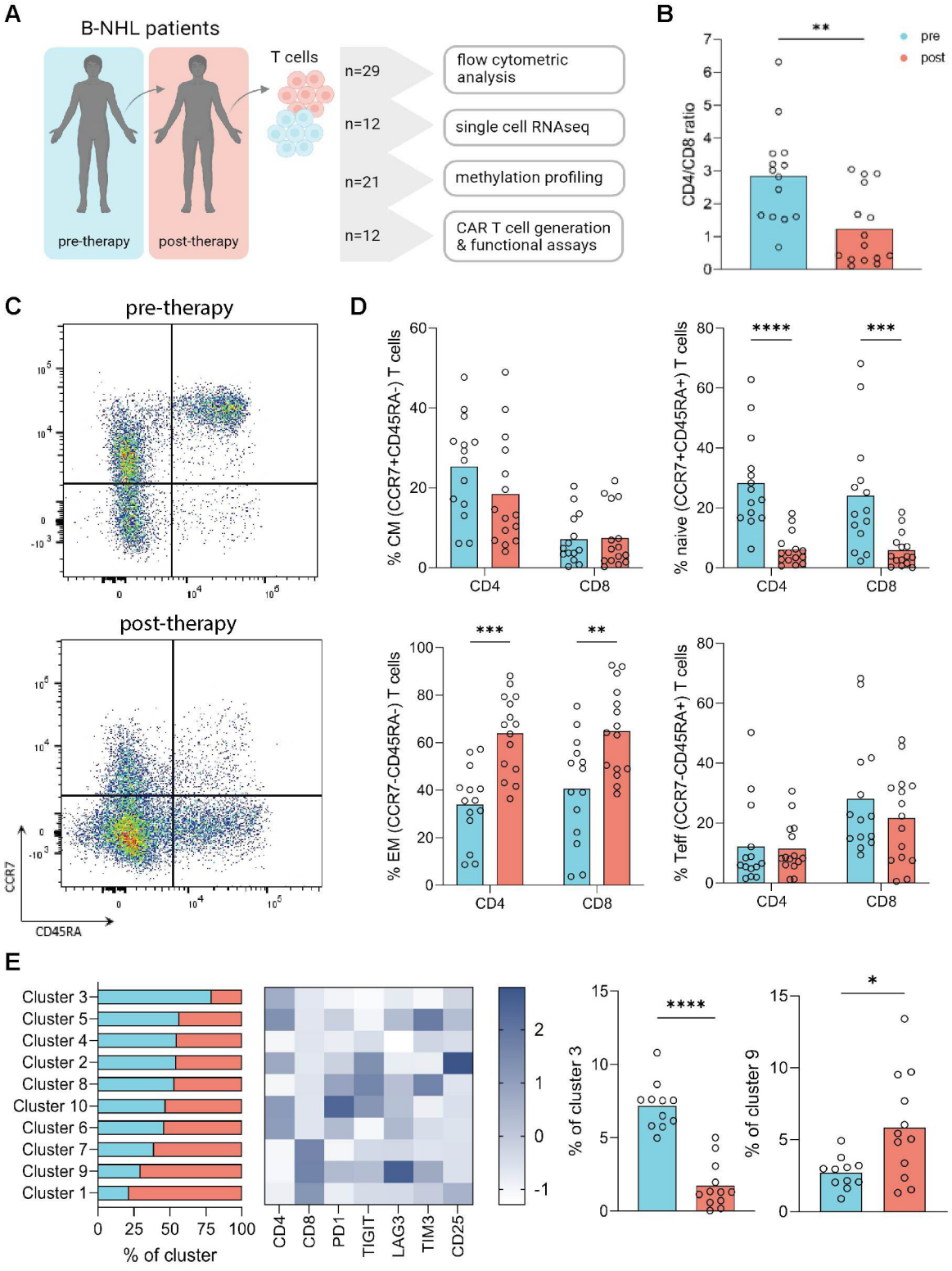

Figure 1 Previous chemotherapy exposure alters T-cell composition of patients with B-NHL. (A) Schematic representation of the implemented analyses. T cells of patients pre- (blue) and post-therapy (red) were isolated and analyzed via flow cytometry, single-cell RNA sequencing, whole-genome DNA methylation screen and functional kill assays. (B) CD4/CD8 ratio of isolated T cells from both cohorts. (C) Representative dot plot depicting the stainings for T-cell phenotypes, using CCR7 and CD45RA. (D) Phenotypes of isolated T cells from both cohorts. t-tests were used for significance testing. (E) Unsupervised clustering of flow cytometry panel, clusters 3 and 9 are significantly different between groups (Mann-Whitney test). B-NHL, B-cell non-Hodgkin's lymphoma; CAR, chimeric antigen receptor; RNA-seq, RNA sequencing.

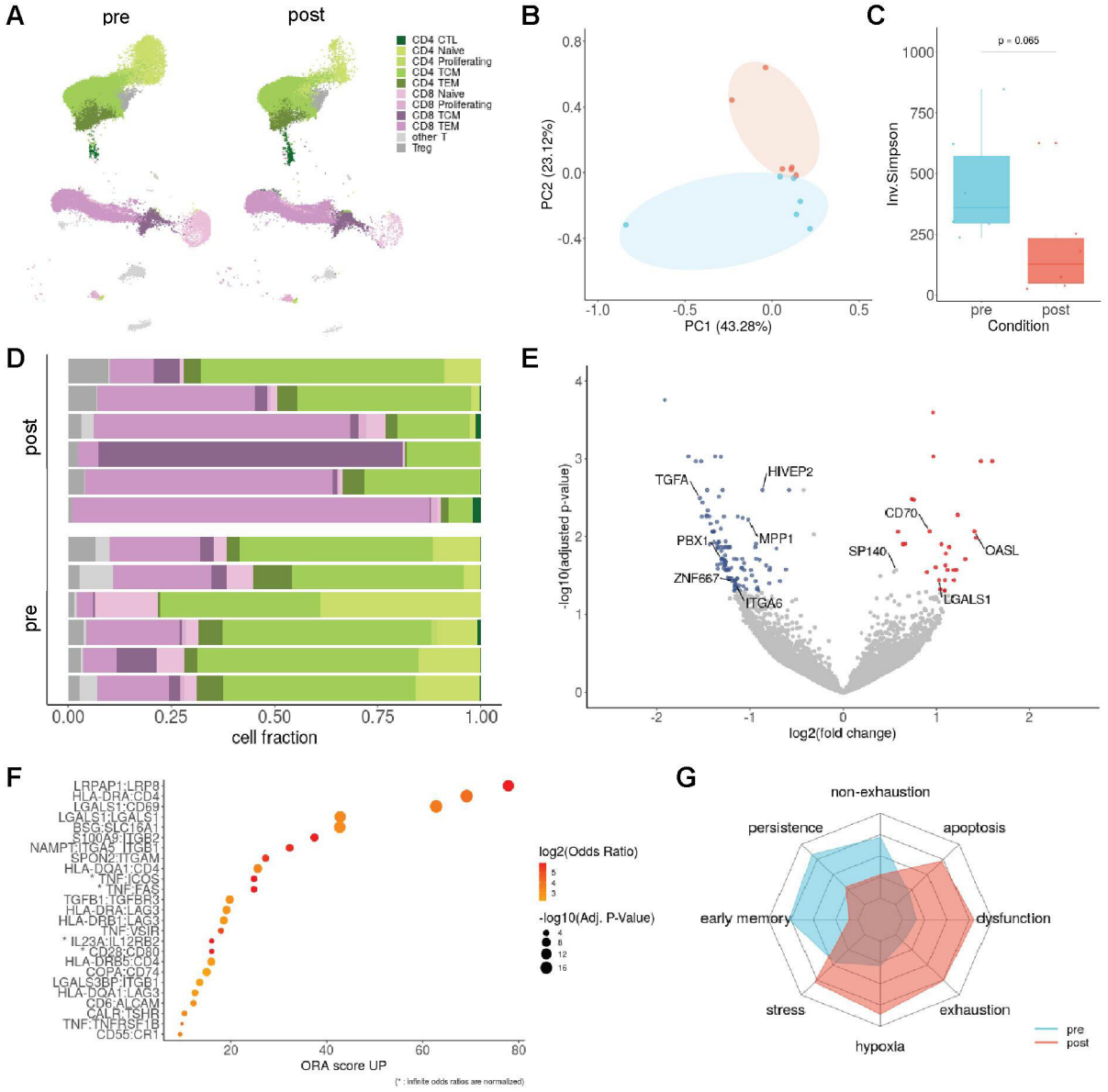

Figure 2 Single-cell RNA sequencing uncovers changes in gene expression of treatment-exposed T cells. (A) Single-cell RNA sequencing data projected into a PBMC reference embedding for both cohorts. (B) Cell type composition of each sample. (C) PCA on the cell type compositions of the different samples. (D) Boxplot visualizing the decreased TCR repertoire in post-therapy samples, as measured by the inverse Simpson score. t-tests were used for significance testing. (E) Volcano plot depicting significantly upregulated (red) and downregulated (blue) genes in post-treatment samples (adj.p<0.05, logFC>0.58). (F) Top ligand-receptor interactions in an over-representation analysis of differential cell–cell signaling between pre-therapy and post-therapy samples. (G) Radar chart depicting the mean AUC score of multiple gene signatures associated with T-cell function. AUC, area under the curve; PBMC, peripheral blood mononuclear cells; PCA, principal component analysis; TCR, T cell receptor.

from a total of 12 patients, divided into pre-therapy and post-therapy samples, respectively. A total of 105,575 T cells, with a median of 8,282 cells per donor after quality control, were analyzed. After annotation of T-cell subtypes via label transfer from a PBMC cell type reference,[23] we performed Uniform Manifold Approximation and Projection (UMAP) for dimension reduction to visualize the cell composition of both cohorts (figure 2A). This data confirmed the significant reduction in naïve T cells and the increase of $CD8^+$ EM T cells post-therapy, with distinct clustering of our cohorts in a principal component analysis (PCA) (figure 2B,C, online supplemental figure S3A). We also observed a trend towards reduced clonal diversity in post-therapy T cells (p=0.065, figure 2D). In a differential gene expression analysis using our pseudo-bulked data, 29 genes were significantly higher and 118 genes significantly lower expressed in post-therapy compared with pre-therapy T cells (adj.p<0.05, abs(logFC)>0.58) (figure 2E, online supplemental table S2). Importantly, these group-specific gene expression patterns could not be attributed solely to the different composition of the T-cell compartment, as the differentially expressed genes

(DEGs) are also reflected at the level of the different T-cell subpopulations (online supplemental figure S2). As no selection of specific T-cell subpopulations is carried out in advance to the manufacturing of approved CAR T-cell products, we focused on the DEGs of all T cells for further analyses. Post-therapy T cells showed higher expression of genes previously reported to be involved in T-cell activation (CD70, OASL), exhaustion (SP140), reduced migration (RGS9) and apoptosis (LGALS1).[30–34] Genes with elevated expression in pre-therapy T cells play a role in T-cell survival (HIVEP2, ZNF667), proliferation (TGFA, MPP1) and migration (ITGA6).[35–38]

To identify pathways which might drive T-cell dysfunction, an over-representation analysis of the top ligand-receptor interactions enriched in post-therapy samples was performed (figure 2F). Among the top hits were interactions between CD4 and MHC class II molecules (HLA-DRA, HLA-DQA1, HLA-DRB5). These interactions are crucial for T-cell activation, suggesting an increasingly activated phenotype post-therapy. This is in line with previous observations of an increase in HLA-DR expression on T cells during chemotherapy treatment.[39] Two interactions involving LGALS1 were also enriched. Given the role of galectin-1, which is encoded by LGALS1, in promoting T-cell apoptosis,[34] this may indicate an increased susceptibility to apoptosis in post-therapy T cells. Another significant interaction involved NAMPT with ITGA5/ITGB. Endogenous NAMPT, also known as visfatin, can promote apoptosis by regulating Bcl2l1, Fas, and caspase-3.[40] In addition, NAMPT has been reported to be involved in chemoresistance by altering the susceptibility to DNA damage, which is consistent with post-therapy T cells that have already been exposed to chemotherapies.[41] A Gene Ontology term analysis of the enriched interactions revealed the response to chemicals or drugs as two of the top hits, again supporting a lasting effect of chemotherapy on the T cells (online supplemental figure S3B).

In order to relate the observed differences in gene expression to available studies, we used previously established gene signatures associated with specific T-cell functions (figure 2G).[6 24 25] This analysis revealed a marked shift towards exhaustion and dysfunction in the post-therapy T cells, while signatures associated with non-exhaustion and persistence were mainly observed in pre-therapy T cells. Together, the transcriptome of post-therapy T cells differs significantly from that of pre-therapy T cells, suggesting that chemotherapy triggers processes that drive T cells into an exhausted, pre-apoptotic and dysfunctional phenotype.

Post-therapy T cells exhibit changes in DNA methylation associated with reduced functionality

Given the recent interest in DNA methylation as a potential regulator of T-cell exhaustion,[42 43] we conducted a genome-wide methylation analysis to identify the epigenomic changes occurring in T cells secondary to chemotherapy. A principal component analysis revealed a distinct separation of the two cohorts (pre-therapy vs post-therapy) based on diverging methylation patterns (figure 3A). Our analysis revealed 88,958 differentially methylated positions (DMPs) between the groups, with 16,764 of these DMPs being hypermethylated and 72,194 hypomethylated in post-therapy T cells (adj.p<0.05, abs(logFC)>0.2) (figure 3B). Additionally, our data revealed a marked enrichment of partially methylated domains (PMDs) in hypomethylated CpGs (5'-C-phosphate-G-3') of post-therapy samples (figure 3C), indicating previous cell activation and differentiation.[44]

To test whether this altered DNA methylation profile after chemotherapy is associated with clinical response to CAR T cells, we applied the recently published EPICART signature to our data, which stratifies response to CAR T-cell therapy (figure 3D).[45] A heatmap illustrating the methylation levels of the 18 CpGs included in the EPICART signature revealed a distinct separation between our groups. The upper panel of the heatmap highlights CpGs for which hypermethylation is associated with a survival benefit, while the lower panel represents positions that require hypomethylation to improve survival outcomes. According to this, pre-therapy T cells show a methylation profile consistent with an improved clinical response. Four single CpG sites of the EPICART signature, which have shown direct correlation with improved survival, also displayed significantly higher methylation levels in pre-therapy T cells (figure 3E).

Although methylation status and gene expression levels do not necessarily correlate due to the complex processes involved in transcription, we observed a remarkable correlation in two genes that are both known to be involved in T-cell functionality. Galectin-1 has been discussed to impede T-cell functionality by impairing migration and recruitment as well as inducing T-cell apoptosis.[33 34] In our data, galectin-1 gene expression was increased in post-therapy T cells. This effect was also observed on a protein level, as we could show a direct correlation of RNA counts and protein abundance for this specific protein (online supplemental figure S3C). Additionally, galectin-1 expression was negatively correlated with methylation at two distinct positions (cg2761953, cg21737444), suggesting a methylation-dependent suppression of galectin-1 expression (figure 3F). HIVEP2 contributes to T-cell development and function by suppressing nuclear factor kappa B (NF-κB) signaling.[41] For this gene, opposing results were obtained with increased methylation and decreased HIVEP2 gene expression in the post-therapy T cells (figure 3G).

Hence, chemotherapy exposure results in significant epigenetic alterations of T cells, which are associated with T-cell dysfunction and worse clinical response to CAR T-cell therapy.

CAR T cells of pre-therapy patients show epigenetic features associated with response to therapy

To determine if the observed differences between pre-therapy and post-therapy T cells persist after the

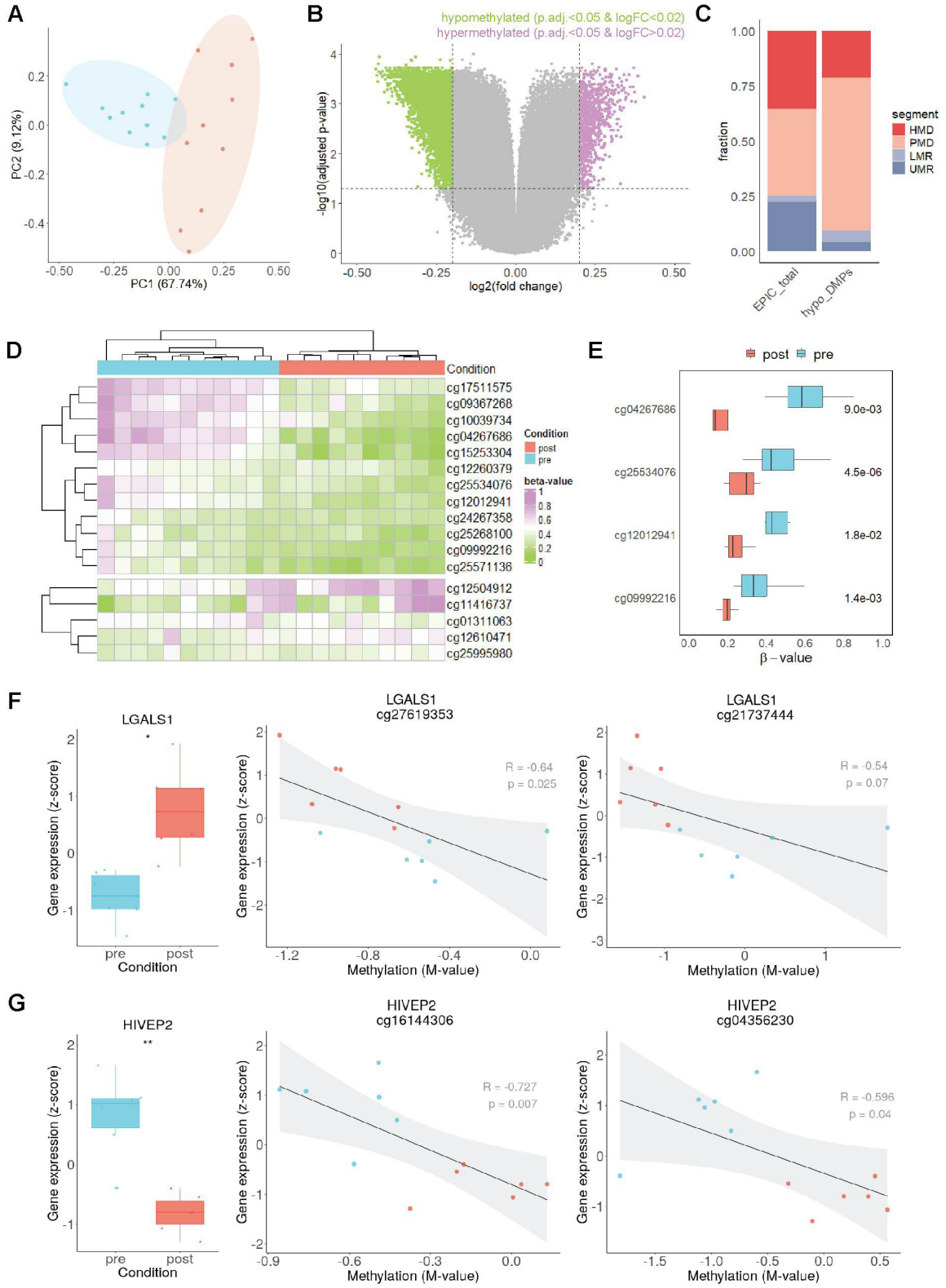

Figure 3 Post-therapy T cells exhibit changes in their DNA methylome associated with reduced functionality. (A) PCA of methylation status of both cohorts. (B) Volcano plot showing the amount of hyper-methylated (violet) and hypo-methylated (green) CpGs. (p.adj<0.05 and abs(logFC)>0.02) (C) Fraction of CpGs that are part of specific regions of the genome. (D) Heatmap showing Euclidean clustering using the CpGs of the EPICART signature. Hypermethylation of CpGs from the upper panel and hypomethylation of CpGs from the lower panel were associated with improved response in the EPICART signature. (E) Methylation levels of four survival-associated CpGs in our cohorts. t-tests were used for significance testing. Gene expression and correlation of methylation and gene expression for (F) LGALS1 and (G) HIVEP2. CpG, 5'-C-phosphate-G-3'; HMD, highly methylated domain; LMR, low methylated region; PCA, principal component analysis; PMD, partially methylated domain; UMR, unmethylated region.

manufacturing of CAR T cells, we genetically modified pre-therapy and post-therapy T cells to express CD19-specific CARs (figure 4A). We observed no differences in lentiviral transduction rate and CD4/CD8 ratio between groups (figure 4B,C).

The phenotypic differences observed ex vivo were largely lost after generation and expansion of CAR T cells, so that the chemotherapy-induced alterations in the T cells as source material were hardly evident anymore. Post-expansion, the number of naïve T cells was comparable, while a minor difference remained in the proportion of EM T cells in the CAR products (figure 4D). Of note, CD27 and CD62L, both associated with a less differentiated phenotype and accordingly an improved response,[6] were significantly increased in pre-therapy $CD4^+$ T cells (figure 4E). Moreover, post-therapy samples exhibited a slightly higher expression of PD-1 and TIGIT.

Since the EPICART signature was originally established on CAR T cells, we performed a genome-wide methylation analysis of our CAR-transduced and expanded T cells, derived pre-therapy and post-therapy, to check whether the differences persist in the final product. While the manufacturing process impacted DNA methylation (online supplemental figure S4), we found that in contrast to the aligned phenotypes, epigenetic differences between our cohorts remained evident after CAR-T cell generation. CAR T cells derived from pre-therapy versus post-therapy T cells still clustered clearly in the principal component analysis (figure 4F), suggesting a functional imprinting on the epigenetic level. Upon application of the EPICART signature to our CAR T-cell methylation data, we observed that even after transduction and expansion, CAR T cells generated from pre-therapy T cells maintained an epigenetic profile associated with improved clinical efficacy (figure 4G).

Prior chemotherapy diminishes CAR T-cell functionality

To functionally validate our findings, we established a repetitive kill assay, using multiple $CD19^+$ DLBCL cell lines as a target (figure 5A). Repeated antigen-specific CAR T-cell activation resulted in a comparable decrease in the production of the effector cytokines interferon-γ and tumor necrosis factor-α in both cohorts (online supplemental figure S5B). Other tested markers, such as PD-1, TIGIT, granzyme B and CD137 also did not differ significantly between cohorts (online supplemental figure S5B and S6A,B). T-cell viability was also similar between cohorts, with an average viability of above 90% at any time (data not shown). However, while CAR T cells from both groups were able to lyse a similar number of cancer cells during the initial stimulation, it became apparent after repeated stimulations that the cumulative killing capacity of CAR T cells derived from post-therapy T cells was reduced (figure 5B, online supplemental figure S6C). For two of the tested cell lines, our analysis uncovered a significant difference between the cohorts in the killing efficacy over time. While CAR T-cell challenge with the other two cell lines did not uncover statistically significant differences, the same trend could be observed. Overall, these results suggest a decreased cytotoxic capacity of post-therapy CAR T cells. Importantly, the differences in cytotoxic potential cannot be explained by differences in expansion alone, as the E:T ratio of 1:1 was restored after each round of stimulation to ensure that both groups had the same starting conditions each time. Thus, pre-therapy CAR T cells show better persistence and efficacy on chronic antigen stimulation, which occurs in the clinical setting of CAR T-cell therapy.

DISCUSSION

Our study uncovers the significant impact of prior chemotherapy on T-cell functionality with respect to CAR T-cell therapy, providing critical insights into optimizing this treatment for patients with B-NHL. We provide a comprehensive data package on multiomic characteristics of T cells derived from pre-therapy and post-therapy patients as well as compare their functionality as CAR T cells, including phenotypic and functional studies.

Both our multiparameter flow cytometry analyses and scRNA-seq data revealed a shift in the T-cell compartment towards more differentiated subpopulations in previously chemotherapy-treated patients with lymphoma. This is consistent with previous reports suggesting that chemotherapy can, in addition to cell depletion, also have an activating effect on the immune system and thus stimulate T cells, which may lead to a decline in naïve T cells.[13 14] In line with this, Zhang *et al* uncovered a chemotherapy-induced reduction in the percentage of naïve T cells in leukemia patients undergoing chemotherapy, as compared with healthy untreated donors.[46] Moreover, it was previously observed that chemotherapy preferentially depletes less differentiated ($CD4^+CD45RA^+$) cells.[39] In children with various cancers it was shown that chemotherapy depleted early lineage T cells, and this also correlated with a reduced ability to respond to ex vivo stimulation.[47] An in vitro study showed that one mechanism by which chemotherapy can affect surviving T cells is by damaging a cells' mitochondria, impacting both energy reserve and proliferation.[48] Our epigenetic data revealed an enrichment of PMDs in hypomethylated CpGs secondary to chemotherapy. PMD hypomethylation reflects a cells' mitotic history and can thus be used as a marker for previous proliferative phases.[44] The association between T-cell differentiation and proliferation aligns with our observation of a higher proportion of differentiated cells in post-therapy T cells. In the context of CAR T-cell therapy, less differentiated T cells have been shown to be associated with improved sustained response, both in the ZUMA-1 and ZUMA-7 trials.[4 49 50] Furthermore, the published methylation signature EPICART used to predict survival following CAR T-cell therapy is also associated with less differentiated cells, with the authors showing that EPICART-positive cells are mainly naïve and early memory T cells.[45] Applying this signature to our data, we observed more favorable methylation patterns

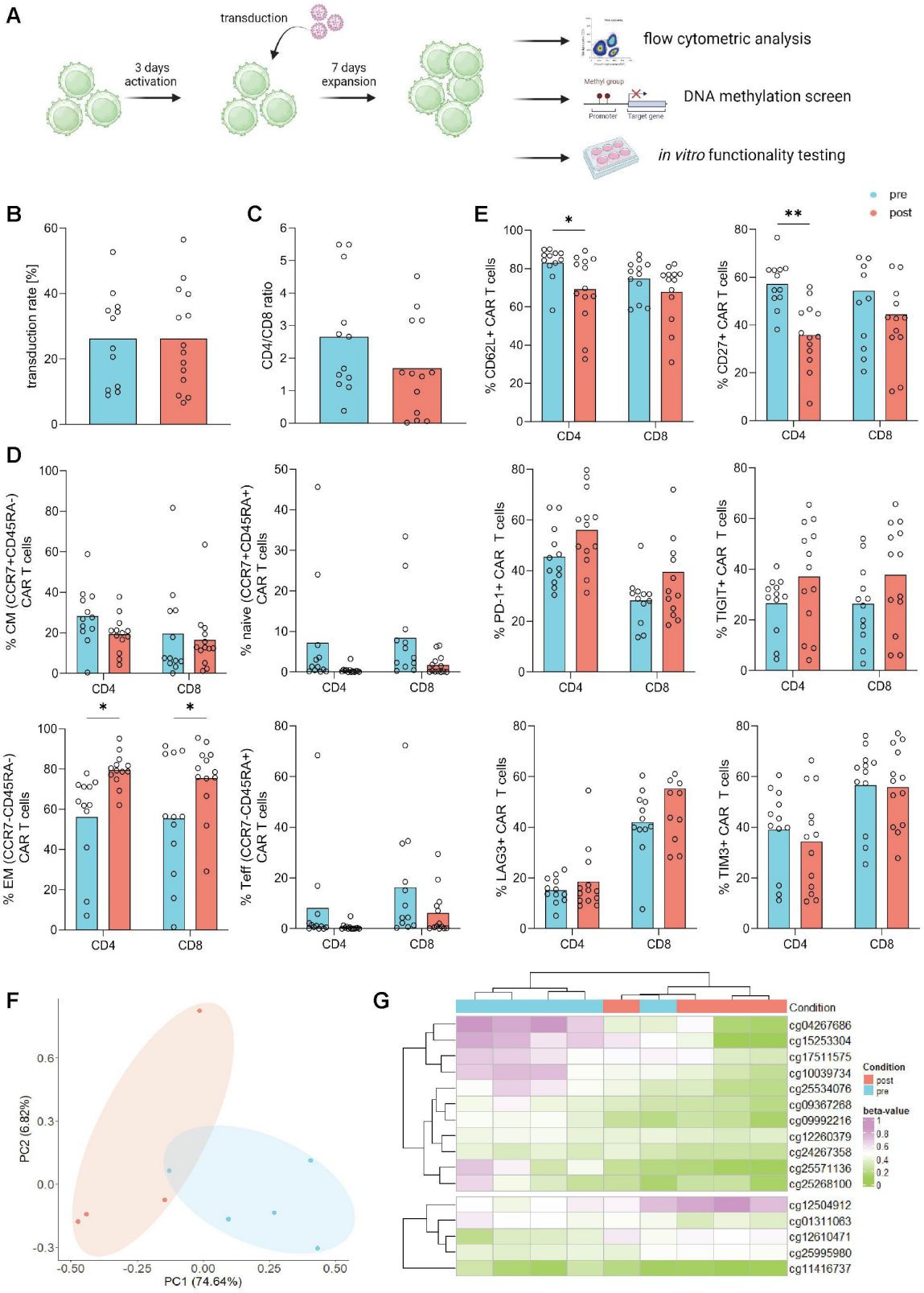

Figure 4 CAR T cells of pre-therapy patients show features associated with durable response. (A) Overview of CAR T-cell generation as well as phenotypic and functional testing workflow. (B) Transduction rate, (C) CD4/CD8 ratio, (D) phenotypes and (E) expression of exhaustion and phenotype markers on pre-therapy and post-therapy samples. t-tests were used for significance testing. (F) PCA of DNA methylation comparing both cohorts. (G) Heatmap showing the clustering of pre-therapy and post-therapy CAR T cells employing the EPICART signature. CAR, chimeric antigen receptor; CM, central memory; EM, effector memory; LAG3, lymphocyte activation gene 3 protein; Teff, effector; TIGIT, T-cell immunoreceptor with immunoglobulin and ITIM domains; TIM3, T-cell immunoglobulin and mucin-domain containing-3; PCA, principal component analysis; PD1, programmed cell death protein 1.

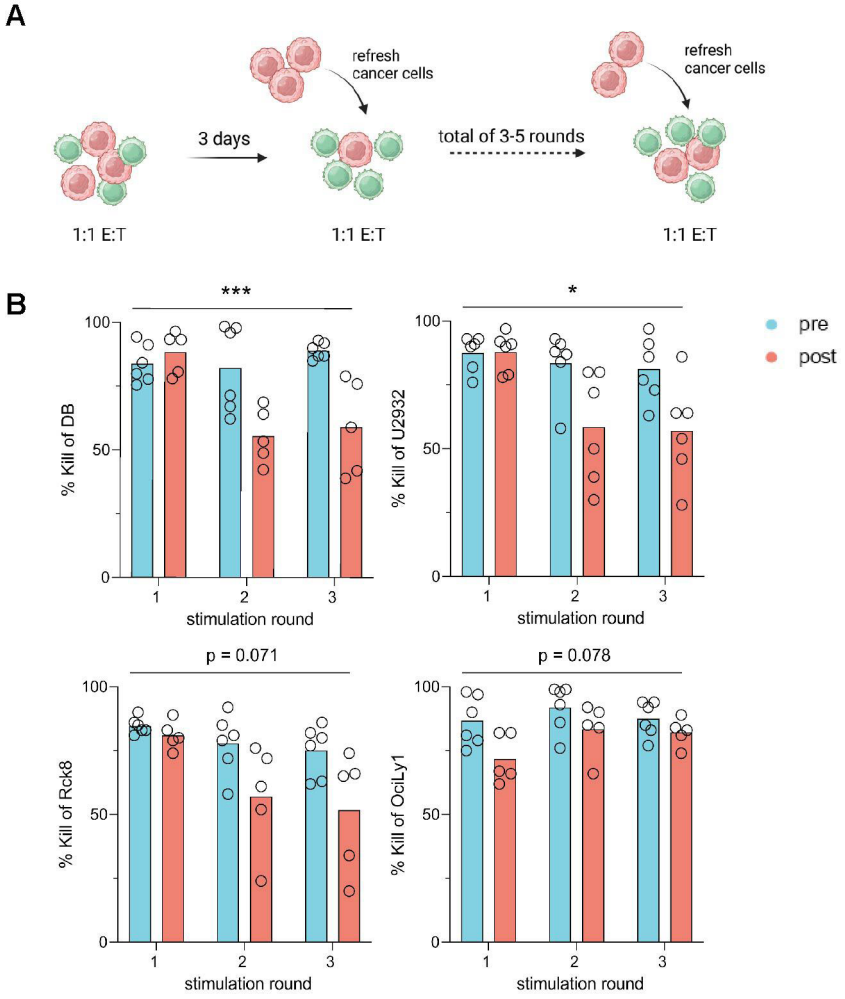

Figure 5 Pre-therapy T cells generate a more effective CAR T product. (A) Overview of the established repetitive kill assay. (B) Percentage of target cell lysis by CAR T cells for each stimulation. Kill is defined as the reduction in viable tumor cells after 72 hours of co-culture. Generalized estimating equations were used for significance testing. CAR, chimeric antigen receptor; E:T, effector:target.

in pre-therapy cells, not only on the ex vivo T cells but also the manufactured CAR T cells. The methylation of these CpGs is thus able to predict an improved clinical response for pre-therapy cells even after transduction and expansion, while the phenotypical differences analyzed by flow cytometry almost leveled off during the course of CAR T-cell manufacturing. This underlines the promise of epigenetic signatures as a predictor of therapy success in CAR T-cell therapy. Epigenetic reprogramming further offers a promising new approach for enhancing cellular products like CAR T cells, which is supported by our data showing that methylation levels and gene expression directly correlate for certain T-cell functionality genes.

T-cell exhaustion due to chronic antigen exposure is one of the main mechanisms of adoptive T-cell therapy failure.[5 6] It was previously demonstrated that the amount of exhausted $CD8^+$ T cells is higher in products of non-responders to CAR T-cell therapy.[51] Gene signatures of hypoxia, apoptosis, exhaustion, stress and dysfunction have been shown to be elevated in T cells of non-responders compared with complete responders.[6 24 25] These signatures are enriched in our post-therapy T cells, suggesting that using pre-therapy T cells as the starting material for CAR T-cell manufacturing may be advantageous. Looking at gene expression differences in more detail, we uncovered genes with higher expression in post-therapy samples that are associated with exhaustion and apoptosis, such as galectin-1 and SP140. Galectin-1 has been implicated in immunosuppression, being involved in T-cell apoptosis as well as cancer immune evasion.[33] In the context of CAR T-cell therapy, leukemia-derived galectin-1 has been shown to reduce activation and efficacy of the CAR T product via CAR downregulation.[52] Additionally, both galectin-1 and SP140 have been linked to non-responsiveness to CAR T-cell therapy, being significantly elevated in T cells of patients with progressive disease.[50] On the other hand, genes with higher expression in pre-therapy samples included HIVEP2, which is a negative regulator of the pro-apoptotic protein Bax, thus dampening the induction of death pathways.[41] These and other differentially expressed genes could be the focus of future

investigations on improving T-cell fitness for adoptive T-cell therapies.

Insufficient persistence is another major challenge of CAR T-cell therapy. Studies could already correlate in vivo CAR T-cell expansion to long-term treatment response.[53] Our data revealed that pre-therapy T cells are enriched for a gene signature associated with T-cell persistence following adoptive cell therapy.[25] This signature was previously identified on clones of tumor-infiltrating lymphocytes that were able to persist in a patient after adoptive transfer and includes genes like KLRB1, CD40LG and ITGB1. Overall, Lu *et al* could show that non-persisting clones had a more effector-like phenotype. In CAR T-cell therapy, the amount of naïve $CD8^+$ T cells, which is higher in pre-therapy samples, correlates with in vivo expansion in patients with lymphoma.[54] In our functional assay we observed an improved expansion of $CD8^+$ CAR T cells of pre-therapy samples. As doubling time in culture in response to stimulation has been shown to be associated with response,[49] this indicates another advantage of T cells from pre-therapy over post-therapy samples for CAR T-cell production. In addition, a more persistent cytotoxic response was observed in the pre-therapy product, hinting towards a more durable efficacy. Of course, in vitro co-culture experiments cannot fully recapitulate the complex interactions of the tumor microenvironment. However, our results are supported by data showing that killing performance in an in vitro assay can be an adequate indicator for clinical efficacy of CAR T-cell products.[50]

The implications of our findings are significant for clinical practice. Using T cells collected before chemotherapy could potentially improve the outcomes of CAR T-cell therapy by enhancing the persistence and efficacy of the engineered T cells. On one hand, this could be applied by performing a T-cell apheresis step in high-risk patients before starting treatment, so that a more functional source material is available for CAR T-cell therapy. On the other hand, these findings strongly underline the importance of the current clinical studies investigating the benefits of moving CAR T cells into first-line therapy for B-NHL.[55]

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