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Long-term in vitro expansion ensures increased yield of central memory T cells as perspective for manufacturing challenges

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Abstract

Adoptive T cell therapy (ATT) has revolutionized the treatment of cancer patients. A sufficient number of functional T cells are indispensable for ATT efficacy; however, several ATT dropouts have been reported due to T cell expansion failure or lack of T cell persistence in vivo. With the aim of providing ATT also to those patients experiencing insufficient T cell manufacturing via standard protocol, we evaluated if minimally manipulative prolongation of in vitro expansion (long-term [LT] >3 weeks with IL-7 and IL-15 cytokines) could result in enhanced T cell yield with preserved T cell functionality. The extended expansion resulted in a 39-fold increase of murine $CD8^+$ T central memory cells (Tcm). LT expanded $CD8^+$ and $CD4^+$ Tcm cells retained a gene expression profile related to Tcm and T memory stem cells (Tscm). In vivo transfer of LT expanded Tcm revealed persistence and antitumor capacity. We confirmed our in vitro findings on human T cells, on healthy donors and diffuse large B cell lymphoma patients, undergoing salvage therapy. Our study demonstrates the feasibility of an extended T cell expansion as a practicable alternative for patients with insufficient numbers of T cells after the standard manufacturing process thereby increasing ATT accessibility.

KEYWORDS
adoptive immunotherapy, cytokines, T lymphocytes, translational medical research

Abbreviations: ATT, adoptive T cell therapy; Bcl-2, B cell lymphoma 2; BLI, BioLuminescent imaging; BLITC, BioLuminescent imaging of T cells; CAR, chimeric antigen receptor; DLBCL, diffuse large B cell lymphoma; GO, gene ontology; IFN-γ, interferon-γ; Lag3, lymphocyte activating 3; LT, long-term; MFI, mean fluorescence intensity; NFAT, nuclear factor of activated T cells; Nr4a1, Nuclear Receptor Subfamily 4 Group A Member 1; PCA, principal component analysis; Pdcd1, programmed cell death 1; Rag, recombination-activating gene; Sca1, stem cell antigen 1; ST, short-term; Tcm, central memory T cells; TCR, T-cell receptor; Teff, primarily effector T cells; Tem, effector memory T cells; TIR, T cell inhibitory receptor; TNF-α, tumor necrosis factor-α; Tox, Thymocyte Selection Associated High Mobility Group Box; Tscm, T memory stem cell.

1 | INTRODUCTION

Adoptive T-cell therapy (ATT) using T cells, genetically redirected with tumor-specific T cell receptor (TCR) or chimeric antigen receptors (CARs), has revolutionized the field of cancer immunotherapy. So far, the most notable success resulted from NY-ESO-1 TCR T cells for multiple melanoma[1] and CD19-CAR T cells for B cell-derived malignancies.[2,3] Part of the success of engineered T cells relates to the effective ex vivo expansion of T cells. Under conventional culture conditions with IL-2, T cells differentiate into central memory T cells (Tcm), effector memory T cells (Tem) and primarily effector T cells (Teff). In recent years, it has been shown that T cell cultivation in the presence of IL-7, IL-15 and IL-21[4-10] or low molecular weight compounds such as the glycogen synthase kinase-3β inhibitor TWS119 promotes the generation of valuable Tcm and T memory stem cells (Tscm) improving ATT efficacy.[11-13] Unlike Tem and Teff, Tcm retain their ability to proliferate and survive for long term (LT).[14-16] Clinical T cell manufacturing protocols for ATT include leukapheresis of $CD8^+$ or $CD4^+$ T cells, followed by ex vivo activation, 9 to 14 days expansion and reinfusion of T cells.[17,18] Several recent preclinical studies have attempted to reduce the duration of ex vivo culture to limit T cell differentiation and enhance the efficacy of ATT.[19-21] Although reduction of the manufacturing time could be beneficial for the differentiation state of T cells and their overall quality, this may represent a limitation for those patients experiencing lymphopenia and lymphocyte dysfunctions subsequent to multiple lines of chemotherapy. ATT is a multistep process and some ATT dropouts are caused by failure to obtain sufficient numbers of T cells to reinfuse. Manufacturing of CD19 CAR T cells failed in 6% to 24% of patients, particularly if they were isolated from older or heavily pretreated diffuse large B-cell lymphoma (DLBCL) patients.[8,21,22] In our study, we aim to address whether a simple extension of T cell expansion in the presence of IL-7 and IL-15 may represent an opportunity to prevent or reduce ATT dropouts in a minimally manipulative manner by sticking to the standard cytokine protocol.

What's new

Adoptive T cell therapy (ATT) is a risky procedure involving the isolation, ex-vivo expansion, and reinfusion of tumor-specific T cells. T cell manufacturing in ATT represents a bottleneck that can limit access to therapy for those patients that experience lymphopenia or lymphocyte dysfunctions. Here, the authors evaluated if minimally manipulative prolongation of in vitro expansion (>3 weeks with IL-7 and IL-15 cytokines) could result in enhanced T cell yields with preserved T cell functionality. The results demonstrate the feasibility of extended T cell expansion as an alternative for patients with insufficient numbers of T cells after the standard manufacturing procedure.

2 | MATERIALS AND METHODS

2.1 | Mice

See Supplemental Table 1. All mice were kept under specific pathogen-free conditions following institutional guidelines.

2.2 | Murine T-cell preparation and culture

T cells were harvested from spleens, using a $CD4^+$ or $CD8^+$ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were cultured in T cell medium (TCM) consisting of RPMI and 10% fetal bovine serum (FBS; both from Life Technologies, Carlsbad, CA, United States) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mmol/L sodium pyruvate, 1x nonessential amino acids, 50 mmol/L β-mercaptoethanol and 2 mmol/L L-glutamine (all from Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). For activation, T cells were stimulated in vitro for 72 hours (polyclonal T cells from albino B6 mice) in TCM plus 1 ng/mL recombinant human IL-2 (PeproTech, Cranbury, NJ, United States) in culture plates coated with 3 μg/mL anti-CD3 (clone 145-2C11) and 2 μg/mL anti-CD28 (clone 37.51; both BD Biosciences, San Jose, CA, United States). Transgenic T cells from Marilyn BioLuminescent imaging of T cells (BLITC) were activated only for 24 hours, since previous results revealed a significant activation-induced cell death after 72 hours (Supplemental Figure 1).

Afterward, T cells were expanded using TCM containing 50 ng/mL recombinant human IL-15 and 10 ng/mL recombinant human IL-7 (both Peprotech, Cranbury, NJ, United States) for further 4 days (short-term culture, ST) or 18 days ($CD4^+$) and 25 days ($CD8^+$) (Long-term culture, LT). Medium was refreshed every third or fourth day.

2.3 | Human T-cell culture

PBMC were isolated by density gradient centrifugation (Ficoll-Hypaque; GE Healthcare, Chicago, IL, United States) from fresh heparinized blood samples from nine healthy donors and from six patients with relapsed/refractory DLBCL which were candidates for high-dose chemotherapy with stem cell support. PBMC were stimulated in vitro for 72 hours in TCM plus 1 ng/mL recombinant human IL-2 (PeproTech, Cranbury, NJ, United States) in culture plates coated with 3 mg/mL anti-CD3 (clone OKT3) and anti-CD28 (clone 15E8, both Miltenyi Biotec, Bergisch Gladbach, Germany). Afterward, human T cells were expanded as murine T cells.

2.4 | Flow cytometry

Surface staining was performed in the presence of Fc-blocking antibodies (#101320, BioLegend, San Diego, CA, United States) according

to standard staining protocols. Intracellular staining for B-cell lymphoma 2 (Bcl-2), interferon-γ (IFN-γ), IL-2, IL-10 and tumor necrosis factor-α (TNF-α) was done with fixation/permeabilization buffer (#420801, #421002, BioLegend, San Diego, CA, United States), according to the manufacturer's instructions. Antibodies are listed in Supplemental Table 2. Samples were acquired on a FACS Canto II (BD Biosciences, San Jose, CA, United States) and CytoFLEX LX (Beckman Coulter, Krefeld, Deutschland), and all analyses were performed using the FlowJo software (FlowJo, LLC, Ashland, OR, United States).

2.5 | In vitro stimulation assay

5 x 10^5 cells/mL expanded T cells were plated into flat 24-well plates coated with anti-CD3/CD28 at 37°C. After 4 hours, granzyme B concentration was determined in the supernatant using the granzyme B Mouse Uncoated ELISA Kit (#88-8022-88, Thermo Fisher Scientific, Waltham, MA, United States), according to the manufacturer's protocol. Expanded T cells were also restimulated in the presence of 10 μg/mL Brefeldin A (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and analyzed by standard flow cytometry staining protocols (see section "Flow cytometry").

2.6 | In vitro proliferation assay

Expanded T cells from albino B6 mice were adjusted to 10^7/mL and labeled with 10 μM CFSE (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 10 minutes at room temperature. Cells were washed with FBS and twice with TCM. To analyze in vitro proliferation, CFSE-labeled T cells were plated (10^5 cells/well) for 24 hours into flat 96-well plates coated with anti-CD3/CD28, respectively, 3 and 2 μg/mL, and then transferred into TCM for 3 days with IL-7/15. CFSE signal was then measured by flow cytometry at 24 hours after labeling and at day 4.

2.7 | Cell preparation for RNA sequencing and transcriptome analysis

$CD8^+$ or $CD4^+$ T cells were sorted for $CD44^+CD62L^+$ (>97%) using a FACS Aria II or Aria III (BD Biosciences). Afterward, RNA was isolated with the RNeasy Mini Kit (QIAGEN, Germantown, MR, United States). The RNA library was generated by using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, United States). Illumina sequencing was performed with NextSeq 500/550 High Output Kit v2 (Illumina, San Diego, CA, United States). RNA-seq expression data were normalized and log-transformed as log2(1 + TPM) (TPM: transcripts per million) for our own data and GSE80306.[23] The sequencing coverage and quality statistics for each sample are summarized in Supplemental Table 5. Microarray data were downloaded from GEO using the R package GEOquery (v2.46.15) for GSE92381,[23] GSE61697,[24] GSE23321,[11] GSE93211[12] and GSE80306[23] or directly extracted from cel files using the packages affy (v1.56), affydata (v1.26) and limma (v.3.34.9) for GSE41909[4] and GSE68003,[13] with rma background correction, quantile normalization and avgdiff summarization. Datasets were summarized over gene names, mapping mouse and human gene names onto each other using orthologues from MGI (HOM_MouseHumanSequence.rpt), and then combined after removing the bottom 5% of expressed genes, quantile normalization and batch correction with respect to assay using ComBat (package sva, v3.26.0). Clustering and principal component analysis (PCA) was performed using row z-scores for the gene set of Gattinoni et al.[11] Differential expression analysis was performed using DESeq2[25] using experimental batch as covariate and GO term analysis with topGO (v2.30.1).

2.8 | In vivo engraftment and persistence assay

Expanded T cells from BLITC mice were sorted for $CD44^+CD62L^+$ (>97%) using a FACS Aria II or Aria III (BD Biosciences, San Jose, CA, United States) and transferred into female albino recombination-activating gene, knock out (RagKO) mice via intravenous injection. Renilla luciferase signals of transferred T cells were measured monthly by BioLuminescent imaging (BLI). Mice were sacrificed after 1 month (engraftment) or 6 months (persistence) to quantify T cell numbers in secondary lymphoid organs using flow cytometry.

2.9 | SV40 TAg tumor model

The cell line TC200.09 was kindly provided by the Thomas Blankenstein's research group and all details are already described in Reference 26. Albino-RagKO mice were challenged with 5×10^6 TAg^+ 200ΔLuc cells, a gastric carcinoma cell line, which was genetically modified from the TC200.09 cell line[26] as described in Reference 27 and was grown in RPMI/FBS plus Dox (0.5 μg/mL). The retroviral packaging cell line PlatE (RRID:CVCL_B488) was grown in DMEM/FBS. All experiments were performed with mycoplasma-free cells. Tumors were grown for 45 days before ATT. $CD8^+$ T cells were transduced with SV40 Tag-specific TCR-I as described in Reference 27. Briefly, $CD8^+$ T cells from BLITC mice were sorted, in vitro activated and transduced with a retroviral vector expressing the alpha- and beta-chain of TCR-I (pMP71-TCR-I).[28] After in vitro expansion for 1 week (ST) or 4 weeks (LT), V_β7-positive $CD8^+$ T cells expressing TCR-I were sorted using a FACS Aria II or Aria III (BD Biosciences) and injected intravenously into tumor-bearing mice (10^5 T cells/mouse). Mean tumor diameter was determined every 2 days by caliper measurement and tumor size was calculated as length × width × weight/2. Mice were sacrificed if tumor volume became 1000 mm^3.

2.10 | H-Y tumor model

The male-derived urothelial carcinoma cell line MB49 (RRID:CVCL_7076) was kindly provided by the Thomas Blankenstein's research group. Cells

were grown in RPMI/FBS[29] and were adjusted to 2×10^5 cells/mL in PBS, mixed 1:1 with matrigel (BD Biosciences, San Jose, CA, United States) and kept on ice until subcutaneous injection of 100 µL (10^4 cells/mouse) into the hind flank of female age-matched albino RagKO mice. Thirteen days later, ST or LT expanded T cells from Marilyn BLITC mice[27] were sorted for $CD44^+$ and $CD62L^+$ (>97%) using a FACS Aria II or Aria III (BD Biosciences) and injected intravenously into tumor-bearing mice (5×10^5 T cells/mouse). Mean tumor diameter was determined every 2 days by caliper measurement and tumor size was calculated as length × width × weight/2. Mice were sacrificed if tumor volume became 1000 mm^3. All experiments were performed with mycoplasma-free cells.

2.11 | BioLuminescent imaging (BLI)

In vivo BLI was performed using a Xenogen IVIS 200 (PerkinElmer, Waltham, MA, United States) after mice were anesthetized with isoflurane (Baxter, Deerfield, IL, United States) in an XGI-8 anesthesia system (PerkinElmer, Waltham, MA, United States). In order to analyze Renilla luciferase signals (T cell engraftment, migration and persistence), mice were imaged 3 minutes after intravenous injection of freshly prepared coelenterazine (Biosynth Ltd, Newbury, United Kingdom) dissolved in 30% DMSO (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and diluted in 70% PBS (#K813-500 mL, VWR) (100 µg/100 µL per mouse). To analyze nuclear factor of activated T cells (NFAT) dependent click-beetle luciferase signals (T cell activation), mice received intraperitoneally D-Luciferin (Biosynth Ltd, Newbury, United Kingdom) (300 µg/g body weight) prepared in PBS and were imaged 10 minutes later. Images were acquired for 5 minutes using small binning. All data were analyzed using Living Image analysis software (PerkinElmer, Waltham, MA, United States). The signal strength was quantified by photon/s/cm^2/steradian after digital setting of equal regions of interest.

2.12 | Generation of genetic modified $CD3^+$ T cells

Isolated human peripheral blood lymphocytes (PBL), isolated from healthy donors, were transduced with a specific tyrosinase-specific TCR-T58 as previously.[30] Briefly, 1×10^6 cells peripheral blood lymphocytes (PBL) were isolated from healthy donors, and seeded in

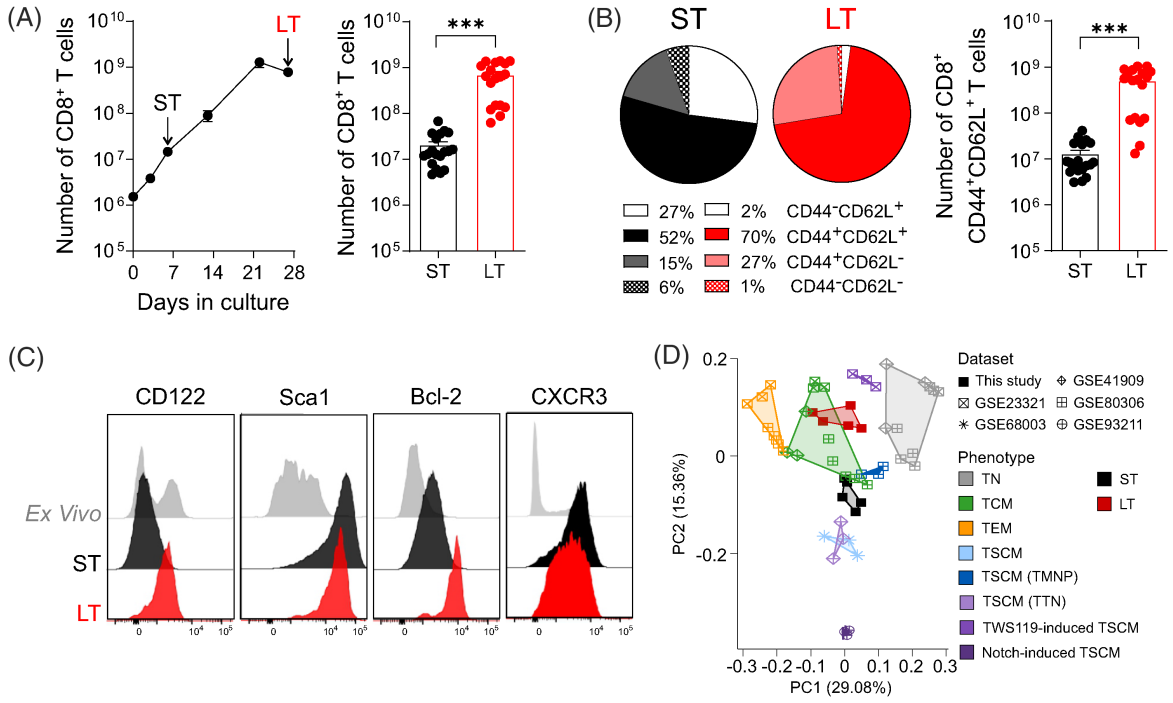

FIGURE 1 Extended expansion of murine T cells leads to increased numbers of $CD8^+$ T cells with Tcm/Tscm phenotype and preserved in vitro functional properties. Polyclonal $CD8^+$ T cells were isolated from C57BL/6 mice, activated via anti-CD3/CD28 and IL-2 for 72 hours and further cultured with IL-7/IL-15 for 4 (ST) or >21 (LT) days. The quantification and phenotypical analysis was done by flow cytometry. A, Growth kinetic of $CD8^+$ T cells is displayed by line diagram as mean values ± SEM. The bar shows mean ± SEM of total $CD8^+$ T cell numbers. B, Distributions of $CD8^+$ T cell subpopulations, defined by CD44 and CD62L, are illustrated in pies. The bar shows mean ± SEM of total $CD8^+CD44^+CD62L^+$ T cell numbers. Data were generated in 5 to 7 independent experiments with n = 5-9 mice. ***$P < .001$, paired t test. C, The $CD8^+CD44^+CD62L^+$ T cell subset resulting from the T cell culture was further characterized by CD122, Sca-1, Bcl-2 and CXCR3 and compared to ex vivo $CD8^+CD44^+CD62L^+$ T cells. Shown are representative histograms from three independent experiments with n = 3 mice. D, Principal component analysis (PCA) of ST and LT Tcm, IL-7/IL-15-generated Tscm cells (TTN,[4] GSE41909), naturally occurring $CD8^+$ T cell subsets (Tnaive/TN, Tem/TEM, Tcm/TCM, Tscm/TSCM,[11,20,34,53] GSE23321), TWS119-enriched Tscm (TSCM,[13] GSE68003) and Notch-induced Tscm (iTSCM,[12] GSE93211), memory T cells with naive phenotype (TMNP,[23] GSE80306) based on 852 genes described by Gattinoni et al from which 427 genes were expressed in all datasets [Color figure can be viewed at wileyonlinelibrary.com]

1 mL T cell medium containing 100 IU/mL IL-2 and on an anti-hCD3/hCD28-coated 24 well plates. Packaging cell line HEK-293T (RRID: CVCL_0063), stably transduced with MLV gag/pol and GALVenv, was transfected with retroviral vector MP71-encoding for tyrosinase-specific TCR-T58. PBLs were transduced by spinoculation with the infected HEK-293T supernatant and containing the virus particles and incubated 48 and 72 hours after activation, on retronectin coated plates in the presence of proteamin sulfate (4 µg/mL) and IL-2. Transduction efficiency was analyzed by flow cytometry by staining for CD8 (APC-H7), mTCRß-constant (PE) and DAPI. Cells were further cultured with IL-7 and IL-15 (each 5 ng/mL). Subsequent specific activation experiments were performed by seeding T cells on a peptid-coated (TCR-tyrosinase$^{369\text{-}377}$: YMDGTMSQV) plate. All experiments were performed with mycoplasma-free cells.

2.13 | Statistics

Statistical analysis was performed using the two-tailed paired *t* test, using GraphPad Prism 7.0 software (GraphPad Software). *P* values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Extended expansion of murine T cells leads to increased numbers of CD8$^+$ T cells with Tcm and Tscm phenotype and preserved in vitro functional properties

In order to evaluate the ability of T cells to expand in a LT culture setting, we used a combined approach of previous published protocols with activation of murine T cells via anti-CD3/CD28 antibodies and consecutive expansion culture in the presence of IL-2 or IL-7/IL-15.[4,11,31] After 2 weeks of expansion, murine T cells exhibited a strong increase in the number of CD8$^+$ T cells. At around week 3 of culture, the CD8$^+$ T cell expansion reached a plateau phase. When we compared CD8$^+$ cell numbers between ST (7 days) and LT (>21 days) expansion, we found a 39-fold increase of CD8$^+$ LT T cells with respect to ST (Figure 1A). The majority of expanded CD8$^+$ T cells were CD44$^+$CD62L$^+$, a phenotype associated with Tcm subpopulation[4,32,33] (Figure 1B). Distribution analyses of subpopulations after 2 and 3 weeks of expansion showed that the increase in Tcm was accompanied by a decrease in naive T cells (data not shown), suggesting

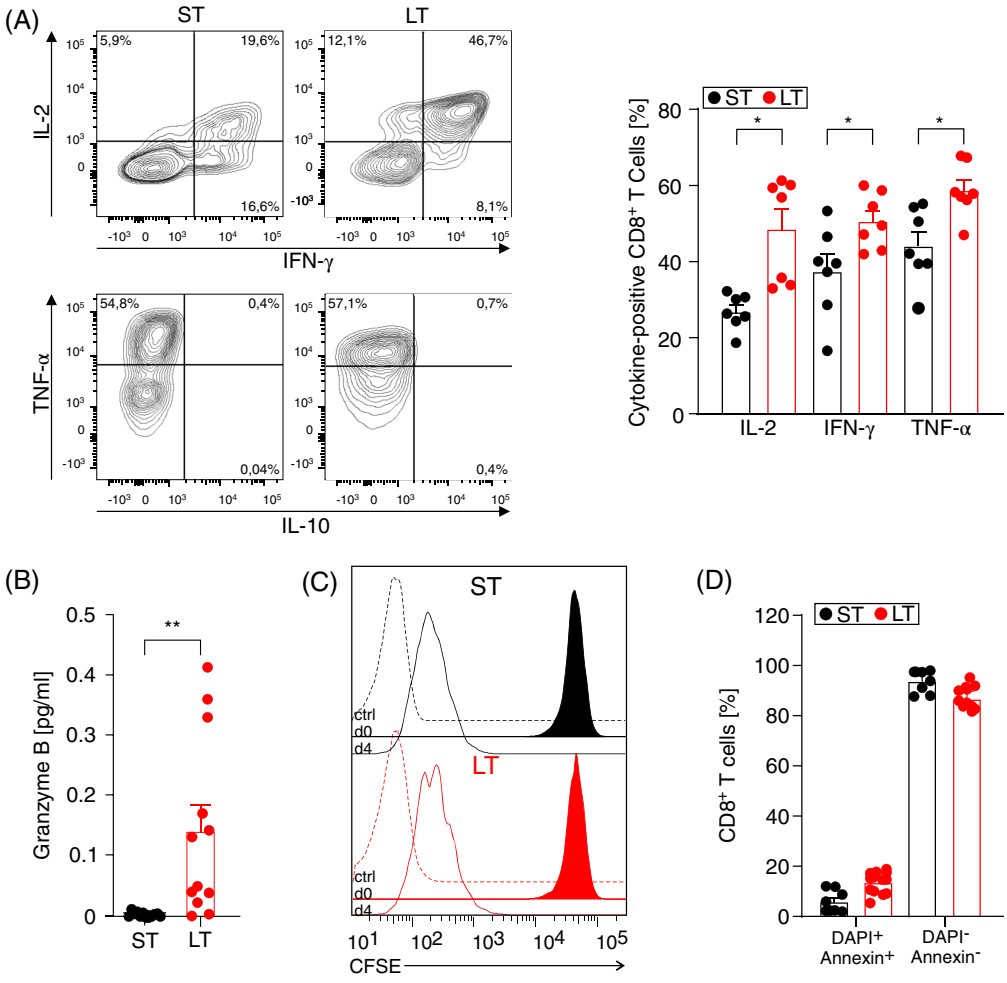

FIGURE 2 LT CD8$^+$ T cells have an increased cytokine expression after in vitro restimulation. ST and LT CD8$^+$ T cells were restimulated with anti-CD3/CD28 for 4 hours (A,B,D) or 24 hours (C). A, Left: Representative counter plots illustrate frequencies of cytokine-expressing CD8$^+$ T cells. Right: The bars show mean values ± SEM. *P < .05, nonparametric paired *t* test. B, The granzyme B concentration in supernatants of restimulated cells was analyzed in triplicates via ELISA. The bars show mean values ± SEM. **P < .01, nonparametric paired *t* test. C, Representative intensity histograms of restimulated CFSE-labeled ST and LT CD8$^+$ T cells. MFI values ± SD: ST-d4 = 233,8 ± 38,8; LT-d4 = 358,5 ± 166,3. D, Representative counter plots illustrate frequencies of apoptotic and/or dead cells (Annexin V referred to as Annexin$^+$ and/or DAPI$^+$) and living cells (Annexin$^-$ DAPI$^-$). The bars show mean values ± SEM. All data were obtained from two independent experiments with n > 2 mice [Color figure can be viewed at wileyonlinelibrary.com]

a conversion of naive to Tcm. The CD44$^+$CD62L$^+$ subset was further characterized for Tscm markers by flow cytometry. ST and LT Tcm revealed an overall increase of stem cell antigen 1 (Sca1), Bcl-2 and CXCR3 expression in culture settings and an even higher expression level of CD122 and Bcl-2 in LT CD8$^+$CD44$^+$CD62L$^+$ T cells (Figure 1C).[12,34]

In order to position LT murine CD8$^+$CD44$^+$CD62L$^+$ T cells into the established hierarchy of T cell subpopulations, we compared their transcriptome profile with published expression data from naturally occurring CD8$^+$ T cell subsets and differently induced Tscm.[4,11,13,23] Unsupervised hierarchical clustering and PCA using the gene set of Gattinoni et al[11] revealed that both ST and LT CD8$^+$CD44$^+$CD62L$^+$ T cells were closely related to previously published CD8$^+$ Tcm/Tscm transcriptomic signatures, whereas the LT signature tended to overlap more with the previously published Tcm datasets (Figure 1D; Supplemental Figure 2).

Afterward, we defined whether LT CD8$^+$ T cell functionality was maintained with respect to ST culture. Upon activation with anti-CD3/CD28 antibodies, LT CD8$^+$ T cells expressed higher amounts of intracellular IL-2, IFN-γ and TNF-α compared to ST CD8$^+$ T cells (Figure 2A). Granzyme B was also significantly higher expressed by LT CD8$^+$ T cells (Figure 2B). On the contrary, IL-10 was not detectable at any time point. We did not observe any significant difference in proliferation capability (Figure 2C, mean fluorescence intensity (MFI) ± SD for ST-d4 = 233,8 ± 38,8 and LT-d4 = 358,5 ± 166,3) and apoptosis susceptibility between the two groups (Figure 2D). Taken together, these data suggested that higher numbers of murine CD8$^+$ T cells with characteristic features related to Tcm and Tscm and preserved functionality could be achieved by LT expansion with IL-7/IL-15.

3.2 | LT CD8$^+$ T cells displayed preserved engraftment, persistence and antitumor capacity in vivo

We also studied the engraftment and persistence capacity of LT expanded T cells under homeostatic conditions in vivo. For this purpose, we isolated T cells from our published bioluminescent dual-luciferase reporter mouse, called BLITC mouse.[27] BLITC cells constitutively express Renilla luciferase and an additional Click Beetle luciferase under the control of the NFAT-responsive promoter, thus allowing to simultaneously analyze T cell migration, expansion and activation in mouse models. ST and LT CD8$^+$CD44$^+$CD62L$^+$ BLITC cells were transferred into Rag1 KO (RagKO) deficient mice, where small lymphoid organs do not contain mature B and T lymphocytes, and we monitored in vivo engraftment and persistence of adoptively transferred T cells over 1 and 6 months, respectively, via BLI analysis (Figure 3A,B). After 1 month, we observed Renilla luciferase signals in cervical and inguinal lymph nodes and quantified the signal intensity by digitally setting regions of interest and computing total flux values. The signal intensities were comparable in both ST and LT groups, suggesting no difference in the engraftment capability of LT T cells (Supplemental Figure 3A). Additionally, we quantified infiltrated CD8$^+$ T cells in peripheral lymph nodes and spleen using flow cytometry underlining the BLI data with comparable absolute numbers of ST and LT CD8$^+$ T cells (Supplemental Figure 3B). In a 6-months follow-up study, comparable Renilla luciferase signals and CD8$^+$ T cell numbers were detectable, hence suggesting similar persistence of transferred ST and LT CD8$^+$ T cells (Figure 3C,D; Supplemental Figure 3C). Since for ATT efficacy, it is essential that transferred T cells maintain or even increase antitumor activity, we tested

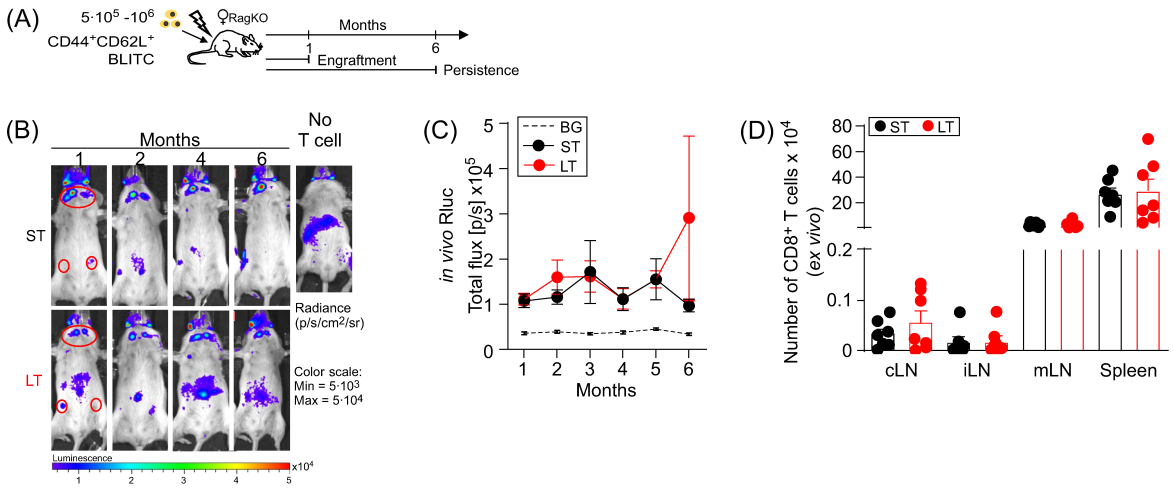

FIGURE 3 Preserved engraftment, persistence and antitumor capacity of LT CD8$^+$ T cells. A, Scheme for T cell transfer into female RagKO mice in order to analyze in vivo engraftment and persistence. B, Representative Renilla luciferase signals of ST or LT CD8$^+$CD44$^+$CD62L$^+$ T cells 1 to 6 months after transfer into female RagKO mice. C, The diagram shows mean ± SEM of Renilla luciferase flux values in the cervical (cLN) 6 months after transfer. BG indicates the background. D, Mice were sacrificed after 1 or 6 months and CD8$^+$ T cells were quantified in different organs via flow cytometry. The bar diagram shows mean data ± SEM of total T cell numbers. All data were generated in two independent experiments with n = 6-8 mice per group [Color figure can be viewed at wileyonlinelibrary.com]

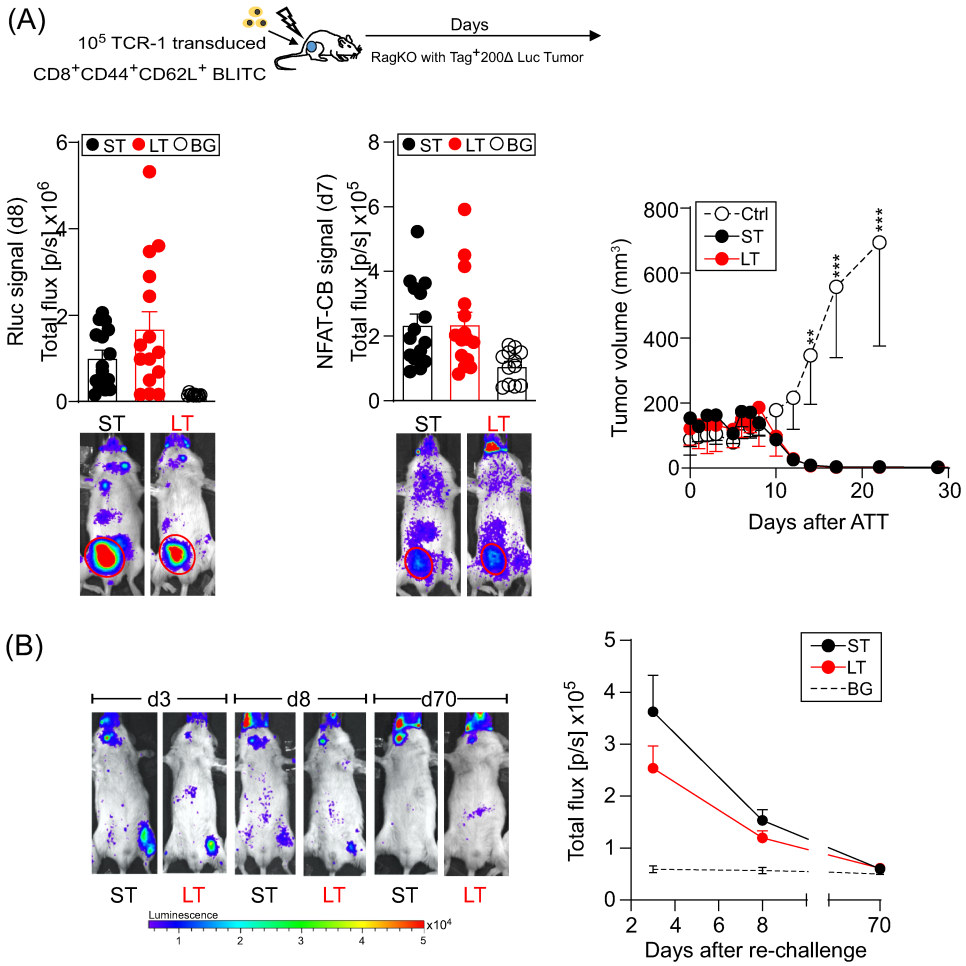

FIGURE 4 Effect of LT CD8$^+$ T cells engraftment on tumor growth. A, Scheme for T cell transfer into Tag$^+$200ΔLuc-tumor bearing female RagKO mice. Shown are representative Renilla and NFAT-click beetle luciferase signals of ST and LT CD44$^+$CD62L$^+$ TCR-1 transduced T cells 7 (NFAT-CB) and 8 (Renilla) days after transfer into Tag$^+$200ΔLuc-tumor bearing mice. Bar diagrams show mean ± SEM of luciferase flux values in the tumor region. Tumor growth kinetics are displayed by line diagram, mean ± SEM. $^{**}P < .01$, $^{***}P < .001$, two-way ANOVA test. All data were generated in two independent experiments with n = 15 mice per group. B, Tag$^+$200ΔLuc-tumor free mice from panel A were rechallenged with 5×10^6 freshly cultured Tag$^+$200ΔLuc tumor cells on the left flank
(note: tumor site of the first challenge was the right flank). Shown are representative Renilla luciferase signals of ST and LT CD44$^+$CD62L$^+$ TCR-1 transduced T cells 3, 8 and 70 days after tumor rechallenge into Tag$^+$200ΔLuc-tumor free mice. The line diagram shows mean ± SEM of luciferase flux values in the tumor region over 70 days. Tumor growth kinetics are not displayed due to missing growth. All data were generated in two independent experiments with n = 12 mice per group [Color figure can be viewed at wileyonlinelibrary.com]

ST and LT CD8$^+$ T cells using the SV40 large T antigen (Tag) tumor model.[26,35] CD8$^+$ BLITC cells were retroviral transduced with TCR-I, a TCR specific for epitope I of Tag protein, then expanded for 7 (ST) to 28 (LT) days and transferred into RagKO mice bearing Tag-positive 200ΔLuc tumors.[27] TCR-I transduced ST and LT CD8$^+$ BLITC cells infiltrated rapidly into the tumor, got activated by the tumor cells and induced a complete tumor rejection within 20 days (Figure 4A). In contrast, in control mice that received no T cells, tumors underwent progressive growth until mice were sacrificed at day 21. In order to test the in vivo persistence of TCR-I CD8$^+$ LT T cells, animals with eradicated tumors from the previous experiments were rechallenged with tumor cells after at least 2 months from ATT. As displayed in Figure 4B, after 3 days from rechallenge, ATT recipients triggered a strong T cell BLI signal at the tumor site and tumor growth was detected, thus demonstrating the persistence of a strong tumor-specific T cell reactivity. In naive-challenged RagKO mice, injected Tag-positive 200ΔLuc tumor cells develop a tumor that shows a constant tumor growth over the time with a tumor volume enriching between 300 and 500 mm^3 after 40 days (data not shown). In summary, these data showed that LT CD8$^+$ T cells retain anti-tumor function and their prolonged expansion does not negatively affect their therapeutic efficacy.

3.3 | LT CD4$^+$ T cells possess central memory/memory stem cell-like phenotype and in vitro functional properties

As for the CD8$^+$ T cell subpopulation, we investigated the feasibility of a prolonged cell culture for CD4$^+$ T cells, by using the same expansion protocol. Although the number of CD4$^+$ T cells was sustained

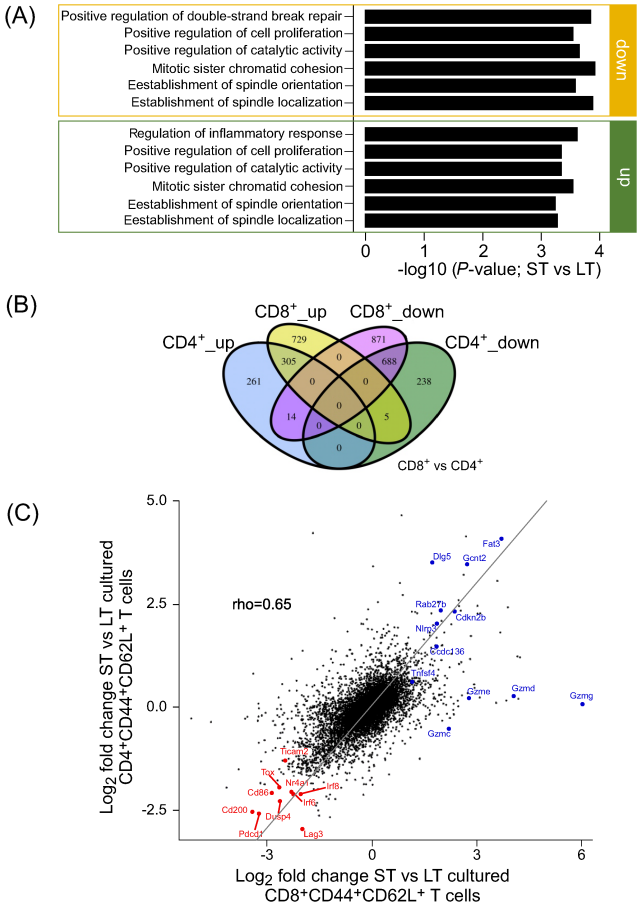

FIGURE 5 Molecular profiles of LT CD8$^+$ and CD4$^+$ T cells identify transcriptional regulators and markers for proliferation, apoptosis and activation. RNA sequencing of polyclonal CD8$^+$CD44$^+$CD62L$^+$ and CD4$^+$CD44$^+$CD62L$^+$ T cells generated via ST or LT expansion with IL-7/IL-15. A, Gene list with GO terms. B, Venn diagram with numbers of differentially regulated genes compared between ST and LT CD8$^+$CD44$^+$CD62L$^+$ and CD4$^+$CD44$^+$CD62L$^+$ T cells. C, Scatter plot with log2 fold changes between ST and LT CD8$^+$CD44$^+$CD62L$^+$ vs CD4$^+$CD44$^+$CD62L$^+$ T cells. Selected genes were highlighted [Color figure can be viewed at wileyonlinelibrary.com]

over time, the overall increase of CD4$^+$ T cells was weaker and maintained from day 7 to day 21 in a sort of stationary state (Supplemental Figure 4A). Moreover, the expansion yield for CD4$^+$ T cells as well as for the CD4$^+$CD44$^+$CD62L$^+$ subpopulation was nearly comparable between ST and LT CD4$^+$ T cells (Supplemental Figure 4B). As shown for CD8$^+$ T cells, also LT CD4$^+$ T cells exhibited phenotypical features of Tcm and Tscm (Supplemental Figure 4C), and CD122, Sca1, Bcl-2 and CXCR3 markers appeared increased in both ST and LT, thus indicating a stable phenotype over time.

We further compared the transcriptome profile of expanded CD4$^+$ cells with published expression data from mouse[12] or human.[24] Consistently, ST and LT CD4$^+$CD44$^+$CD62L$^+$ T cells grouped closely with CD4$^+$ Tcm, and with naturally or Notch-induced CD4$^+$ Tscm subsets (Supplemental Figures 4D and 5). Notably, the grouping is similar for both murine[12] and human[24] T cells.

We also tested the in vitro and in vivo properties of ST and LT CD4$^+$ T cells. After activation via anti-CD3/CD28, LT CD4$^+$ T cells expressed intracellular IL-2 and IFN-γ at higher level than ST, while TNF-α expression remained comparable (Supplemental Figure 4E). The in vitro proliferation and apoptotic rate after restimulation was also comparable between the two groups (Supplemental Figure 4F,G). Furthermore, we investigated the antitumor activity of ST and LT CD4$^+$CD44$^+$CD62L$^+$ H-Y TCR transgenic T cells isolated from Marilyn-BLITC mice (Marilyn-BLITC Tcm).[27] ST and LT Marilyn-BLITC Tcm were transferred into female RagKO mice bearing H-Y positive MB49 tumors. In contrast to the antitumor activity mediated by CD8$^+$ T cells, we recorded only a delay in tumor progression for CD4$^+$ comparably for ST and LT T cells (Supplemental Figure 4H).

3.4 | Longitudinal transcriptional alterations during T-cell expansion with IL-7/IL-15

We compared the transcriptome of ST and LT murine CD8$^+$CD44$^+$CD62L$^+$ and CD4$^+$CD44$^+$CD62L$^+$ T cells for differently expressed genes, in order to identify longitudinal transcriptional alterations. For this, we performed differential gene expression analysis using DESeq2.[25] Comparing ST and LT Tcm, we found 2612 altered genes for CD8$^+$ and 1511 altered genes for CD4$^+$ T cells (adjusted $P < .05$ and fold change >.5). The majority of these genes showed moderate but significant expression alterations with absolute log2 fold change between 1.5 and 2.0. To identify gene expression patterns that elucidate biological functions or pathways affected in LT CD8$^+$ and CD4$^+$ T cells, gene ontology (GO) term enrichment analysis was performed for both, in order to assign the differently expressed genes to biologically meaningful GO categories (Figure 5A). Top significant genes for each GO term are listed in Supplemental Table 3. Genes encoding key regulators of proliferation were downregulated in both T cell subsets, whereas regulators of cellular responses and signaling were upregulated over time. Furthermore, we compared the overlap of altered genes in CD8$^+$ and CD4$^+$ T cell expansion and found 688 of 931 (74%) downregulated genes in LT CD4$^+$ T cells similarly downregulated in CD8$^+$ T cells. Three hundred five of 580 (53%) upregulated genes in LT CD4$^+$ T cells were also upregulated in LT CD8$^+$ T cells (Supplemental Table 4). This relatively high correlation is illustrated by the Venn diagram and a scatter plot with selected genes highlighted (Figure 5B,C).

3.5 | An extended expansion of human T cells from healthy donors and DLBCL patients results in increased numbers of CD8$^+$ and CD4$^+$ Tcm cells with preserved in vitro function

As the clinical application of ATT has markedly increased and the number of infused T cells is critical to the outcome, our final aim was to transfer our findings on T cell LT expansion to the human setting.

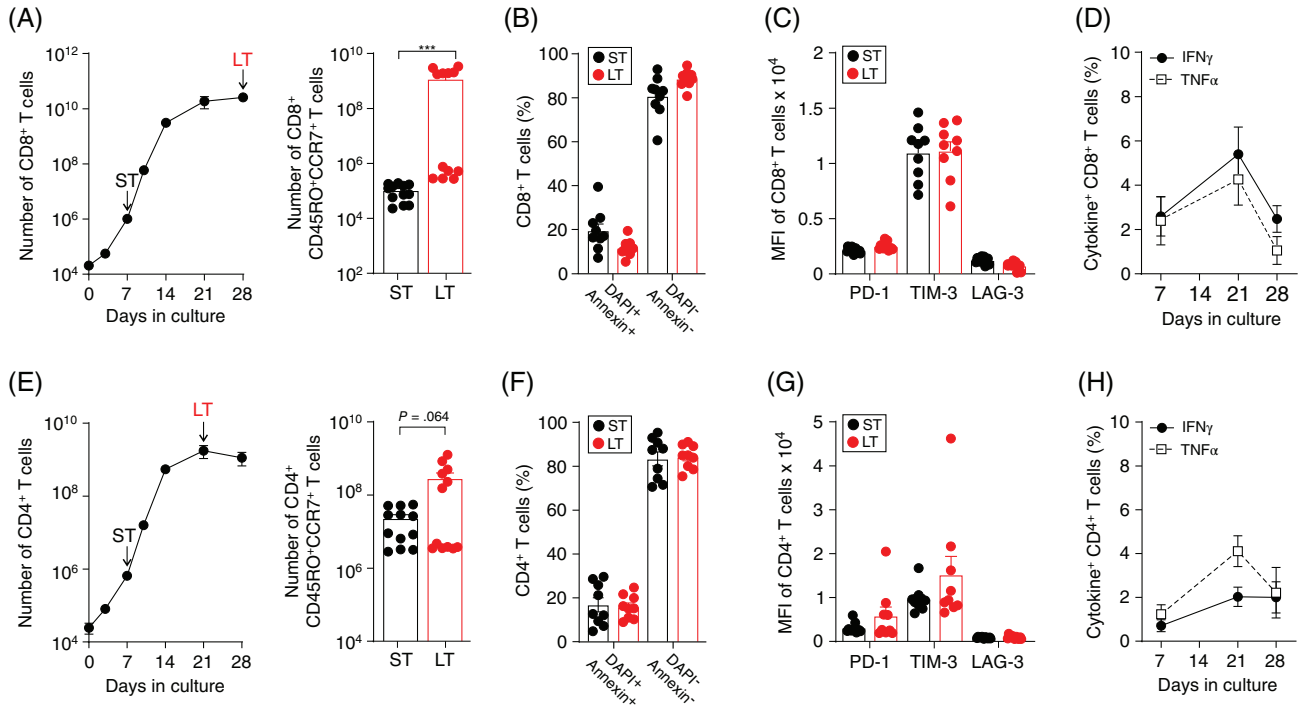

FIGURE 6 Extended T cell culture results in increased numbers of $CD8^+$ and $CD4^+$ T cells from healthy donors with preserved in vitro function. T cells from healthy donors were enriched using a Ficoll density gradient, activated via anti-CD3/CD28 and IL-2 for 72 hours and further cultured with IL-7/IL-15 for 4 (ST) or 25 (LT) days. The quantification and phenotypical analysis was done by flow cytometry. A, Growth kinetic of $CD8^+$ T cells is displayed by line diagram as mean values ± SEM. Before-after diagram shows mean ± SEM of total $CD8^+CD45RO^+CCR7^+$ T cell numbers. ***$P < .001$, nonparametric paired *t* test. B, ST and LT $CD8^+$ T cells were restimulated for 4 hours using anti-CD3/CD28. The bars show mean values ± SEM of frequencies of apoptotic and/or dead cells (Annexin V referred as $Annexin^+$ and/or $DAPI^+$) and living ($Annexin^-DAPI^-$) $CD8^+$ T cells. C, The bars show mean values ± SEM of inhibitory T cell receptor expression on $CD8^+$ T cells after restimulation for 10 days. D, Expanded $CD8^+$ T cells were restimulated at the indicated time points as described in B. The line diagram shows mean values ± SEM of IFN-γ and TNF-α expressing $CD8^+$ T cells over time. E, Growth kinetic of $CD4^+$ T cells is displayed by line diagram as mean values ± SEM. Before-after diagram shows mean ± SEM of total $CD4^+CD45RO^+CCR7^+$ T cell numbers. $P = .064$, nonparametric paired *t* test. F, ST and LT $CD4^+$ T cells were treated as described in B. The bars show mean values ± SEM of frequencies of apoptotic and/or dead cells ($Annexin^+$ and/or $DAPI^+$) and living ($Annexin^-DAPI^-$) $CD4^+$ T cells. G, The bars show mean values of the mean fluorescence intensity (MFI) ± SEM of inhibitory T cell receptor expression on $CD4^+$ T cells after restimulation for 10 days. H, The line diagram shows mean values ± SEM of IFN-γ and TNF-α expressing $CD4^+$ T cells upon restimulation. Growth kinetics were generated in two independent experiments with n = 4 healthy donors. Residual data were generated in three independent experiments with n = 9 healthy donors [Color figure can be viewed at wileyonlinelibrary.com]

We firstly analyzed the expansion and in vitro properties of T cells from healthy donors. T cells were enriched by density gradient centrifugation, activated and cultured as described for murine T cells. In line with our murine data, we observed a marked increase in human $CD8^+$ T cell numbers during the first 3 weeks and, subsequently the $CD8^+$ T cell expansion kinetic slowed down, reaching a plateau phase (Figure 6A, left graph). We also analyzed the phenotype of cultured $CD8^+$ T cells regarding the expression of CD45RO and CCR7 via flow cytometry. The LT expansion resulted in a 10^4-fold increase of human $CD8^+CD45RO^+CCR7^+$ Tcm cells (Figure 6A, right graph; Supplemental Figure 6). We then tested possible differences in $CD8^+$ T cell functionality after ST and LT expansion by restimulating them with anti-CD3/CD28 antibodies for either 4 hours or 10 days, in order to and analyze cell death/apoptosis and cytokine production as well as the expression of exhaustion markers (T cell inhibitory receptors, TIRs), respectively. The apoptosis and expression levels of TIRs were comparable between ST and LT $CD8^+$ T cells (Figure 6B,C). $CD8^+$ T cells expressed comparable amounts of IFN-γ and TNF-α after 1 and 4 weeks of expansion with a clear peak of production after 3 weeks (Figure 6D). Interestingly, human $CD4^+$ T cell expanded much stronger and longer than murine $CD4^+$ T cells in LT cultures, thus resulting in 50-fold increase of human $CD4^+CD45RO^+CCR7^+$ T cells after 3 weeks (Figure 6E; Supplemental Figure 6). Similarly to human $CD8^+$ T cells, the extent of apoptosis and TIRs expression upon in vitro restimulation were comparable between both groups (Figure 6F,G), and at the same time, ST and LT $CD4^+$ T cells expressed comparable amounts of IFN-γ and TNF-α cytokines with a peak for IFN-γ at week 3 (Figure 6H). Noteworthy, IL-2 was also measured but hardly detectable at any time point for $CD4^+$ and $CD8^+$ (data not shown).

In order to estimate the impact of genetic modification on LT T cell culture, we transduced human peripheral lymphocytes isolated from a healthy donor with tyrosinase-specific TCR-T58. Transduced $CD8^+$ T cells exhibited increased growth over time

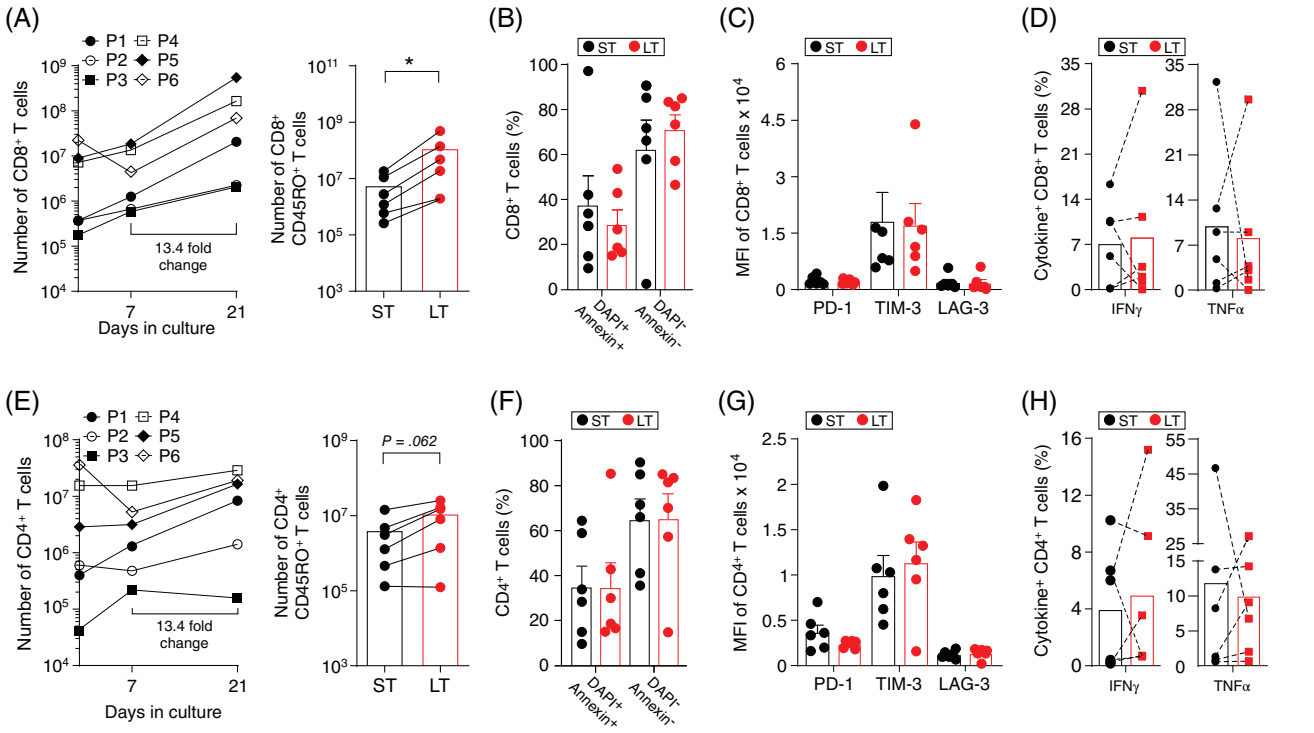

FIGURE 7 Extended T cell culture results in increased numbers of $CD8^+$ and $CD4^+$ T cells from DLBCL patients with individual in vitro function. T cells from DLBCL patients were enriched using a Ficoll density gradient, activated via anti-CD3/CD28 and IL-2 for 72 hours and further cultured with IL-7/IL-15 for 18 days. The quantification and phenotypical analysis were done by flow cytometry. A + E, Growth kinetics of $CD8^+$ and $CD4^+$ T cells are displayed by line diagrams for each patient. Before-after diagram shows the mean + individual values of total $CD8^+CD45RO^+$ and $CD4^+CD45RO^+$ T cell numbers. *$P < .05$, nonparametric paired *t* test. B + F, ST and LT $CD8^+$ and $CD4^+$ T cells were restimulated for 4 hours using anti-CD3/CD28 to analyze cell death and cytokine expression. The bars show mean values ± SEM of frequencies of apoptotic and/or dead cells (Annexin V referred as $Annexin^+$ and/or $DAPI^+$) and living ($Annexin^-DAPI^-$) $CD8^+$ T cells. C + G, ST and LT $CD8^+$ and $CD4^+$ T cells were restimulated for 10 days using low-dose anti-CD3/CD28 to analyze the expression of inhibitory T cell receptors. The bars show mean values ± SEM of the mean fluorescence intensity (MFI) ± SEM of inhibitory T cell receptor expression on $CD8^+$ and $CD4^+$ T cells after restimulation for 10 days. D + H, Expanded $CD8^+$ and $CD4^+$ T cells were restimulated at the indicated time points as described in B + F. The before-after diagram shows mean values and individual values of IFN-γ and TNF-α expressing $CD8^+$ and $CD4^+$ T cells over time [Color figure can be viewed at wileyonlinelibrary.com]

while $CD4^+$ to a lesser extent (Supplemental Figure 7A). In addition, transduction did not affect proliferation of $CD45RO^+CCR7^+$ $CD8^+$ and $CD4^+$ T cells (Supplemental Figure 7B). After 28 days, T cell numbers increased by more than 10-fold and about 10-fold, respectively. TIR expressions by transduced T cells were also comparable to nontransduced human T cells shown in Figure 6C,G (Supplemental Figure 7C). We also defined the ability of LT transduced $CD8^+$ and $CD4^+$ T cells to be activated. As for T cells in Figure 6D,H, transduction did not affect the ability of $CD8^+$ and $CD4^+$ to produce the proinflammatory cytokines TNFα and IFNγ after activation with anti CD3/CD28 antibodies when comparing ST and LT T cells (Supplemental Figure 7D). Transduced $CD8^+$ T cells were also able to respond to TCR-tyrosinase (T58) specific activation (Supplemental Figure 7D, black line). As last, we tested the ability of permanent stimulation to evoke exhaustion and TIR expression in transduced $CD8^+$ and $CD4^+$ T cells. After 11 days of stimulation with anti CD3/CD28 antibodies only lymphocyte activating 3 (Lag3) expression increased respect day 0, while the other TIRs were unaffected (Supplemental Figure 7E). At the same time, no significant increase of tumor death was identified after 11 days of anti CD3/CD28 stimulation (Supplemental Figure 7F) in LT cultured $CD4^+$ and $CD8^+$.

Since in clinical practice treatment of relapsed/refractory lymphoma patients with CAR T cells is hampered in some cases by the failure to generate sufficient numbers of T cells,[8,21,22] we next evaluated T cell expansion in DLBCL patients undergoing salvage therapy as potential candidates for CAR T-cell therapy. We obtained samples from six patients, enriched T cells from peripheral blood mononuclear cells (PBMCs) and cultured them following the same procedure as described for the healthy donors. We determined the main representative T cell phenotype thorough flow cytometric analysis of CD45RO and CCR7 cell membrane markers, and in four of the six patients we found a significant reduced numbers of early lineage T cells, naive T cell (Tn) ($CD8^+/CD4^+$ $CD45RO^+$ $CCR7^-$) and Tcm ($CD8^+/CD4^+$ $CD45RO^+$ $CCR7^+$) (Supplemental Figure 8). After in vitro expansion, five patients exhibited a comparable initial T cell proliferation with an averaged 2.3-fold T cell expansion within the first week, while for one patient (patient number 6), the absolute number of T cells dropped to about 17%. However,

after further expansion, an averaged 13.4-fold and 3.4-fold increase for $CD8^+$ and $CD4^+$ T cells, respectively, was achieved (Figure 7A,E). The number of $CD45RO^+$ $CD8^+$ and $CD4^+$ T cells has also expanded over time (Figure 7B, F). In order to test T cell exhaustion after prolonged culture condition, we measured TIRs in ST and LT T cells. TIR expressions were comparably expressed and their expression did not increase over time for both $CD4^+$ and $CD8^+$ T cells (Figure 7C,G). On the other hand, the stable expression of exhaustion markers did not correlate with the functionality of T cells, which instead expressed IFN-γ and TNF-α with high variability upon activation and over time (Figure 7D,H).

4 | DISCUSSION

ATT has developed into an important new tool in the treatment of malignancies, particularly hematologic malignancies, with CAR-T cell therapies possibly revolutionizing treatment of relapsed/refractory lymphoma and multiple myeloma. In solid tumors, CAR-T cells are only beginning to be used, most likely TCRs addressing tumor associated antigens will play a major role in the future in these entities. Despite the impressive results in the early outcome of ATT patients, the clinical success is far from being achieved in all patients. Beside escape mechanisms involving downregulation of targeted antigens and engraftment failure, ATT has further encountered several limitations during the manufacturing process due to insufficient T cell collection, generation and/or expansion. This is particularly evident in older and in heavily pretreated patients.[8,21,22] In our study, we investigated whether a limited number of T cells after manufacturing procedure could be circumvented by simply extending the time of current protocols with the aim of achieving an adequate T cell graft to avoid ATT dropout while being minimally manipulative. Certainly, this would only be a viable option if the Tcm/Tscm properties could be maintained despite extended expansion, which is why we also tested for potential loss of T cell function. We firstly provided the proof of concept of a LT IL-7/IL-15 expansion protocol and preserved T cell functionality for murine T cells in an ATT tumor model. Several studies have shown that IL-7 and IL-15 offer better growth support than IL-2[10] and promote a Tcm/Tscm phenotype,[4,32,33,36] thus ultimately improving ATT efficacy.[37] In all these studies, T cells were cultured for 1 or 2 weeks, while only Cieri et al[4] investigated T cell expansion over 4 weeks in order to test the expansion potential of individual T cell subsets, however longitudinal changes in T cell function and transcriptome were not addressed. Therefore, we not only investigated the extent of T cell expansion, but simultaneously examined whether T cell functionality could be maintained despite an extended expansion culture protocol. Murine $CD8^+$ T cells steadily expanded up to 4 weeks when cultured with IL-7/IL-15, while murine $CD4^+$ T cells expanded to a lesser extent without significant differences between ST and LT culture. To date, a comprehensive analysis of the gene expression profile of LT expanded murine Tcm remains missing. As few murine datasets of $CD4^+$ and $CD8^+$ are available we included in our study some selected human data set, and the analysis was performed using batch correction to remove systematic differences between datasets of different origin, and caused by the species differences. Unsupervised hierarchical clustering and PCA of RNA sequencing of data derived from murine $CD8^+$ and $CD4^+$ Tcm after ST and LT expansion and published signatures T cell subtypes revealed that after LT expansion, $CD8^+$ Tcm were closely related to a typical $CD8^+$ Tcm and Wnt-induced Tscm expression signature thus demonstrating the maintenance of a Tcm phenotype in LT condition. At the same time similarly to the ST $CD4^+$ Tcm, LT $CD4^+$ Tcm cells positioned most closely to $CD4^+$ or Notch-induced $CD4^+$ Tscm cells, thus indicating a stable profile over time. The (GO) term enrichment analysis between ST and LT culture of murine T cells revealed that among the 2612 and 1511 altered genes identified in $CD8^+$ and $CD4^+$, respectively, over half of the gene overlap between $CD8^+$ and $CD4^+$, thus suggesting the existence of a specific cluster of genes that are similarly regulated by IL-7/IL-15 over time. On the other hand, the downregulation of a set of genes encoding for key regulators of cell cycle and proliferation may be explained with a possible enrichment of a plateau-phase in LT culture. This is in line with previous findings showing a similar cell cycle arrest with the induction of a resting phase in IL-15-induced memory-like $CD8^+$ T cells.[32] In addition, LT Tcm cells downregulated genes associated with immune checkpoint inhibition (*Cd200*, *Pdcd1*, *Lag3*)[38,39] and T cell dysfunction (*Dusp4*, *Tox* and *Nr4a1*)[40-43] thus suggesting that a prolonged in vitro expansion with IL-7/IL-15 does not induce T cell exhaustion with consequent loss of T cell functions.

All data derived from the longitudinal phenotypical and molecular analyses revealed that an extended expansion using IL-7/IL-15 is feasible and increases T cell numbers with favorable Tcm/Tscm phenotype and largely preserved transcriptional activity.

Regarding the in vitro functionality, proliferative capacity, apoptosis susceptibility and upregulation of TIRs upon polyclonal stimulation with anti-CD3/CD28 have been shown to be comparable between ST and LT culture. Upon restimulation, LT $CD8^+$ and $CD4^+$ T cells could even produce larger amounts of IL-2, IFN-γ and granzyme B than after ST culture. The higher granzyme B expression is noteworthy, since the proportion of Tcm/Tscm is predominant. Here, it must be mentioned that no isolated Tcm were stimulated, so that it can be assumed that the smaller fraction of Teff also contributes to the measured granzyme B production. Interestingly, Chattopadhyay et al have shown granzyme B expression by Tcm, but only by those, which are CD27 negative and CD57 bright.[44]

In respect to in vivo functionality, multiple studies have shown a correlation between engraftment and persistence of adoptively transferred T cells and clinical outcome.[45-47] We clearly show here that T cell function is preserved when T cells are expanded for a longer period in the presence of IL-7/IL-15, and that such LT T cells are able to engraft, expand, persist and develop antitumor activity. This has been demonstrated differently for IL-2,[19,48,49] where shortly IL-2 expanded T cells could outperform the longer cultured T cells in expansion, survival capacity and in vivo antitumor activity. These data underline the clinical relevance of our findings.

In order to evaluate whether such minimally manipulative good manufacturing practice-feasible procedure might be an option for patients with hampered T cell proliferation, we assessed the effect of

IL-7/IL-15 LT expansion on T cells derived from healthy donors as well as from DLBCL patients. As for murine T cells, we observed a long-lasting expansion of human $CD8^+$ T cells over 4 weeks. Differently from murine $CD4^+$ T cells, human $CD4^+$ T cells from healthy donors significantly increased over time. Such differences are in line with previous findings about the diverse priming and differentiation between murine and human $CD4^+$ T cells.[34] The ability of human $CD4^+$ T cells to expand is critical for medical treatment since the impact of $CD4^+$ T cells for the antitumor immunity as either supporting partner or as effector cells has been increasingly acknowledged.[50-52] In our experimental settings, LT expansion of human T cells provided less differentiated $CD45RO^+CCR7^+$ $CD8^+$ and $CD4^+$ T cells, respectively. Functional assays displayed that ST and LT human T cells possess similar apoptosis susceptibility and upregulation of TIRs upon polyclonal stimulation with anti-CD3/CD28. Activation and cytokine expression of ST and LT T cells were comparable. Interestingly, IFNγ and TNFα expression level peaked after 3 weeks of expansion and declined shortly after. When the LT expansion protocol was applied to T cells derived from DLBCL patients, improved numbers of T cells could also be achieved, albeit the expansion was not as vigorous as seen for the healthy donors, possibly due to the heavy pretreatment of DLBCL patients. In addition, over the stimulation period, T cells from DLBCL patients behave inconsistently, with large variations in the pattern of response to stimulation between patients. This likely reflects different functional capability of T cells, possibly reflecting the influence of different therapies, age, comorbidity and disease status on T cell function. DLBCL patients can lack T cells with early lineage phenotype (Tnaive or Tcm), and dropouts in clinical trials can occur due to failure of CAR T cell expansion[8,21,22] underlying the need for strategies to improve the proliferative and functional properties of T cells from heavily pretreated and/or older patients. However, LT culture induced an averaged increase of $CD45RO^+$ $CD8^+$ and $CD4^+$ in DLBCL patients of our study, which may represent a perspective for those patients who experience manufacturing failure. At the same time, cytokine expression by LT T cells was overall preserved although with great variability in the production of IFNγ and TNFα for the individual patients.

In conclusion, we provide data showing that the T cell yield can be significantly increased and in addition to that, we demonstrate that a prolonged expansion may be a feasible treatment option since T cell functionality is preserved. T cell manufacturing is indisputably a resource consuming and cost-intensive process and certainly, we are aware that prolonged time until ATT entails the risk of disease progression. However, dropout in T cell manufacturing still represent an unmet medical need for some patients, and an extended manufacturing protocol may signify a therapeutic opportunity to circumvent otherwise ATT dropout. Further investigations are required to identify possible markers or clinical conditions that could help to identify those patients who might be in need of a prolonged T-cell expansion.

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CONFLICT OF INTEREST

Lars Bullinger declares being on the advisory committees of Abbvie, Amgen, Bristol-Myers Squibb, Celgene, Daiichi Sankyo, Gilead, Hexal, Janssen, Jazz Pharmaceuticals, Menarini, Novartis, Pfizer, Sanofi, Seattle Genetics. The other authors declared no potential conflicts of interest.

ETHICS STATEMENTS

All mice experiments were conducted in compliance with the institutional guidelines of the Max Delbrück Center for Molecular Medicine (Berlin, Germany) and approved by the Landesamt für Gesundheit und Soziales Berlin, Germany (G0048/14, G0005/15, G0307/15). Human samples were obtained from healthy donors referred to our Hematology, Oncology and Tumorimmunology Unit (Charité-Berlin) after signed an informed consent. The study was approved by the Charité-Berlin local ethics committee (no. EA1/070/17).

CONSENT FOR PUBLICATION

The risk of identification of the healthy donors/patients is minimized by anonymization to prevent the identity of the healthy donors/patients.

DATA AVAILABILITY STATEMENT

The sequencing data generated are available in Gene Expression Omnibus under the accession GSE140250. The other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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