

# IL15 Enhances CAR-T Cell Antitumor Activity by Reducing mTORC1 Activity and Preserving Their Stem Cell Memory Phenotype



Darya Alizadeh<sup>1</sup>, Robyn A. Wong<sup>1</sup>, Xin Yang<sup>1</sup>, Dongrui Wang<sup>1,2</sup>, Joseph R. Pecoraro<sup>1</sup>, Cheng-Fu Kuo<sup>1,2</sup>, Brenda Aguilar<sup>1</sup>, Yue Qi<sup>3</sup>, David K. Ann<sup>2,3</sup>, Renate Starr<sup>1</sup>, Ryan Urak<sup>2</sup>, Xiuli Wang<sup>1</sup>, Stephen J. Forman<sup>1</sup>, and Christine E. Brown<sup>1</sup>

## Abstract

Improvements in the quality and fitness of chimeric antigen receptor (CAR)-engineered T cells, through CAR design or manufacturing optimizations, could enhance the therapeutic potential of CAR-T cells. One parameter influencing the effectiveness of CAR-T cell therapy is the differentiation status of the final product: CAR-T cells that are less-differentiated and less exhausted are more therapeutically effective. In the current study, we demonstrate that CAR-T cells expanded in IL15 (CAR-T/IL15) preserve a less-differentiated stem cell memory (Tscm) phenotype, defined by expression of CD62L<sup>+</sup>CD45RA<sup>+</sup>CCR7<sup>+</sup>, as compared with cells cultured in IL2 (CAR-T/IL2). CAR-T/IL15 cells exhibited reduced expression of exhaustion markers, higher antiapoptotic properties, and increased proliferative capacity upon antigen challenge. Furthermore, CAR-T/IL15 cells

exhibited decreased mTORC1 activity, reduced expression of glycolytic enzymes and improved mitochondrial fitness. CAR-T/IL2 cells cultured in rapamycin (mTORC1 inhibitor) shared phenotypic features with CAR-T/IL15 cells, suggesting that IL15-mediated reduction of mTORC1 activity is responsible for preserving the Tscm phenotype. CAR-T/IL15 cells promoted superior antitumor responses *in vivo* in comparison with CAR-T/IL2 cells. Inclusion of cytokines IL7 and/or IL21 in addition to IL15 reduced the beneficial effects of IL15 on CAR-T phenotype and antitumor potency. Our findings show that IL15 preserves the CAR-T cell Tscm phenotype and improves their metabolic fitness, which results in superior *in vivo* antitumor activity, thus opening an avenue that may improve future adoptive T-cell therapies.

## Introduction

Chimeric antigen receptor (CAR)-T cell therapy is a promising therapeutic approach that delivers antitumor responses in some patients, especially in the setting of hematologic malignancies. However, not all patients respond, and the durability of the therapy can be limited. Ongoing efforts aim to enhance the antitumor potential of the CAR-T cells, especially for more challenging therapeutic settings such as solid tumors. Several parameters can affect the potency of the CAR-T cell product, including the use of specific T-cell subsets [CD8<sup>+</sup> or CD4<sup>+</sup> T cells,

central memory T cells, or bulk peripheral blood mononuclear cells (PBMC)], the type of costimulatory molecules integrated in the CAR construct, and the *ex vivo* culture conditions (1–5). CAR-T cells are usually generated from PBMCs and expanded *ex vivo* using IL2 (6). However, T-cell products obtained using these procedures are phenotypically heterogeneous, and may largely be composed of antigen-experienced, highly differentiated T-cell subsets such as effector-memory (Tem) and effector (Teff) T cells (7). Starting with less-differentiated T cells such as naïve (Tn), stem cell memory (Tscm), and central memory (Tcm) T cells for CAR-engineering results in more potent antitumor immune responses than Tem- and Teff-engineered CAR products (8–11). However, although less-differentiated cells may be more beneficial, *ex vivo* culture methods (cytokine composition and culture duration) often promote T-cell differentiation.

The  $\gamma$ c-cytokine IL2, as a T-cell growth factor, remains the most common cytokine used for expansion of therapeutic T-cell products being administered to patients (6). However, repetitive stimulation of T cells with IL2 during *ex vivo* expansion can result in T-cell exhaustion and reduced T-cell persistence (10). The inclusion of other  $\gamma$ c-cytokines, such as IL7 and IL15, has shown some benefit during *ex vivo* expansion of T cells (2). Indeed, this class of cytokines has broad effects on lymphocyte development, differentiation, and their homeostasis (12). Some studies have shown that use of IL7 and IL15 together may preserve the Tscm phenotype and enhance the potency of CAR-T cells (2, 13). Others have reported that IL21 promotes expansion of CD27<sup>+</sup>CD28<sup>+</sup> CD8<sup>+</sup> T cells (14) and enhances potency of CD19-CAR-T

<sup>1</sup>Department of Hematology and Hematopoietic Cell Transplantation, T Cell Therapeutics Research Laboratory, City of Hope, Duarte, California. <sup>2</sup>Irell and Manella Graduate School of Biological Sciences, Duarte, California. <sup>3</sup>Department of Diabetes Complications and Metabolism, Diabetes and Metabolism Research Institute, City of Hope Beckman Research Institute and Medical Center, Duarte, California.

**Note:** Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

S.J. Forman and C.E. Brown share senior authorship of this article.

**Corresponding Author:** Christine E. Brown, Beckman Research Institute of the City of Hope Comprehensive Cancer Center, 1500 East Duarte Road, Duarte, CA 91010. Phone: 626-256-4673, ext. 63977; Fax: 626-471-3602; E-mail: cbrown@coh.org

**doi:** 10.1158/2326-6066.CIR-18-0466

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cells (15), as compared with other cytokines such as IL2. Despite these observations, the mechanisms by which these cytokines enhance T-cell potency remain poorly understood.

Cellular metabolism regulates T-cell differentiation as well as the retention of memory characteristics (16). Metabolic profiling and functional analyses have indicated that terminally differentiated Teff cells are characterized by high glycolytic activity whereas less-differentiated cells primarily rely on fatty acid oxidation (FAO) for energy production (17). Skewing cellular metabolism toward FAO by overexpressing carnitine palmitoyltransferase 1a (CPT1A, an enzyme in FAO) or by inhibiting glycolysis in T cells increases the number of memory CD8<sup>+</sup> T cells (16). Glycolysis and glucose transport is regulated by mammalian target of rapamycin (mTOR) activity (18). In this context, studies have indicated that the inhibition of the mTOR pathway using rapamycin results in the generation of CD8<sup>+</sup> memory T cells (19–21). Thus, targeting pathways controlling T-cell metabolism represents an attractive strategy to control the differentiation status of these cells.

With the goal of preserving T cells with a stem-like phenotype during *ex vivo* expansion and to prevent terminal differentiation and activation-induced cell death, we compared cytokine conditions of our standard manufacturing platform (IL2/IL15low) with the use of IL15 alone. In this study, we demonstrate that *ex vivo* culture of CAR-T cell products in IL15 (CAR-T/IL15) was superior for maintaining the Tscm phenotype. Upon tumor challenge, CAR-T/IL15 cells showed fewer apoptotic features, higher proliferative capacity, and superior antitumor activity *in vivo*. Exposure to IL15 resulted in reduced activity of mTORC1, a regulator of glycolysis. IL15-mediated reduction of mTORC1 activity plays a role in preventing T-cell differentiation, as CAR-T/IL2 cells treated with rapamycin exhibit phenotypic characteristics similar to CAR-T/IL15 cells. Further assessment of the metabolic function confirmed that CAR-T/IL15 cells exhibit greater mitochondria oxygen consumption rate (OCR) and spare respiratory capacity, which confers a metabolic advantage for survival and recall upon antigen challenge. These phenotypic characteristics were observed in both CD28-CAR- and 4-1BB-CAR-T cells. Together, these findings reveal that IL15 reduces mTORC1 activity and improves the potency of CAR-T cells.

## Materials and Methods

### Tumor cell lines

Raji-ffluc (CD19<sup>+</sup>; a kind gift from Dr. Michael Jensen in 2014), LCL [CD19<sup>+</sup>; generated by Epstein-Barr virus (EBV) transformation of human B cells as described previously; ref. 8], KG1A (CD19<sup>-</sup>; a kind gift from Dr. Ravi Bhatia in 2008), and the human glioma cell line PBT030-2-ffluc (IL13R $\alpha$ 2<sup>+</sup>; low passage tumor sphere line derived from human GBM tissue) were maintained as previously described (22). Cell banks of tumor lines were authenticated for the desired antigen/marker expression by flow cytometry and tested for *Mycoplasma*. Thawed cells from banks were maintained in culture for 1 to 3 months.

### T-cell isolation, lentiviral transduction, and *ex vivo* CAR-T cell expansion

Blood products were obtained from healthy donors or discard kits with informed written consent (whenever necessary) after protocols were approved by the City of Hope (COH) Internal Review Board. All studies were conducted in accordance with U.S.

Common Rule ethical guidelines. The PBMCs were isolated by density gradient centrifugation over Ficoll-Paque (GE Healthcare) and then underwent CD14 depletion (PBMC population). For the CD62L<sup>+</sup> population, additional CD25 depletion (Miltenyi Biotec) followed by CD62L<sup>+</sup> selection was performed. T cells were stimulated with Dynabeads Human T-Expander CD3/CD28 (Invitrogen) at a 1:3 ratio (T-cell:bead) overnight in X-VIVO-15 (Lonza) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine and different cytokine cocktails as follows: IL2/IL15 [50 U/mL IL2 (Novartis), 0.5 ng/mL IL15 (CellGenix)]; IL15/IL7 [10 ng/mL IL15, 10 ng/mL IL7 (Miltenyi Biotec)]; IL15/IL7/IL21 [10 ng/mL IL15, 10 ng/mL IL7, 10 ng/mL IL21 (Miltenyi Biotec)]; IL15 [10 ng/mL] or IL2 titrations [25, 20 or 10 U/mL IL2 (Novartis) 0.5 ng/mL IL15 (CellGenix)]. Stimulated T cells were then transduced or not with a lentivirus vector encoding the CD19-CAR or IL13R $\alpha$ 2-CAR as previously described (8, 22, 23). Mock and transduced cell cultures were given the indicated cytokines three times a week for 18 to 21 days of culture before subsequent analyses. For time point analyses comparing CAR-T/IL2 with CAR-T/IL15, exogenous cytokines were replaced three times per week for up to 32 days, and cells were cryopreserved at an early (days 14–16), mid (day 23), or late (day 32) time point post-bead stimulation for further detailed analyses. For rapamycin-treated groups, CAR-T/IL2 cells were incubated with rapamycin (100 nmol/L) (LC Laboratories) beginning at 4 days after bead stimulation and refreshed with media containing rapamycin three times per week.

### Flow cytometry

CAR-T cell lines were stained with fluorochrome-conjugated antibodies against the following markers: CD3, CD8, CD45, CD62L, CD27, CD45RA, CD45RO, CD127, CD95 (BD Biosciences); Lag3, PD-1 (eBioscience); Tim3 (R&D Systems); CCR7, or 2B4 (BioLegend). Staining with anti-EGFR (BioLegend) or anti-CD19 (BD Biosciences) was used as a surrogate for CAR expression in cells transduced to express CD19-CAR or IL13R $\alpha$ 2-CAR, respectively. For the assessment of active caspase-3, CAR-T cell lines at the early, mid, and late time points were fixed and stained using the PE-Anti-Active Caspase-3 Kit (BD Biosciences) according to the manufacturer's instructions. For p-STAT5 staining, cells were fixed with 4% paraformaldehyde, followed by 10 minutes of incubation in chilled methanol. Cells were stained with p-STAT5-Alexa Fluor 488 (BD Biosciences).

For the assessment of CAR-T cell proliferation and cytotoxic activity, 25,000 CD19-CAR-T cells and 25,000 tumor cells (Raji-ffluc, LCL, or KG1A) were incubated for 7 days. The cell mixture was stained with anti-CD3, -CD8, and -EGFR. The absolute number of viable tumor cells and CAR-T cells was measured using flow cytometry.

For the degranulation assay, 50,000 CD19-CAR-T cells and 50,000 tumor cells (Raji-ffluc, LCL, or KG1A) were cocultured for 5 hours in the presence of the GolgiStop Protein Transport Inhibitor (BD Biosciences). The cell mixture was stained with anti-CD3, -CD8, and -EGFR followed by intracellular staining with anti-IFN $\gamma$  (BD Biosciences). Degranulation assay post adoptive T-cell therapy was performed as previously described (5). Briefly, intratumoral CAR-T cells were isolated post therapy and cocultured *ex vivo* with tumor at a 1:1 ratio for 5 hours in the presence of the GolgiStop Protein Transport Inhibitor (BD Biosciences). The cell mixture was stained with anti-CD45, -CD8, and

-CD107a followed by intracellular staining with anti-IFN $\gamma$  and anti-TNF $\alpha$  (BD Biosciences).

Recursive killing assay was performed as previously described (5). Briefly, CAR-T/IL2 or CAR-T/IL15 cells were cocultured with tumor (1:4 for IL13R $\alpha$ 2-CAR:tumor; 1:3 for CD19-CAR:tumor). CAR-T cells were rechallenged with additional tumor cells as described in the results. At the end of the repetitive tumor challenge (5–7 days), viable tumor cells and CAR-T cells were counted using flow cytometry.

All samples were acquired on MACSQuant Analyzer 10 (Miltenyi Biotec) and analyzed with FlowJo software (v10.1, TreeStar) and GraphPad Prism Software (v5).

### Metabolism assays

Mitochondrial OCR was measured using the Seahorse Bioscience XF96 Extracellular Flux Analyzer (Agilent). Briefly, 0.2 or 0.4 million cells were seeded in cell culture microplates on the day of the experiment. Cells were suspended in the OCR XF Assay medium (Agilent, 102365-100) supplemented with glucose (25 mmol/L, Sigma) and sodium pyruvate (1 mmol/L, Gibco). The pH value of the assay medium was adjusted to 7.4. OCR was measured following sequential injections of oligomycin (1 mmol/L, Sigma), FCCP (0.5 mmol/L, Sigma), and rotenone (2.5 mmol/L, Sigma) according to the manufacturer's instructions. OCR measurements were normalized to cell numbers.

To measure mitochondria membrane potential, T cells were incubated with tetramethylrhodamine methyl ester (TMRM; Invitrogen) at a final concentration of 25 nmol/L for 15 minutes at 37°C. To quantify glucose uptake, T cells were incubated with 2-NBDG (fluorescent glucose analogue; Invitrogen) at a final concentration of 50  $\mu$ mol/L for 30 minutes at 37°C. The mean fluorescent intensity (MFI) of cells was measured using flow cytometry.

### RNA-sequencing (RNA-seq) analysis

Thawed CD19-CAR-T cells were labeled with fluorescently labeled anti-CD4, -CD8 (BD Biosciences), and -EGFR (BioLegend). CAR-expressing (EGFR<sup>+</sup>) CD4<sup>+</sup> or CD8<sup>+</sup> cell populations were separated using a BD FACSAriaII (BD Biosciences). RNA was isolated from pure CD4<sup>+</sup> or CD8<sup>+</sup> populations using the RNeasy Mini Kit (Qiagen), and cDNA was reverse transcribed using the SuperScript VILO Mastermix (Life Technologies) according to the manufacturer's instructions. RNA-seq library preparation was conducted as previously described (5). The 51-bp single-ended sequence reads were mapped to human genome (hg19) using TopHat (Version 2.0.8), and the expression of Refseq genes was counted with customized R scripts. A gene was considered expressed with an RPKM (reads per kilobase of gene per million reads mapped to exons) value more than one. Only genes expressed in at least one sample were kept for differential expression analysis. The raw counts were then normalized using the TMM (weighted trimmed mean of M-values) method, and differentially expressed genes were identified by 2-fold change ( $\geq 2$  and  $\leq 0.5$  for up- and downregulated genes, respectively) via exactTest using the Bioconductor package "edgeR" (24).

### Multidimensional scaling (MDS) analysis

Classic MDS plot was generated using the plotMDS function in the edgeR Bioconductor package (24). Briefly, the distance matrix was computed from RNA-seq data in units of RPKM and used as

input for MDS analysis. The first two coordinates of the configuration matrix are plotted with numbers corresponding to the differentially expressed genes (including both up- and down-regulated genes) with a log<sub>2</sub> (fold change)  $\geq 1$  or  $\leq -1$  in gene expression between indicated samples. Graph corresponds to four samples: CAR-T/IL2, day 14; CAR-T/IL2, day 32; CAR-T/IL15, day 14; CAR-T/IL15, day 32.

### Immunoblot analysis

CD19-CAR-T cells were lysed in ice-cold RIPA buffer (Thermo Scientific) containing protease inhibitor cocktail (Roche). Supernatants from centrifuged whole-cell lysates were prepared in 4 $\times$  LDS Sample Buffer (Life Technologies) and run on a 4%–12% Bis-Tris gel (Life Technologies). Gels were transferred to a 0.45- $\mu$ m PVDF membrane (Life Technologies) and incubated overnight at 4°C using primary antibody to Bcl2, pAkt-S473, Akt, prpS6-S235/236, rpS6, Glut1, and (Cell Signaling Technology), p-STAT5 (BD Biosciences), actin (Abcam), or GAPDH (Millipore), then incubated with HRP-conjugated secondary antibody (Millipore) and imaged using ECL detection reagent (GE Healthcare) and TI-BA Series 2000A film processor on autoradiography film (Denville Scientific).

### q-RT-PCR analysis

At the early and late time points, CAR-T cells were separated into CD4<sup>+</sup> or CD8<sup>+</sup> populations using the EasySep CD8 or CD4 Positive Selection Kits (Stem Cell Technologies), respectively. RNA was isolated using the RNeasy Mini Kit (Qiagen). cDNA was reverse transcribed using the SuperScript VILO Mastermix (Life Technologies) according to the manufacturer's instructions. qPCR reactions were performed as previously described (5). Primers are described in Supplementary Table S1.

### In vivo xenograft models

All mouse experiments were approved by the COH Institutional Animal Care and Use Committee. For CD19-CAR-T studies, NOD/Scid IL2R $\gamma$ C<sup>null</sup> (NSG) mice (6–10-week-old) were injected with  $0.5 \times 10^6$  Raji-ffluc cells (CD19<sup>+</sup>) intravenously (i.v.) on day 0. CD19-CAR-T cells or mock-transduced T cells ( $1 \times 10^6$ ) that were expanded *in vitro* under different cytokine conditions were injected i.v. into mice 3 days after tumor inoculation. For IL13R $\alpha$ 2-CAR-T studies, NSG mice (6–10-week-old) were injected with  $0.1 \times 10^6$  human glioma cell line (IL13R $\alpha$ 2<sup>+</sup>) intracranially (i.c.) as described before (22). On day 8, IL13R $\alpha$ 2-CAR-T cells or mock ( $5 \times 10^4$  cells), expanded in different cytokine conditions, were administered intratumorally. Tumor burden was monitored with Xenogen IVIS (Xenogen) or SPECTRAL Ami X (Spectral Instruments Imaging) and analyzed using Living Image 2.50 (PerkinElmer) or AMIView software (v1.7.061, Spectral Instruments Imaging). Survival curves were generated by GraphPad Prism Software (v5). For some experiments, post-therapy, retro-orbital blood samples were RBC-lysed using PharmLyse buffer (BD Biosciences), then assessed using anti-human CD45 and anti-human CD3 antibodies (BD Biosciences) to measure the frequency of adoptively transferred T cells by flow cytometry. Assessment of CAR-T function post-therapy was conducted as previously described (5). Briefly, tumors were injected subcutaneously, ( $1 \times 10^6$  cells), after 7 days CAR-T/IL15 or CAR-T/IL2 was injected intratumorally. T cells were isolated and recultured with tumor at 1:1 ratio and degranulation assay was conducted as described above.

### Statistical analysis

Statistical significance was determined using Student *t* test (two groups) or one-way ANOVA analysis with a Bonferroni test (three or more groups). Survival was plotted using a Kaplan–Meier survival curve, and statistical significance was determined by the log-rank (Mantel–Cox) test. All analyses were carried out using GraphPad Prism Software (v5). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  as significant; ns, not significant.

## Results

### CAR-T cells expanded *ex vivo* in IL15 maintain a less-differentiated phenotype

Previous studies have indicated that IL2 promotes the generation of highly differentiated T-cell subsets such as Tem and Teff (10, 25). By comparison, IL15 is reported to promote the generation of memory T cells (11, 26, 27) and promote T-cell survival and antitumor activity in mice (28, 29). To assess whether the replacement of IL2 by IL15 may overcome this pitfall for human CAR-T cells expanded under clinically relevant manufacturing processes, we compared *ex vivo*-expanded CD19-CAR-T cells cultured with either IL2 (CAR-T/IL2) or IL15 (CAR-T/IL15). The CD19-CAR is a second-generation CD28-CAR (30), which is currently being evaluated clinically (NCT02051257, NCT02146924, and NCT02153580). For these studies, CAR-T cells were cultured in 10 ng/mL IL15 or our current clinical cGMP manufacturing cytokine condition, which uses 50 U/mL IL2 with low levels of IL15 (0.5 ng/mL; IL2/IL15low; refs. 23, 31). CAR-T cells cultured with IL2/IL15low (manufacturing platform) show no phenotypic differences when compared with 50 U/mL IL2 alone; therefore, CAR-T cells generated from IL2/IL15low were labeled as CAR-T/IL2 (Supplementary Fig. S1). For these studies, unless otherwise stated, we also utilized our current clinical manufacturing platform, which enriches CD62L<sup>+</sup> T cells, primarily consisting of naïve (Tn), stem cell memory (Tscm), and central memory (Tcm) subsets, for CAR engineering. Flow cytometry analysis of selected T cells post-enrichment confirms that >85% of the T cells are CD62L<sup>+</sup> and 55% ± 10% are CD3<sup>+</sup>CD45RA<sup>+</sup> CD62L<sup>+</sup> Tn or Tscm (3 representative healthy donors; Supplementary Fig. S2).

To compare IL2 with IL15 *ex vivo* expansion conditions, we expanded cells over an extended period of time (up to 32 days) as this process allows the two populations to be compared for differentiation, exhaustion, and apoptosis status (32). Our data indicate that throughout extended culture, CAR-T/IL15 maintains a higher proportion of T cells (both CD4<sup>+</sup> and CD8<sup>+</sup>) that exhibit a Tscm phenotype (defined as CD95<sup>+</sup>, CD45RA<sup>+</sup> CCR7<sup>+</sup>, CD62L<sup>+</sup> CD27<sup>+</sup>, or CD62L<sup>+</sup> CD127<sup>+</sup>) as compared with CAR-T/IL2 (Fig. 1A and B; Supplementary Fig. S3A), which corresponded to a higher CD45RA:CD45RO ratio for CAR-T/IL15 (Supplementary Fig. S3B). Further qPCR analysis demonstrated an upregulation of key memory stem-like-associated transcription factors (i.e., *LEF1* and *TCF7*) in CAR-T/IL15 as compared with CAR-T/IL2, which was confirmed with the RNA-seq analysis conducted on purified CD4<sup>+</sup> and CD8<sup>+</sup> subsets (Fig. 1C and D). In line with this finding, CAR-T/IL15 displayed reduced expression of genes regulating effector differentiation such as eomesodermin (*EOMES*), T-box 21 (*TBX21*), as well as cytotoxic molecules (for example, granzyme B and perforin) as compared with CAR-T/IL2 (Fig. 1E and F). Furthermore, CAR-T/IL15 cells expressed less IFN $\gamma$  (a marker of Teff cells) following antigen

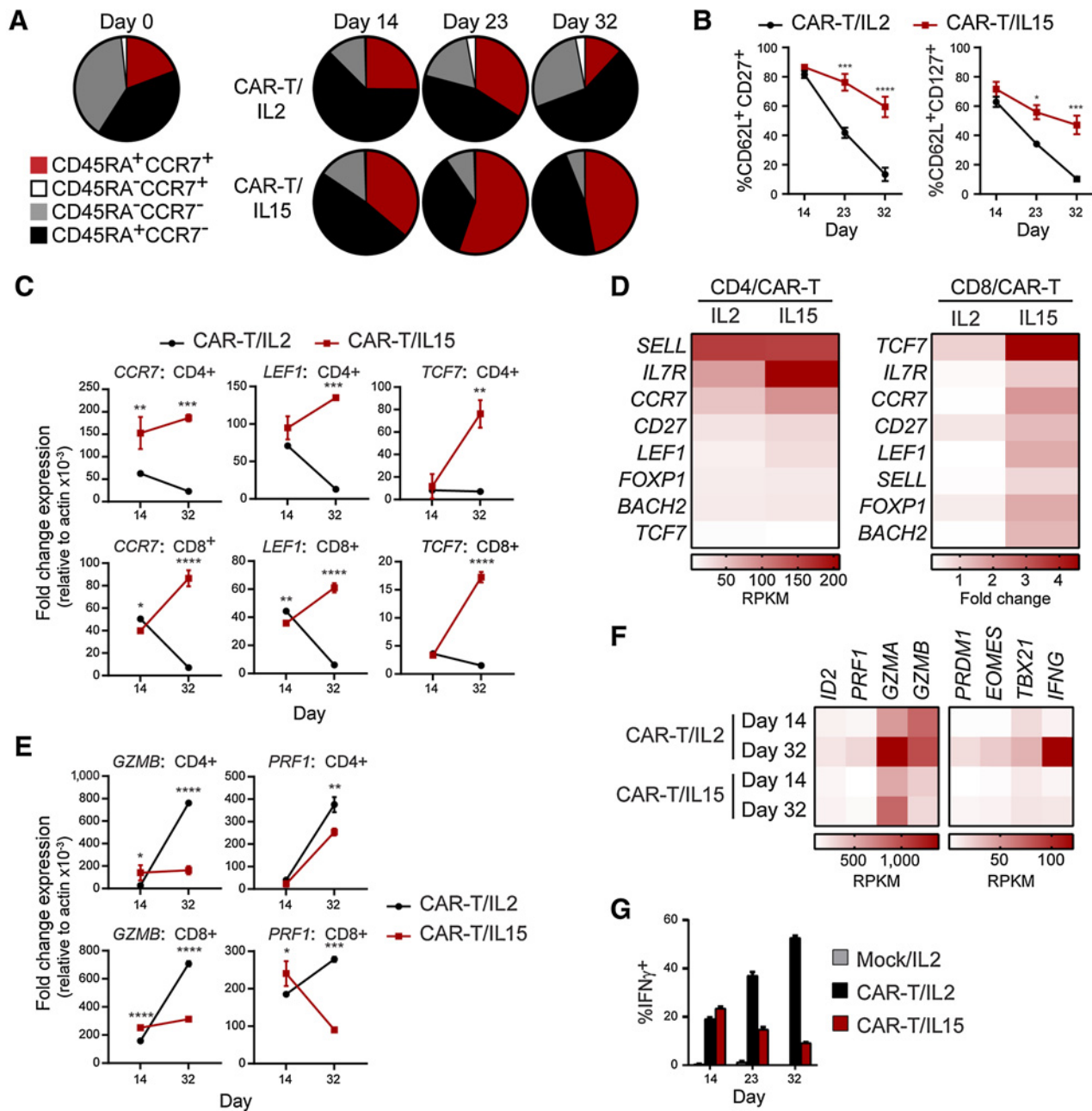
stimulation with CD19<sup>+</sup> Raji cells compared with CAR-T/IL2 cells (Fig. 1G and Supplementary Fig. S3C). With extended culture, we observed a decrease in the frequency of CD4<sup>+</sup> T cells in both culture conditions, which resulted in insufficient coverage by RNA-seq for analysis on day 32 (Fig. 1D–F).

Next, to further assess the influence of IL15 and IL2 on CAR-T cells during *ex vivo* expansion, CD8<sup>+</sup>CAR<sup>+</sup> cells from each culture condition at early and late time points (days 14 and 32) were compared for global gene expression changes. Hierarchical clustering highlighted extensive differences between the two culture conditions (Fig. 2A). MDS analysis showed that by day 14 in culture, CAR-T/IL2 and CAR-T/IL15 exhibited different expression profiles (721 differentially expressed genes) and even greater differences by day 32 (1,687 differentially expressed genes,  $P < 0.01$  and greater than 2-fold change in expression; Fig. 2B). Extended culture over time altered the gene-expression profiles more for CAR-T/IL2 cells (1,674 differentially expressed genes) than CAR-T/IL15 cells (782 differentially expressed genes). Furthermore, as early as day 14, 123 genes were differentially expressed in the CD4<sup>+</sup> T-cell population cultured in IL2 as compared with IL15 (Supplementary Fig. S4). These data thus confirm that CAR-T/IL15 cells retain their phenotypic characteristics in both CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations compared with their CAR-T/IL2 counterparts.

### IL15 promotes T-cell survival and inhibits T-cell exhaustion

The role of IL2 in proliferation and activation-induced cell death is well established (33), and IL15 is a known antiapoptotic factor in several systems (34). Inhibition of caspase-3 activity through IL15-mediated posttranslational modifications improves T-cell survival (28). Further, IL15 enhances the antioxidant capacity of T cells, thus leading to increased T-cell persistence (35). To extend these studies to the *ex vivo* expansion of human CAR-T cells, we evaluated the CD19-CAR-T cell apoptotic phenotype under these conditions. Our data indicate that CD19-CAR-T/IL15 cells expressed significantly less active caspase-3 compared with CAR-T/IL2 (Fig. 3A). Upregulation of the antiapoptotic molecule Bcl2 in CAR-T/IL15 compared with CAR-T/IL2 further confirms that IL15 exerts an antiapoptotic effect on T cells (Fig. 3B).

T-cell exhaustion is a state of dysfunction that at early and intermediate stages involves upregulation of inhibitory receptors such as PD-1, Lag3, and 2B4 and is accompanied by reduced antitumor function (36). Exhaustion affects CAR-T cell function and persistence, which is a barrier for effective CAR-T cell responses (37). Methods that overcome or prevent T-cell exhaustion could improve the effectiveness of CAR-T therapy. To determine whether IL15 affects T-cell exhaustion, T cells were cultured for up to 32 days to allow for induction of inhibitory molecules. Although no difference in the frequency of PD-1<sup>+</sup> T cells was observed (Supplementary Fig. S5A), a significant increase in the frequency of Lag3<sup>+</sup> and 2B4<sup>+</sup> T cells in CAR-T/IL2 over the extended culture period was detected. Upregulation of Lag3 and 2B4 also corresponded to an increase in *LAG3* and *CD244* (2B4) gene expression in the CD8<sup>+</sup> population (Fig. 3C and D). As early as 14 days after *ex vivo* culture, CD4<sup>+</sup> T cells cultured in IL2 showed an overall higher expression of inhibitory molecules such as CTLA4 (*CTLA4*), PD-1 (*PDCD1*), Lag3 (*LAG3*), PD-L1 (*CD274*) and suppressive cytokines and transcription factors such as IL13 and FOXP3, respectively (Fig. 3D). Taken together, these studies demonstrate that human CAR-T cells expanded in IL15 are programmed for survival and sustain a less-exhausted phenotype.

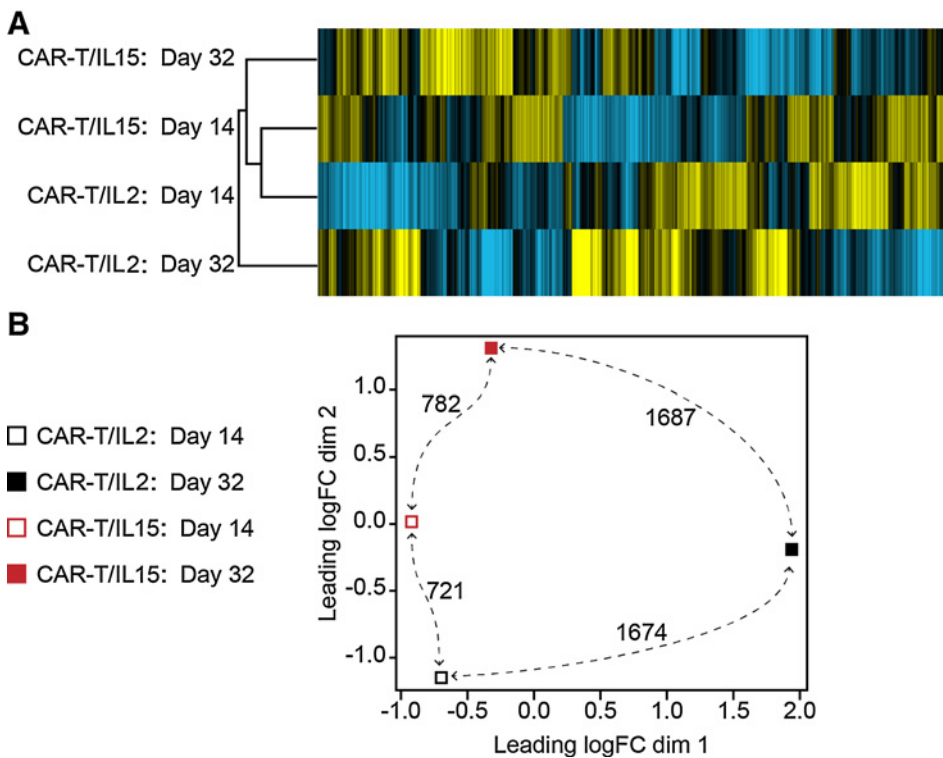


**Figure 1.** IL15 enriches for CAR-T cells with Tscm phenotype. Flow-cytometric analysis compares the frequency of Tscm population over time in CD19-CAR-T cells cultured in IL2 or IL15 as (A) CD45RA<sup>+</sup> CCR7<sup>+</sup> T cells summarized in pie charts and (B) CD62L<sup>+</sup>CD27<sup>+</sup> and CD62L<sup>+</sup>CD127<sup>+</sup> shown in graphs. Data shown are representative of three independent donors. C, Quantitative RT-PCR analysis of key genes upregulated in T cells with Tscm phenotype for CD4<sup>+</sup> and CD8<sup>+</sup> CAR-T cells. D, Heat map depicts global changes in the expression of genes regulating T-cell differentiation shown as robust multichip analysis (RPKM)-normalized intensity for CD4<sup>+</sup> T cells on day 14 (left) and fold change from days 14 to 32 for CD8<sup>+</sup> T cells (right). E, Quantitative RT-PCR analysis of indicated effector genes in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. F, Robust multichip analysis (RPKM)-normalized intensity of genes involved in effector function in CD8<sup>+</sup> T cells. G, Effector function measured by flow-cytometric analysis of IFN $\gamma$ <sup>+</sup> CAR-T cells after coculturing CAR-T cells with target cells (CD19<sup>+</sup>; Raji.) at a 1:1 effector:target ratio for 5 hours. Data are representative of two independent studies. Data, mean  $\pm$  SEM; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

**IL15-cultured CAR-T cells exhibit less mTORC1 activity and reduced expression of glycolytic enzymes**

We next sought to identify alterations in signaling pathways that may explain the phenotypic and functional differences

observed in cells generated in IL15 versus IL2 cytokine conditions. Multiple signal transduction pathways have been implicated in regulating cell differentiation and preserving Tscm phenotype. Although Akt plays a role in T-cell effector differentiation (38), our



**Figure 2.** IL15-cultured T cells represent a distinct, less-differentiated T-cell subset. **A**, Hierarchical clustering shows global gene-expression changes in CD8<sup>+</sup> T cells between the two culture conditions ( $P < 0.01$ , false discovery rate  $< 5\%$ , Benjamini-Hochberg method). Yellow and blue colors indicate increased and decreased expression, respectively. **B**, MDS analysis depicts the number of genes differentially regulated in CD8<sup>+</sup> T cells cultured in IL2 or IL15.  $P < 0.01$  ( $t$  test), false discovery rate  $< 5\%$ ; 2-fold change in expression.

results indicated that phosphorylation of Akt (pAkt; Fig. 4A) in CAR-T/IL2 cells did not differ from that in CAR-T/IL15 cells. Similarly, STAT5 activation (p-STAT5), which functions downstream of cytokine signaling was also not different between the IL2 and IL15 products (Supplementary Fig. S5B). However, mTORC1 activity was decreased in CAR-T/IL15 by more than 90%, as measured by reduced phosphorylation of ribosomal protein S6 (rpS6; Fig. 4A). The mTORC1 signaling pathway is involved in metabolic changes and regulation of glucose transport (39). Specifically, mTOR signaling increases glycolysis by increasing glucose transporter GLUT1 (*SLC2A1*) expression and stimulating glycolytic activity (40). Consistent with this observation, IL15 reduced GLUT1 expression in CAR-T cells and increased expression of CPT1A an enzyme that regulates FAO (Fig. 4A). To determine if decreased mTORC1 activity in CAR-T/IL15 cells leads to commensurate changes in expression of genes in the metabolism of T cells, we evaluated expression of other enzymes involved in glycolysis and FAO pathways. CD19-CAR-T/IL15 had reduced expression of glycolytic enzymes and conversely increased expression of enzymes involved in the FAO pathway (Fig. 4B and C) and exhibited overall low mitochondrial potential ( $\Delta\psi_m$ ; Fig. 4D), suggesting that IL15-mediated decrease of mTORC1 activity is associated with metabolic changes in CAR-T cells. Additionally, a decrease in GLUT1 expression correlated with reduced glucose uptake in CAR-T/IL15 (Fig. 4D). To determine if these gene expression and metabolic measurements were associated with functional changes in cellular metabolism, we evaluated the mitochondria OCR, a measure of overall mitochondrial respiration and also an indicator of oxidative phosphorylation (OXPHOS). Consistent with the observed phenotypic changes, CAR-T/IL15 cells exhibited greater OCR and spare respiratory capacity, a feature of long-lived memory T cells (Fig. 4E). These findings are in line with previous studies indicating that

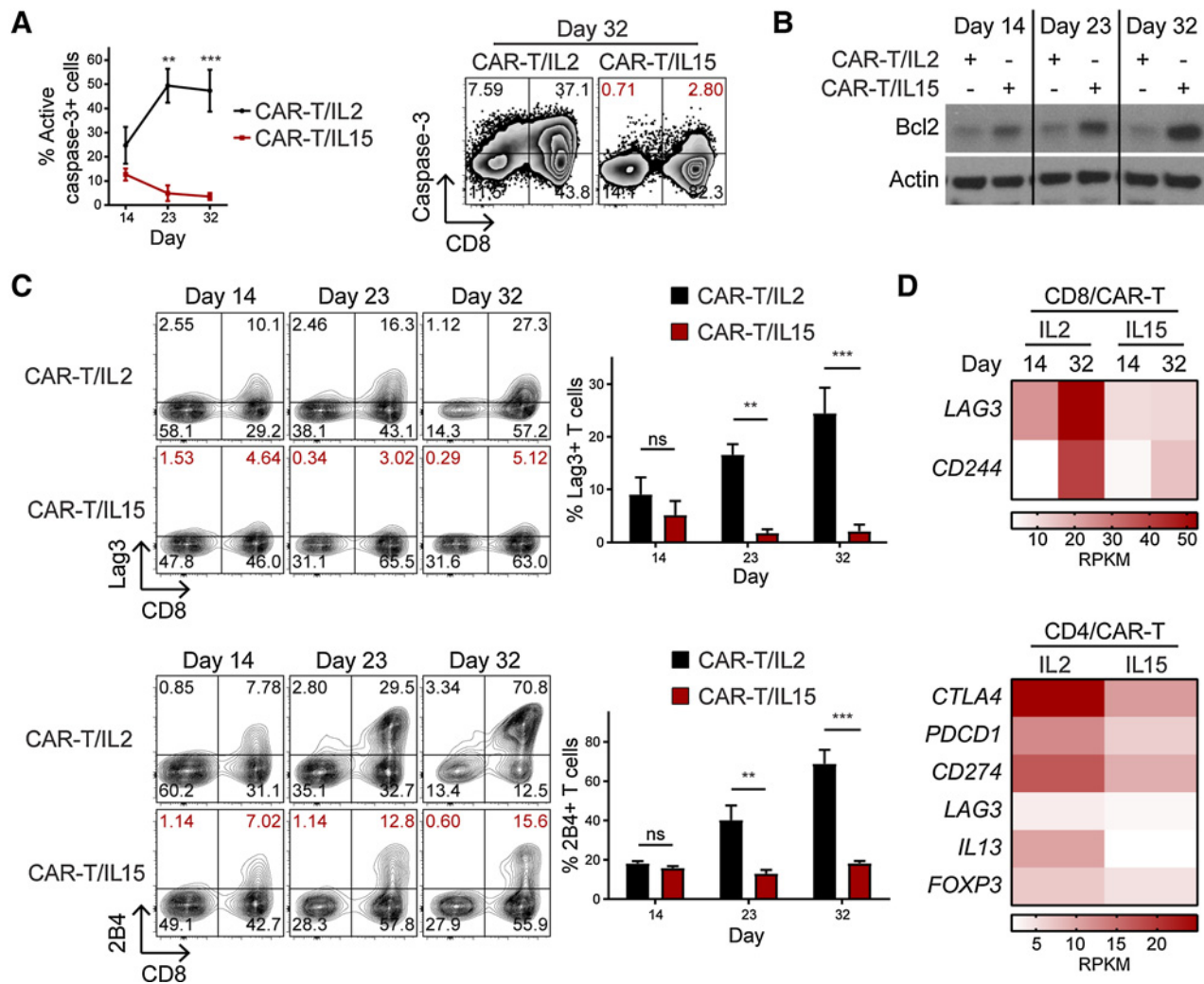
Tscm and Tcm subsets are characterized with higher OCR and exhibit low- $\Delta\psi_m$ , which correlate with superior antitumor activity and *in vivo* persistence (41).

Lastly, to determine whether IL15-mediated reduction in mTORC1 activity is the factor that preserves the Tscm phenotype, CAR-T/IL2 cells were cultured in the presence or absence of rapamycin (mTORC1 inhibitor) and compared with CAR-T/IL15. Expanding CAR-T/IL2 in the presence of rapamycin promoted a Tscm phenotype, similar to CAR-T/IL15 (Fig. 5A and B). Western blot analysis confirmed downregulation of p-rpS6 and GLUT1 and upregulation of CPT1A and Bcl2 in CAR-T/IL15 and IL2 plus rapamycin-cultured cells (Fig. 5C). Collectively, these data demonstrate that IL15-mediated reduction in mTORC1 activity leads to decreased glycolysis and prevents T-cell differentiation.

#### Enhanced proliferative capacity of CAR-T/IL15 cells correlates with antitumor activity and persistence

The phenotypic differences observed in CAR-T/IL15 compared with CAR-T/IL2 indicated that IL15 was superior at preserving the stem-like properties of T cells, which has been previously shown to correlate with improved antitumor potency (11). We therefore compared the *in vitro* antitumor effects of CD19-CAR-T/IL2 and CAR-T/IL15 cells against CD19<sup>+</sup> Raji tumor cells in a 7-day coculture assay. Upon tumor antigen stimulation, CAR-T/IL15 exhibited 1.5-fold higher proliferative capacity and persistence and maintained superior overall *in vitro* killing capacity as compared with CAR-T/IL2 (Fig. 6A and B). To further evaluate the recursive killing capacity of each population, CAR-T cells were rechallenged with additional tumor cells. At the end of repetitive tumor challenge, CAR-T/IL15 cells outperformed CAR-T/IL2 cells in tumor killing capacity (Supplementary Fig. S5C).

Lastly, we compared how extended culture conditions in either IL15 or IL2 can affect CAR-T cell antitumor activity *in vivo*.



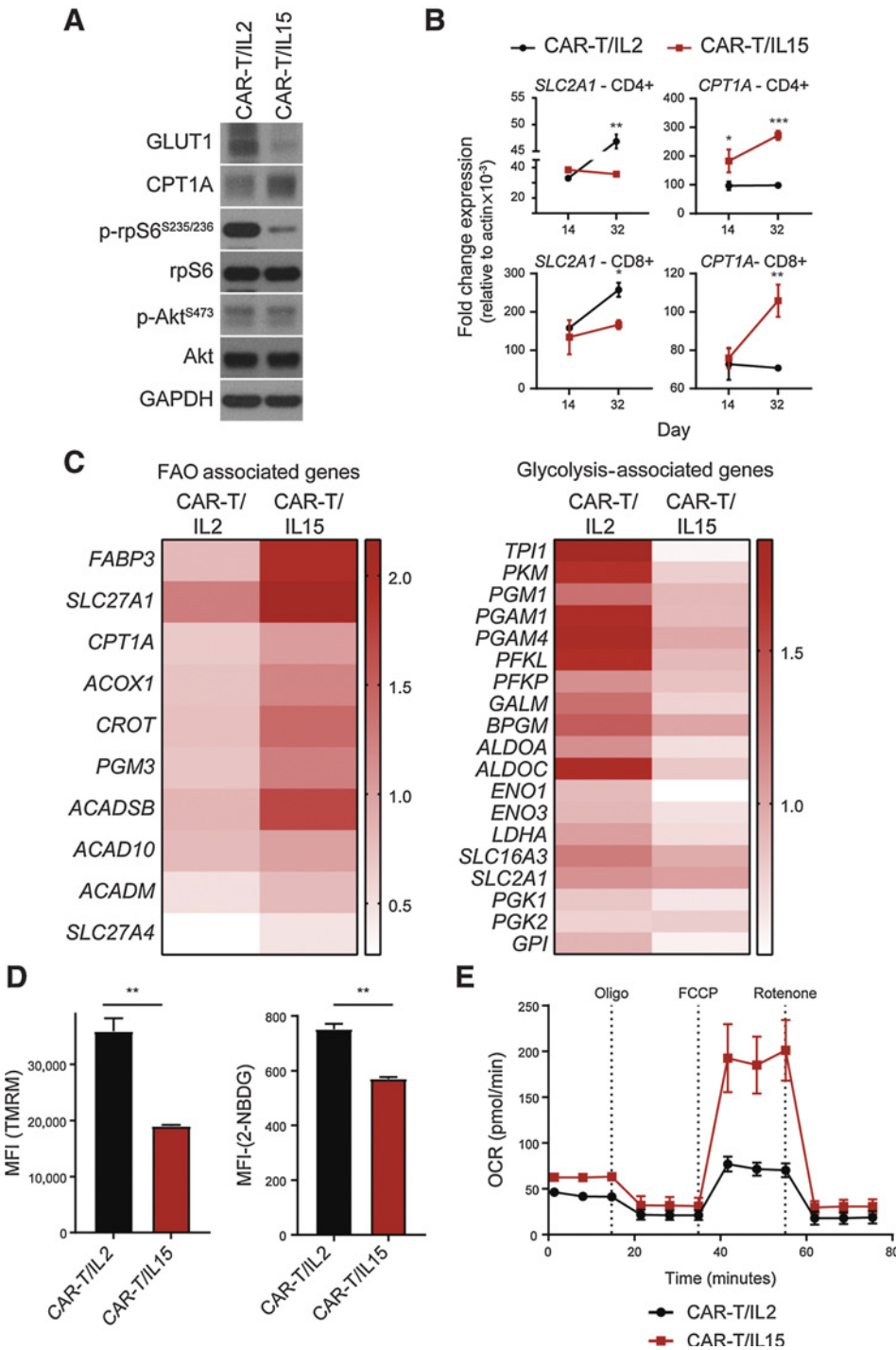
**Figure 3.** IL15 promotes T-cell survival and prevents upregulation of inhibitory receptors associated with T-cell exhaustion. **A**, Intracellular active caspase-3 was measured by flow cytometry in CD19-CAR-T cells cultured in IL2 or IL15. Graph displays the percentage of CD3<sup>+</sup> cells expressing active caspase-3 from three different donors (left), and one representative density plot of intracellular caspase-3 staining on day 32 (right). **B**, Western blot analysis shows the amount of antiapoptotic protein Bcl2 in CAR-T cells cultured in IL2 or IL15 over time. **C**, Flow-cytometric analysis shows the frequency of CAR-T cells positive for inhibitory receptors Lag3 (top) and 2B4 (bottom). Flow cytometry plots over time from one representative donor are shown (left), and bar graphs are presented as mean  $\pm$  SEM from three independent donors. **D**, Robust multichip analysis (RPKM)-normalized intensity of the *LAG3* and *2B4* (*CD244*) genes in CD8<sup>+</sup> T cells at day 14 or 32 (top) and genes involved in inhibitory and suppressive function in CD4<sup>+</sup> T cells on day 14 (bottom). Data are representative of three independent studies. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

CD19-CAR-T/IL2 or CAR-T/IL15 cells expanded for 14 or 32 days were administered to mice bearing Raji-ffluc tumors, an aggressive CD19<sup>+</sup> lymphoma mouse model. The adoptive transfer of CAR-T/IL15 cells mediated superior antitumor activity as measured by bioluminescent flux and promoted significantly greater survival advantage than CAR-T/IL2 at both culture time points (Fig. 6C–E). CAR-T/IL15 cells expanded for 32 days showed similar efficacy as CAR-T/IL2 cells expanded for only 14 days. In line with these findings, CAR-T/IL15 cells exhibited significantly longer persistence than CAR-T/IL2 cells at both early and late expansion periods (Fig. 6F and G). CAR-T/IL2 cells taken from extended culture condition (day 32) exhibited the lowest persistence 7 days after infusion, which correlated with the least efficacious survival outcome (Fig. 6E). Together, these data indi-

cate that CAR-T/IL15 cells outperform CAR-T/IL2 cells in antitumor potency both *in vitro* and *in vivo* and furthermore, the negative impact of extended culture is partially rescued by culture in IL15.

**IL15-mediated effects are observed across various manufacturing processes and CAR designs**

To evaluate whether the phenotypic profile of CAR-T/IL15 cells was also observed in PBMC-derived CAR-T cell products, we also assessed the phenotypic changes for PBMC-expanded CAR-T cells cultured in IL2 compared with IL15. As anticipated, PBMC-derived CD19-CAR-T cells, which contain Tn, Tscm, and Tcm, also exhibited IL15-mediated phenotypic changes, which include maintenance of the Tscm phenotype (CCR7<sup>+</sup>CD45RA<sup>+</sup>; CD62L<sup>+</sup>CD27<sup>+</sup>; CD62L<sup>+</sup>CD127<sup>+</sup>) and enhanced proliferative

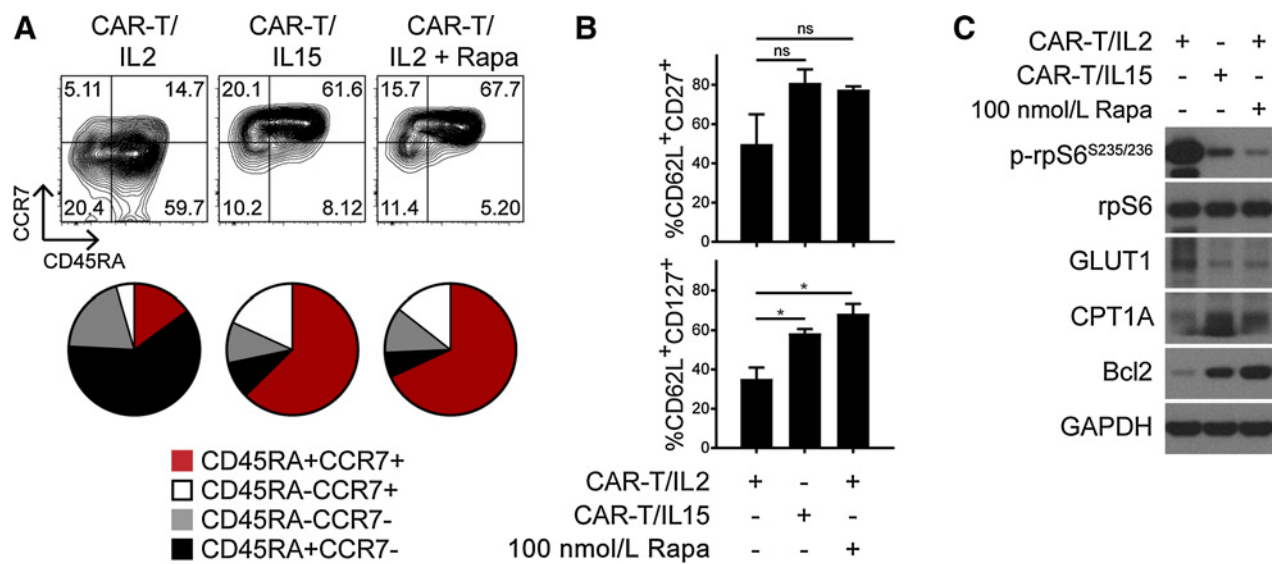


**Figure 4.** IL15 reduces mTORC1 activity and decreases the expression of glycolytic enzyme genes with a concomitant increase in FAO genes. **A**, Immunoblot analysis of indicated proteins in CD19-CAR-T cells cultured in IL2 or IL15 on day 23. GAPDH was used as a loading control. **B**, Quantitative RT-PCR analysis of Glut1 (*SLC2A1*) and *CPT1A* expression in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Results are presented relative to the actin gene. **C**, Heat map of RNA-seq analysis of sorted CD8<sup>+</sup> T-cell subsets, highlighting changes in the canonical genes associated with FAO (left) or glycolysis (right) represented as fold change between days 14 and 32. Red indicates greatest increase and white indicates no change. **D**, Bar graph represents the differences in MFI of mitochondria potential (TMRM; left) and glucose uptake (2-NBDG) measurements in CAR-T cells. **E**, OCR of CD19-CAR-T/IL2 and CD19-CAR-T/IL15 cells in response to indicated mitochondrial modulators: oligomycin; FCCP; rotenone. Data are representative of two independent studies. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

capacity upon antigen stimulation (Supplementary Fig. S6A and S6B). Furthermore, PBMC-derived CAR-T cells cultured in IL15 also exhibited increased expression of Bcl2 and reduced mTORC1 activity as indicated by reduced expression of p-rpS6 (Supplementary Fig. S6C). *In vivo*, both PBMC- and CD62L<sup>+</sup>-derived CAR-T cells cultured in IL15 showed superior survival benefit compared with IL2-cultured cells (Supplementary Fig. S6D). Together, these data suggest that IL15 has a similar biological impact on CAR-T cells derived from unselected T-cell populations.

To determine whether the phenotypic and functional characteristics observed for CAR-T/IL15 cells were generalizable to other CAR designs, similar studies were conducted using our glioma-targeted IL13R $\alpha$ 2-CAR-T cells, which utilize a 4-1BB costimulatory domain (22). IL13R $\alpha$ 2-CAR-T cells cultured with IL15 also sustained their stem-like phenotype and remained less exhausted when compared with IL2-cultured cells (Supplementary Fig. S7A and S7B). Similar to CD19-CAR-T cells, IL13R $\alpha$ 2-CAR-T cells cultured in IL15 also exhibited reduced mTORC1





**Figure 5.**

Reduced mTORC1 activity is responsible for the IL15 maintenance of a Tscm phenotype. **A**, Flow cytometry analysis shows changes in CD45RA<sup>+</sup> CCR7<sup>+</sup> CD19-CAR-T cells cultured in IL2, IL15, or IL2 + rapamycin (100 nmol/L; top), summarized in a pie chart (bottom). **B**, Bar graph shows changes in the frequency of CD62L<sup>+</sup>CD27<sup>+</sup> and CD62L<sup>+</sup>CD127<sup>+</sup> T cells cultured in the above conditions. Data, mean  $\pm$  SEM of two experiments. **C**, Immunoblot analysis of p-rpS6, rpS6, GLUT1, CPT1A, and Bcl2 proteins confirms reduced mTORC1 activity and phenotypic similarities in T cells cultured in IL15 and IL2 + rapamycin. GAPDH was used as a loading control. Data are representative of two independent studies. \*,  $P < 0.05$ .

activity and GLUT1 expression, which correlated with greater OCR, low  $\Delta\psi_m$ , and decreased glucose uptake (Supplementary Fig. S7C and S7D). Furthermore, intratumoral CAR-T/IL15 cells exhibit a greater polyfunctional phenotype (TNF $\alpha$ <sup>+</sup> CD107 $\alpha$ <sup>+</sup> and IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup>) as compared with CAR-T/IL2 cells (Supplementary Fig. S7E). IL13R $\alpha$ 2-CAR-T cells cultured with IL15 exhibited greater antitumor activity upon multiple tumor rechallenge *in vitro* as well as *in vivo* against orthotopic GBM tumors (Supplementary Fig. S7F and S7G). Together, these studies suggest that the effect of IL15 is independent of CAR design (CD28 or 4-1BB costimulatory domain) as both CD19- and IL13R $\alpha$ 2-CAR-T products exhibited similar phenotypic and metabolic advantages when cultured in IL15 alone as compared with expansion in IL2.

#### Inclusion of other $\gamma$ -cytokines with IL15 does not improve antitumor activity

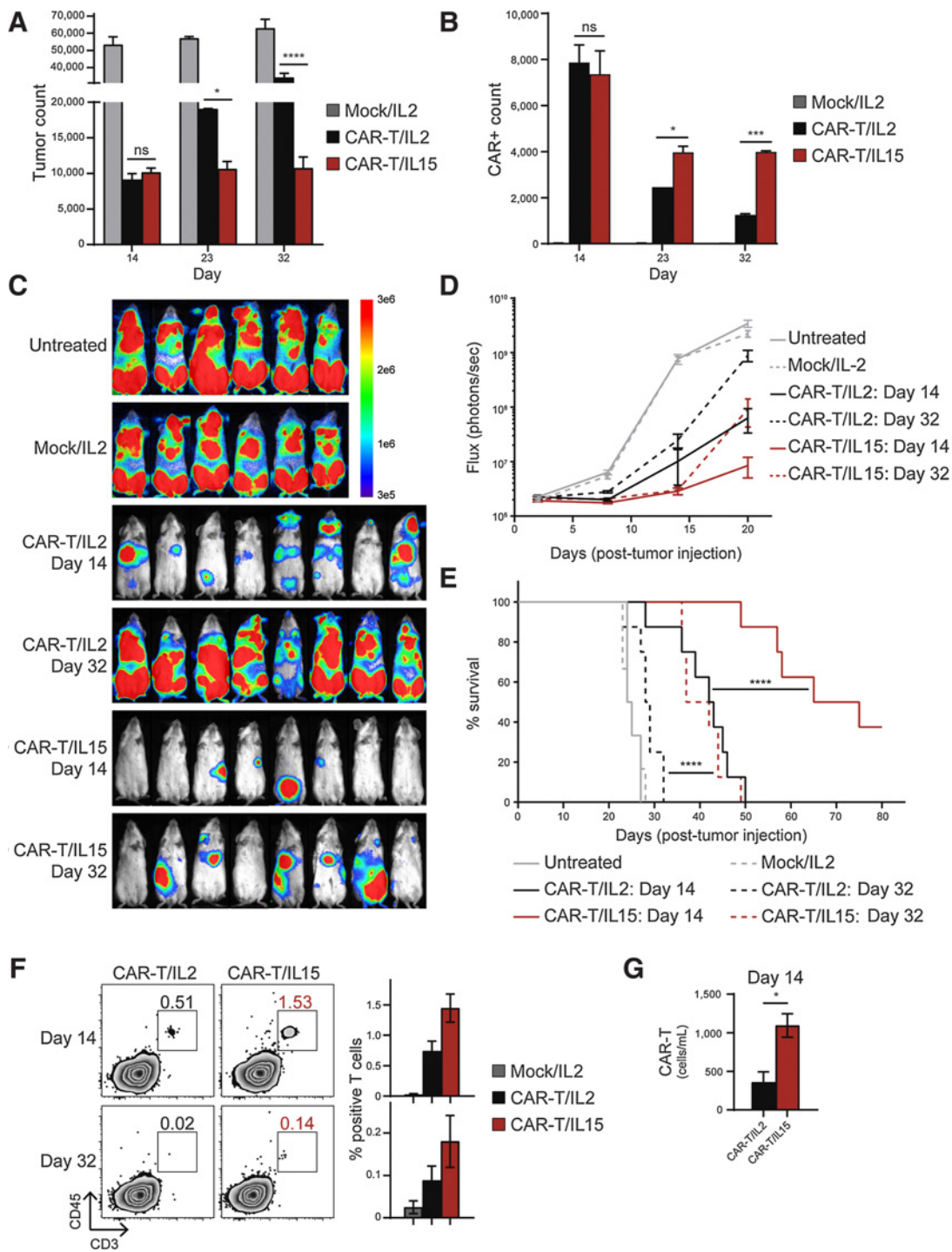
Our studies highlight the benefit of culturing in IL15 as compared with IL2. Previous reports demonstrate a benefit in combining IL15 with other cytokine combinations for the *ex vivo* expansion of human T cells, particularly IL21 (42) or IL7 (2). With the goal of defining the optimal condition for the production of improved CAR-T cells, additional cytokine combinations (IL15/IL7 and IL15/IL7/IL21; refs. 2, 13–15) were assessed. CAR-T/IL15 cells expressed a more prominent CD45RA<sup>+</sup>CCR7<sup>+</sup> and CD62L<sup>+</sup>CD27<sup>+</sup> less-differentiated memory phenotype compared with cells in other culture conditions (Fig. 7A). Further, the addition of IL7 and/or IL21 with IL15 increased expression of inhibitory receptors such as Lag3 and 2B4 as compared with IL15 alone (Fig. 7B). The observed changes were detected in both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets, with CD8<sup>+</sup> T cells showing a larger difference in phenotypes between the cytokine culture conditions (Fig. 7A and B). T cells cultured in IL15/IL7/IL21 exhibited the least favorable phenotype, defined as increased expression of inhibitory molecules and very low frequency of CD45RA<sup>+</sup>CCR7<sup>+</sup>

T cells (7%  $\pm$  5%; Fig. 7A and B). These studies indicate that the addition of other  $\gamma$ -cytokines to the culture condition does not result in a superior CAR-T product.

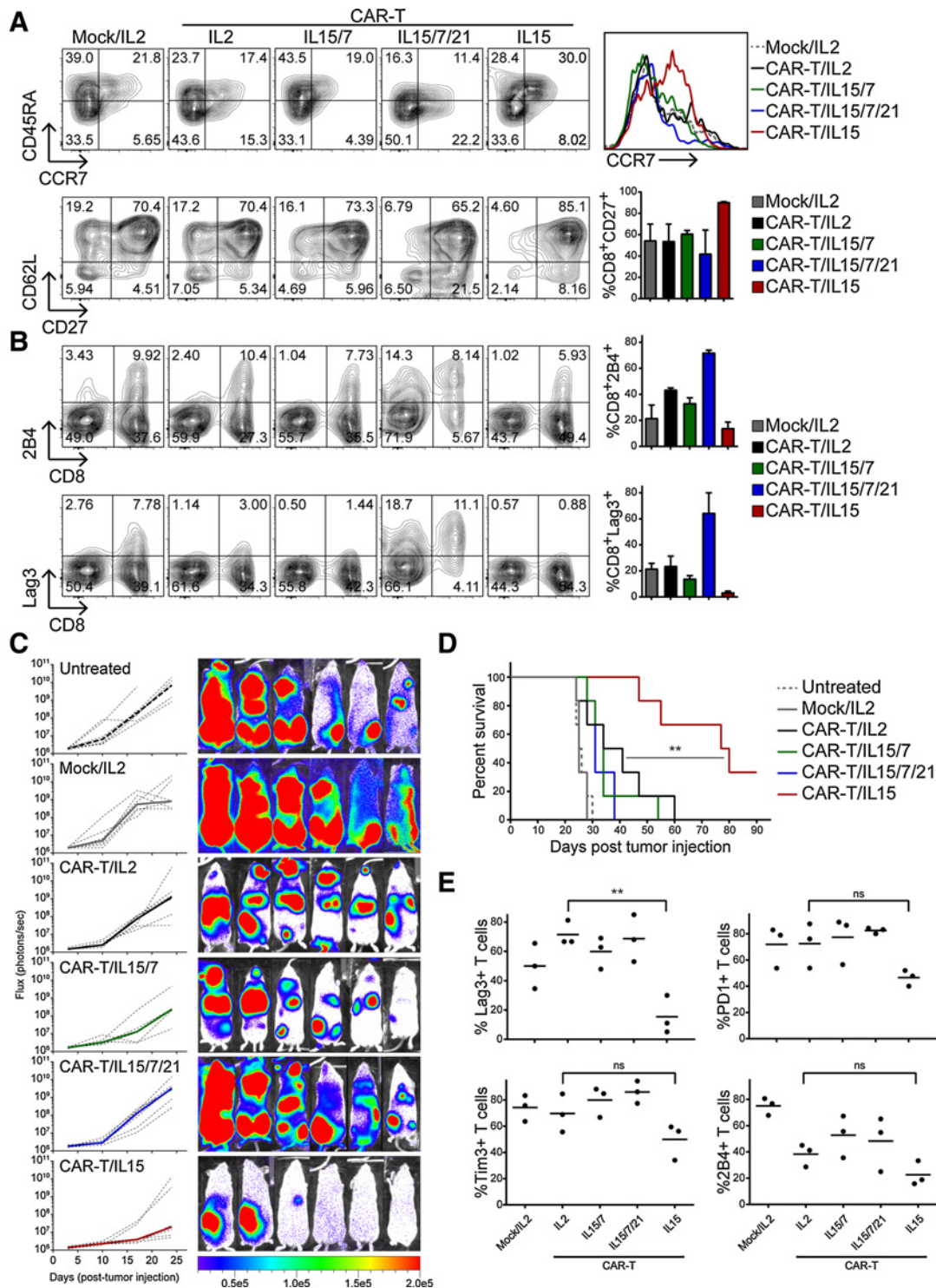
To compare how CAR-T cells cultured in various cytokine conditions perform *in vivo*, CD19-CAR-T cells cultured in IL2 (i.e., clinical cGMP manufacturing condition IL2/IL15low), IL15, IL15/IL7, and IL15/IL7/IL21 were adoptively transferred at a suboptimal dose against mice bearing CD19<sup>+</sup> Raji-ffluc tumors. *In vivo* assessment of CAR-T cell products showed superior antitumor activity of CD19-CAR-T cells cultured in IL15, as compared with other cytokine conditions (Fig. 7C and D). Additional  $\gamma$ -cytokines in culture condition resulted in a less efficacious CAR product even with relatively comparable CAR and CD4/CD8 T-cell composition (Supplementary Fig. S8A–S8D). Furthermore, assessment of T cells isolated from blood post CAR-T cell therapy showed reduced expression of inhibitory molecules (PD-1, Lag3, and 2B4) in the CAR-T/IL15 group (Fig. 7E), as compared with other cytokine combinations. Reducing the IL2 concentration did not enhance antitumor activity of CAR-T cells. The phenotypic characteristics of low-dose IL2 was similar to standard IL2 (Supplementary Fig. S9A–S9C). Together, these data highlight the impact of IL15 on T cells as compared with other cytokine culture conditions and suggest that inclusion of other  $\gamma$ -cytokines with IL15 can reduce the beneficial properties of IL15 alone.

## Discussion

Considerable progress has been made in improving the CAR-T cell technology to obtain cell products that result in enhanced persistence/expansion and antitumor response. Studies have shown benefits for using IL15/IL7 in culture (2) or IL15 as part of the CAR cassette (43, 44). However, little is known about the IL15-mediated intracellular signaling that modulates the formation and maintenance of a less-differentiated T-cell phenotype,



**Figure 6.** CAR-T cells cultured in IL15 exhibit enhanced proliferative capacity with superior antitumor activity: **A**, CD19-CAR-T cells were cocultured with tumor cells (CD19<sup>+</sup>; Raji) at a 1:1 effector:target ratio for 7 days, then number of **(A)** tumor cells and **(B)** CAR-T cells were counted by flow cytometry and graphed. Mice bearing Raji-ffluc lymphoma ( $0.5 \times 10^6$ ) were untreated or treated with  $1 \times 10^6$  mock or CD19-CAR-T cells three days after tumor engraftment. T cells were thawed and injected i.v. after cryopreservation at the indicated number of days in *ex vivo* culture. **C**, Bioluminescent images compare tumor progression 19 days after adoptive transfer of T cells ( $n = 6-8$  mice per group). **D**, Bioluminescent flux plot quantifying tumor burden in response to different treatment groups over time. Data are shown as mean  $\pm$  SEM. **E**, Kaplan-Meier survival curve depicts overall survival. **F**, Frequency of circulating CAR-T cells 10 days after CAR-T cell therapy identified by flow cytometry using anti-human CD3 and CD45 (left) and bar graph summarizes the frequency of human T cells identified in each group (right). **G**, Bar graph summarizes the number of CAR-T cells/mL of blood from day 14 groups. Data are presented as mean  $\pm$  SEM of 6 to 8 individual animals and two independent studies. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .



**Figure 7.** Inclusion of additional  $\gamma c$  cytokines with IL15 reduces antitumor activity. **A**, Flow cytometric analysis of CD19-CAR-T cells cultured in different cytokine combinations 18 to 20 days after the initiation of culture showing the frequency of CD45RA<sup>+</sup> CCR7<sup>+</sup> and CD27<sup>+</sup> CD62L<sup>+</sup> CAR-T cells (left). Histogram plot showing CCR7 expression in total CD8<sup>+</sup> T cells (right). Bar graph shows the percentage of CD27 expressing CD8<sup>+</sup> T cells. **B**, Flow-cytometric analysis of indicated inhibitory molecules gated on CAR-T cells (left). Bar graphs showing the frequency of CD8<sup>+</sup> T cells expressing 2B4 (top) and Lag3 (bottom). **C**, Bioluminescent flux plot (left) and images (right) and **(D)** Kaplan-Meier survival curves compared tumor progression over time in treated and untreated groups ( $n = 6-8$  mice per group). Representative of two independent experiments. **E**, Comparison of inhibitory receptor expressions on CAR-T cells harvested from animals 17 days after therapy ( $n = 3-5$  mice per group). Data are representative of two independent studies. \*\*  $P < 0.01$ .

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including Tscm, and its role in antitumor activity (11). Sustaining the Tscm population during *ex vivo* expansion prior to adoptive T-cell therapy is challenging as culture conditions often result in an undesired effect of promoting the development of terminally differentiated T cells. Here, we demonstrate that the use of IL15 alone in the culture conditions for CAR-T cell generation preserves Tscm phenotype and results in enhanced antitumor activity and self-renewing capacity. We further show that CAR-T/IL15 downregulates mTOR activity, which results in a global decrease in the expression of glycolytic enzymes and subsequent increase in the expression of FAO-related enzymes. Together, these phenotypic characteristics resulted in CAR-T cells with superior cellular metabolism. Furthermore, we show that IL15-mediated reduction of mTORC1 is responsible for preserving the Tscm population, which is the first reported in the context of *ex vivo* expansion of CAR-T cells.

Efforts have been made to pharmacologically induce formation of memory T cells, using TWS119 (Gsk3b blockade; activator of Wnt signaling), rapamycin (mTORC1 inhibitor; refs. 20, 21), metformin (AMPK activator; ref. 17), or 2DG (Hk2 inhibitor; ref. 16). Although the physiologic role of these molecules in post-thymic T-cell development remains unknown, studies have suggested that these pathways can regulate T-cell differentiation and memory T-cell formation. In particular, targeting the mTOR pathway in mice results in the increased formation of memory T cells, characterized by the cell-surface expression of CD62L and CD127 and more of the antiapoptotic molecule Bcl2 (21). mTORC1 activity regulates the expression of genes involved in glycolysis (39). mTOR-mediated induction of glycolysis also promotes T-cell differentiation and formation of terminally differentiated effector T cells (21, 45). In our study, we observed that CAR-T/IL15, but not CAR-T/IL2, exhibits reduced mTORC1 activity, which was associated with the downregulation of key glycolytic enzymes. This metabolic switch was associated with improved persistence and antitumor activity of the CAR-T cells in two different tumor models (i.e., lymphoma and glioblastoma). In order to directly test that reduced mTORC1 and global glycolytic enzyme expression and changes in the metabolic activity is responsible for preserving the Tscm population, T cells cultured in IL2 were treated with rapamycin, an mTORC1 inhibitor. CAR-T/IL2 cultured in the presence of rapamycin exhibited similar phenotypic characteristics to CAR-T/IL15, suggesting that IL15 preserves the Tscm population by reducing mTORC1 activity. Overall, these findings are in line with now mounting evidence that metabolic pathways in T cells play a role in the regulation of T-cell longevity and function (46).

Another undesired effect of *ex vivo* expansion of T cells is the potential to generate exhausted T cells, which leads to limited persistence and antitumor efficacy *in vivo* (37). Here, we report that in CAR-T/IL15 cells, exposure to IL15 prevented the upregulation of the inhibitory molecules 2B4 and Lag3. We also demonstrate that the percentage of cells exhibiting apoptotic features (increased active caspase-3 and reduced Bcl2 expression) was reduced in CAR-T/IL15, which is consistent with previous reports on the antiapoptotic functions of IL15 (28, 33). Together, these phenotypic changes may account for the superior antitumor activity observed in CAR-T/IL15 cells in tumor-bearing hosts.

The effect of IL15 on CD8<sup>+</sup> T cells has been documented (29, 47). Conversely, the influence of IL15 on CD4<sup>+</sup> T cells remains unclear. Although some reports have indicated that IL15 promotes the expansion of effector CD4<sup>+</sup> T-cell and its require-

ment for maintenance of memory-like CD4<sup>+</sup> T cells, others have shown that IL15 may increase the proliferation of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (48, 49). In our study, when comparing CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations in the bulk T-cell product (via flow cytometry, qPCR and RNA-seq), it was evident that both CD8<sup>+</sup> and CD4<sup>+</sup> T cells shared similar phenotypic characteristics such as decreased caspase-3 activity, sustained Tscm phenotype, and reduced expression of exhaustion markers. On the basis of these observations, we believe that although there may be intrinsic differences between CD4<sup>+</sup> and CD8<sup>+</sup> T cells in response to IL15, CD4<sup>+</sup> and CD8<sup>+</sup> T cells share regulatory pathways that contribute to the superior antitumor activity observed in the final bulk CAR-T cell product.

The arrest of lymphocyte differentiation to maintain long-lived, self-renewing antigen-experienced T cells with stem-like properties remains a goal for the development of efficient antitumor adoptive T-cell therapy (11, 50). Selection of less-differentiated memory T-cell populations prior to *ex vivo* expansion and use of signaling inhibitors that prevent T-cell differentiation are other strategies for increasing the number of less-differentiated T cells available for adoptive T-cell therapy. Our study shows that signaling mediated by IL15 preserves the Tscm phenotype in CAR-T cells during *ex vivo* expansion and subsequent adoptive transfer *in vivo*. We demonstrate that these phenotypic differences are relevant in the context of our second-generation CD19- and IL13R $\alpha$ 2-CARs that utilize CD28 and 4-1BB costimulation, respectively, and thus appear to be intrinsic to the T cells and independent of CAR design. We also find that the Tscm phenotype is preserved for both PBMC- and CD62L<sup>+</sup>-derived CAR-T cell products cultured in IL15. The ability to modulate the *ex vivo* expansion conditions to promote and sustain Tscm population has considerable implications for the field of adoptive T-cell immunotherapy, specifically CAR-T therapy. Apart from the role of IL15 in preserving Tscm, IL15 has also been shown to "rescue" tolerant T cells by restoring their proliferative and antitumor activity (47), which also has implications as T-cell tolerance affects antitumor immune response. Lastly, the use of IL15 to induce long-lived T-cell memory subsets via reduced mTORC1 activity furthers the advancement of successful T-cell-based therapies.

#### Disclosure of Potential Conflicts of Interest

C.E. Brown is a consultant/advisory board member for Mustang Bio. No potential conflicts of interest were disclosed by the other authors.

#### Authors' Contributions

Conception and design: D. Alizadeh, C.E. Brown

Development of methodology: D. Alizadeh, D. Wang, X. Wang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Alizadeh, R.A. Wong, X. Yang, J.R. Pecoraro, C.-F. Kuo, B. Aguilar, Y. Qi, R. Starr, R. Urak, X. Wang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Alizadeh, R.A. Wong, D. Wang, J.R. Pecoraro, C.-F. Kuo, Y. Qi, D.K. Ann, X. Wang, S.J. Forman, C.E. Brown

Writing, review, and/or revision of the manuscript: D. Alizadeh, R.A. Wong, D. Wang, Y. Qi, S.J. Forman, C.E. Brown

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Alizadeh, B. Aguilar

Study supervision: D. Alizadeh, C.E. Brown

#### Acknowledgments

This work was supported in part by Mustang Bio., Inc., City of Hope Lymphoma SPOR grant P50 CA107399, Cancer Center Support Grant P30

CA33572 and R01CA220693-01. Patents associated with CAR design, T-cell manufacturing and delivery have been licensed by Mustang Bio., Inc., for which S.J. Forman and C.E. Brown receive licensing and consulting payments. The authors acknowledge the editorial assistance provided by Dr. Julie R. Ostberg and would also like to thank City of Hope Bioinformatics core specially Drs. Shu Tao and Xiwei Wu for their help with the RNA-sequencing analysis.

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Received July 11, 2018; revised December 6, 2018; accepted March 12, 2019; published first March 19, 2019.

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