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Manufacturing Chimeric Antigen Receptor (CAR) T cells for Adoptive Immunotherapy

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Corresponding Author:	Saba Ghassemi, PhD University of Pennsylvania Philadelphia, PA UNITED STATES
Corresponding Author's Institution:	University of Pennsylvania
Corresponding Author E-Mail:	ghassemi@pennmedicine.upenn.edu
Order of Authors:	Saba Ghassemi, PhD
	Michael C. Milone
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Penn Medicine
Center for Cellular Immunotherapies

Department of Pathology and Laboratory Medicine

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Ronald Myers, PhD.,
Science Editor
JoVE

Dear Dr. Myers:

On behalf of my co-author I wish to submit to JoVE our manuscript entitled “Approaches to Manufacture Chimeric Antigen Receptor (CAR) T cells and Assess their Function for Adoptive Immunotherapy” for consideration. Our work builds upon our extensive experience in the development and clinical evaluation of chimeric antigen receptor (CAR) therapies for B cell malignancies and the discovery of biomarkers predictive of response to CD19-specific CAR T (CTL019) cells, including the ability of these cells to expand and persist post-infusion (Sci Transl Med. 2015 Sep 2;7(303):303ra139). This work is based on our recently published manuscript (Cancer Immunol Res. 2018 Sep;6(9):1100-1109) that showed the culture duration is an important determinant of function of CAR T cells in vivo. Importantly, the manufacturing process presented in this paper has been validated under current Good Manufacturing Process conditions, used in our clinical trials.

We believe that our manuscript will be of great interest to investigators in the field of cellular therapy as it comprehensively presents the process of generating CAR T cells which is one major factor affecting T cell immunotherapy. We hope that you will appreciate its value and consider our manuscript for publication in your journal.

Sincerely,

Saba Ghassemi

Saba Ghassemi, PhD

8th Floor, Room 8-303 • 3400 Civic Center Boulevard, Bldg. 421SPE • Philadelphia, PA 19104-5156
215-573-9684 • ghassemi@pennmedicine.upenn.edu

TITLE:

Manufacturing Chimeric Antigen Receptor (CAR) T cells for Adoptive Immunotherapy

AUTHORS AND AFFILIATIONS:

Saba Ghassemi[1,2], Michael C. Milone[1,2]

[1]Center for Cellular Immunotherapies, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

[2]Department of Pathology and Laboratory Medicine, Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA, USA

Corresponding author:

Saba Ghassemi (ghassemi@pennmedicine.upenn.edu)

Email address of co-author:

Michael C. Milone (milone@pennmedicine.upenn.edu)

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SUMMARY:

We describe an approach to reliably generate chimeric antigen receptor (CAR) T cells and test their differentiation and function in vitro and in vivo.

ABSTRACT:

Adoptive immunotherapy holds promise for the treatment of cancer and infectious disease. We describe a simple approach to transduce primary human T cells with chimeric antigen receptor (CAR) and expand their progeny ex vivo. We include assays to measure CAR expression as well as differentiation, proliferative capacity and cytolytic activity. We describe assays to measure effector cytokine production and inflammatory cytokine secretion in CAR T cells. Our approach provides a reliable and comprehensive method to culture CAR T cells for preclinical models of adoptive immunotherapy.

INTRODUCTION:

Chimeric antigen receptors (CARs) provide a promising approach to redirect T cells against distinct tumor antigens. CARs are synthetic receptors that bind an antigen target. While their precise composition is variable, CARs generally contain 3 distinct domains. The extracellular domain directs binding to a target antigen and is typically comprised of a single chain antibody fragment linked to the CAR via an extracellular hinge. The second domain, commonly derived from the CD3ζ chain of the T cell receptor (TCR) complex, promotes T cell activation following CAR engagement. A third costimulatory domain is included to enhance T cell function, engraftment, metabolism, and persistence. The success of CAR T cell therapy in various hematopoietic malignancies including B cell acute lymphoblastic leukemia (ALL), chronic

lymphocytic leukemia (CLL) and multiple myeloma highlights the therapeutic promise of this approach[1-6]. The recent Food and Drug Administration (FDA) approvals for two CD19-specific CAR T cell therapies, tisagenlecleucel for pediatric and young adult ALL and axicabtagene ciloleucel for diffuse large B-cell lymphoma, reinforces the translational merit of CAR T cell Therapy.

CAR T-based approaches involve the isolation of T cells from peripheral blood, activation, genetic modification, and expansion ex vivo. Differentiation is an important parameter regulating CAR T cell efficacy. Accordingly, restricting T cell differentiation during ex vivo culture enhances the ability of the infused product to engraft, expand, and persist, providing long term immunosurveillance following adoptive transfer[2,7-9]. T cells consist of several distinct subsets including: naïve T cells (Tn), central memory (Tcm), effector memory (Tem), effector differentiated (Tte) and stem cell memory (Tscm). Effector differentiated T cells have potent cytolytic ability; however, they are short lived and engraft poorly[10-12]. In contrast, T cells with a less-differentiated phenotype including naïve T cells and Tcm exhibit superior engraftment and proliferative abilities following adoptive cell transfer[13-18]. The composition of the collected T cells in the premanufactured product can vary across patients and correlates with the therapeutic potential of CAR T cells. The proportion of T cells with a naïve-like immunophenotype in the starting apheresis product is highly correlated with both engraftment and clinical response[19].

Culture duration is an important parameter influencing differentiation in CAR T cells prepared for adoptive transfer. We recently developed an approach to generate superior quality CAR T cells using an abbreviated culture paradigm[20]. Using our approach, we showed that limited culture gives rise to CAR T cells with superior effector function and persistence following adoptive transfer in xenograft models of leukemia. Here, we present the approaches to reliably generate CART19 cells (autologous T cells engineered to express anti-CD19 scFv attached to CD3ζ and the 4-1BB signaling domains) and include a detailed description of the assays that provide insight into CAR T bioactivity and efficacy prior to adoptive transfer.

PROTOCOL:

All animal studies are approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

1. T cell activation, transduction, and expansion

1.1. Activate fresh or cryopreserved primary human T cells by mixing with anti CD3/CD28 magnetic beads (e.g., dynabeads) at a ratio of 3 beads per T cell in 6-well cell culture dishes. Culture T cells in X-VIVO 15 medium supplemented with 5% normal human AB serum, 2 mM L-glutamine, 20 mM HEPES, and IL2 (100 units/mL). Maintain T cells at a concentration of 10^6 T cells/mL during expansion. Culture T cells at 37 °C, 20% O_2, and 95% humidity with 5% CO_2.

1.2. After overnight stimulation, add lentiviral supernatant to activated T cells. Calculate the volume of supernatant necessary to achieve a multiplicity of infection (MOI) of 3–5.

NOTE: The CD19-BBζ CAR lentivirus plasmid consists of a CD8 hinge, 4-1BB costimulatory domain, and CD3ζ signaling domain[21]. CD19-BBζ lentiviral supernatant was generated as previously described[21].

1.3. On day 3, collect a representative aliquot of cells for cryopreservation. Prior to cryopreservation, remove the magnetic beads by gentle pipetting and magnetic separation. Prepare freezing medium containing phosphate-buffered saline (PBS) with 0.5% dimethyl sulfoxide (DMSO) and store in 4 °C until use.

1.3.1. Centrifuge T cells at 300 x *g* for 5 min. Discard the supernatant and add 5 mL of PBS. Centrifuge cells at 300 x *g* for 5 min and discard the PBS.

1.3.2. Resuspend the cell pellet in 1 mL of cold cryopreservation medium. Freeze T cells in a chilled freezing container and store at -80 °C for 48 h. Transfer the frozen cells to liquid nitrogen.

1.4. Wash the rest of the T cells once in 5 mL of PBS to eliminate residual vector. Centrifuge at 300 x *g* for 5 min. Decant the PBS and resuspend the cell pellet in T cell culture medium at a concentration of 0.5 x 10^6 cells/mL.

1.5. Split the T cells into two cultures, designated for day 5 and day 9. Count T cells by flow cytometry using counting beads (**Table of Materials**) and monoclonal antibodies to human CD4 and CD8, as well as a viability dye (**Table of Materials**).

1.5.1. To measure T cell concentration, prepare a master mix containing 500 µL of PBS, 5 µL of counting beads, 10 µL of 7-Amino-actinomycin D cell viability solution, 4 µL of CD4-FITC and 4 µL of CD8-APC. Add 40 µL of T cells to the master mix and measure cell concentration by flow cytometry based on number of live T cells/bead counts. Refeed to maintain the cultures at a concentration of 0.5 x 10^6 cells/mL every other day.

1.6. On day 5, count and cryopreserve day 5 cultures as described in steps 1.3 and 1.5.

1.7. On day 7, wash 0.5 x 10^6 T cells in PBS and resuspend in 100 µL of fluorescence activated cell sorting (FACS) buffer. Detect CAR surface protein expression by immunostaining with a fluorescently-conjugated anti-CAR19 idiotype by flow cytometry.

1.8. On day 9, count day 9 cultures and cryopreserve as described in step 1.3.

2. Phenotypic assessment of T cell differentiation

2.1. Prepare a master mix containing pre-titrated antibodies for anti-CD3–BV605 (clone OKT3), anti-CD14–Pacific Blue (PB) (clone HCD14), anti-CD19–PB (clone HIB19), anti-CD4–BV510 (clone OKT4), anti-CD8–H7APC (clone SK1), anti-CCR7–FITC (clone 150503), anti-CD45RO–PE (clone UCHL1), anti-CD27–PE-Cy7 (clone 1A4CD27), anti-CD95–PerCP-Cy5.5 (Clone DX2), and anti-CAR19-APC.

2.2. Prepare individual fluorescence minus one (FMO) controls for anti-CD45RO–PE, anti-CCR7–FITC, anti-CD27–PE-Cy7 and anti-CD95–PerCP-Cy5.5 to distinguish positively stained cells from background.

2.3. Prepare dead cell staining solution by diluting live/dead stock reagent (**Table of Materials**) 1:10,000 in PBS.

2.4. Thaw day 3, day 5, and day 9 T cells that were previously cryopreserved. Centrifuge 1 x 10^6 T cells from each group at 300 x *g* for 3 min. Discard the supernatant. Wash the cells once with PBS. Centrifuge at 300 x *g* for 3 min and discard the PBS.

2.5. Mix T cells with dead staining solution for 15 min at room temperature (RT), protected from the light.

2.6. Add 1 mL of FACS buffer to quench the dead cell staining dye. Centrifuge at 300 x *g* for 3 min, discard the supernatant and resuspend the cell pellet in 100 µL of FACS buffer containing the antibody cocktail described in step 2.1 and 2.2. Incubate for 1 h at 4 °C.

2.7. Add 1 mL of FACS buffer and centrifuge at 300 x *g* for 3 min to wash off unbound antibody. Repeat three times with FACS buffer.

2.8. Resuspend cells in 1% paraformaldehyde (PFA) and store at 4 °C.

2.9. As CD45RO expression decreases after fixation, analyze the samples by flow cytometry after immunostaining. To assess differentiation, gate in the following order: singlets (FSC-H vs FSC-A), live CD3+ T cells (Dump [live-dead violet, CD14-PB and CD19-PB vs CD3-BV605], CD4-BV510 vs CD8-APC-H7). In CD4+ and CD8+ subsets, gate on CD45RO-PE vs CCR7-FITC to define naïve-like T cells (CD45RO-CCR7+), Tcm (CD45RO+CCR7+), Tem (CD45RO+CCR7-), and Tte (CD45RO-CCR7-). To identify Tscm, gate on CD27+ T cells in naïve-like T cells population. In this compartment, Tscm are CD95+ and Tn are CD95-.

3. In vitro functional analysis

3.1. **CAR T cell proliferation and cytokine secretion**

3.1.1. Verify CAR expression as well as cell viability as described in steps 1.5 and 1.7, by flow cytometry.

3.1.2. Wash 5 x 10^6 T cells from each group (day 3, day 5 and day 9) with PBS and resuspend in a 1 µM solution of carboxyfluorescein diacetate succinimidyl ester (CFSE) in PBS for 3.5 min at RT.

3.1.3. Immediately add 10 mL of PBS containing 10% fetal bovine serum (FBS) to quench the reaction.

3.1.4. Centrifuge the solution at 300 x *g* for 3 min. Discard the supernatant and repeat this wash step three times. Count the cells at the conclusion of CFSE staining using a Coulter counter (**Table of Materials**).

3.1.5. Harvest CFSE-stained cells (unstimulated), resuspend in 1% PFA and store at 4 °C for analysis by flow cytometry.

3.1.6. Incubate the desired number of CFSE-stained CAR T cells with irradiated K562-CD19 (target) as well as K562-wild type (control) cells at a ratio of 1:1 for 120 h in cytokine free culture medium and culture conditions as described in step 1.1.

3.1.7. After 24 h, centrifuge the culture vessel at 300 x *g* for 5 min. Collect 120 µL of cellular supernatant. Assess activation-dependent production of IL2, IFNγ, TNFα, GM-CSF, and other inflammatory cytokines (IL1β, IL4, IL5, IL6, IL8, and IL10) by Luminex analyses in accordance with the manufacturer's recommendations.

3.1.8. Replace the volume of supernatant that was collected in step 3.1.7 with an equivalent volume (120 µL) of fresh medium.

3.1.9. On day 3 (i.e., after 96 h), count and re-feed at a concentration of 0.5 x 10^6 cells/mL using bead-based flow cytometry as previously in step 1.5.

3.1.10. On day 5, count the live CD3+ cells and calculate the fold change of live T cells relative to the live T cell count at day 0.

3.1.11. Perform a comprehensive analysis of CFSE dilution in dividing CAR T cells using FlowJo software to highlight successive rounds of cell division.

NOTE: Proliferation assays are well described in the FlowJo manual.

3.2. **Cytotoxicity assay**

NOTE: The ability of CART19 cells to kill target cells expressing CD19 is evaluated using a ^{51}Cr release-assay.

3.2.1. Label the target cells by mixing 5 x 10^5 K562-CD19, K562-wild type control cells, or NALM6 leukemia cells with 50 µL of $Na_2{}^{51}CrO_4$ and 0.5 mL of RPMI supplemented with 10% FBS for 90 min in the incubator at 37 °C.

3.2.2. Centrifuge cells at 300 x *g* for 2.5 min. Discard the radioactive supernatant in appropriate disposal bins and wash the target cells in 5 mL of PBS. Repeat the wash steps twice.

3.2.3. Resuspend the target cells in phenol red-free medium containing 5% FBS. Use this medium for the rest of the procedure to reduce background.

3.2.4. After evaluating CAR expression and cell viability by flow cytometry (as described in section 5.1), mix CAR T cells with labeled target cells at effector:target (E:T) ratios of 10:1, 3:1 and 1:1, in triplicate. Transfer to a 96-well U bottom plate.

3.2.5. In parallel, include target cells alone, and target cells with 1% sodium dodecyl sulfate (SDS), to determine spontaneous (S) and maximum (M) ^{51}Cr release, respectively.

3.2.6. Centrifuge cells at 300 x *g* for 5 min and incubate for 4 h or 20 h in a 37 °C incubator with 5% CO_2.

3.2.7. After the designated time, centrifuge the culture vessel at 300 x *g* for 5 min. Collect 35 µL of cellular supernatant and transfer to a reader plate. Avoid bubbles. Let the plate dry overnight.

3.2.8. Seal the plate with a standard plate seal and count with a liquid scintillation counter. Chromium abundance in the supernatant provides a proxy of target cell killing. Calculate the percentage of specific lysis as follows: 100 x (counts per minute [cpm] experimental release – cpm S release)/ (cpm M release – cpm S release).

4. In vivo functional analysis

4.1. Obtain 6−10-week-old NOD-SCID $\gamma_c^{-/-}$ (NSG) mice, which lack an adaptive immune system, and assign them to treatment/control group randomly.

4.2. Inject animals intravenously via tail vein with 1 x 10^6 NALM6 cells in 0.1 mL sterile PBS.

4.3. After 5−7 days, confirm tumor engraftment by bioluminescence imaging (BLI). Inject 150 mg/kg of D-luciferin to mice which have been anesthetized with isoflurane (volume is dependent on body mass).

4.3.1. Measure bioluminescence values using an imaging system. Quantify total flux using the corresponding software by drawing rectangles of identical area around mice, reaching from head to 50% of the tail length. Subtract the background for each image individually.

4.4. After establishing leukemia, inject 3 x 10^6 day 9 CART19 cells, 0.5 x 10^6 day 3 CAR T cells, or corresponding non-transduced (NTD) human T cells via tail vein in a volume of 100 µL of sterile PBS/Ca^{2+}.

NOTE: As the bioactivity of cells harvested at various intervals during the culture process is different, the chosen concentration of day 3 cells is lower than day 9.

4.5. To determine disease progression, measure the bioluminescence values twice a week as described above in step 4.3.

4.6. To determine CAR T cell engraftment, collect 75 µL blood via retro-orbital bleeding in an EDTA-coated tube. Transfer 50 µL of blood to absolute counting tubes.

4.7. Stain blood with antibodies against CD45, CD4, CD8 and CAR for 30 min at RT. Add 400 µL of 1x FACS lysing solution to the tubes and vortex thoroughly. After staining, analyze surface marker expression by flow cytometry.

NOTE: Other surface markers can be used to assess the differentiation and exhaustion status of the T cells in blood.

4.8. To measure cytokines levels in blood, centrifuge blood at 1200 x *g* for 30 min at 4 °C to separate serum from the upper layer of blood.

4.9. Collect serum and measure the cytokines with a designated reader plate according to the manufacturer's instructions.

REPRESENTATIVE RESULTS:

Using the methods described above, we stimulated and expanded T cells for either 3 or 9 days (**Figure 1A,B**). We also analyzed their differentiation profile, as indicated by the gating strategy outlined in **Figure 1C**, by measuring the abundance of distinct glycoproteins expressed on the cell surface. We show a progressive shift towards effector differentiation over time during ex vivo culture (**Figure 1D**). We assessed the effector function and proliferative capacity of CAR T cell in response to antigen. We show that cells that were expanded less (harvested earlier) were functionally superior compared to the cells extensively cultured over a longer duration. Day 3 CART19 cells have enhanced proliferative and cytolytic ability upon re-stimulation with their cognate ligand relative to day 9 (**Figure 2A,B**).

In a human xenograft moue model of ALL, we compared the potency of CAR T cells harvested at 3 days versus 9 days (**Figure 3A**). We showed a dose dependent anti leukemic response for the CART19 cells generated for 9 days with a complete response for high dose of 3×10^6 and a loss of efficacy for the low dose of 0.5×10^6. The day 3 CART19 cells showed persistent tumor control in both high and low doses of CART19 cells (**Figure 3B**). This response was associated with the absolute count of CART19 in the peripheral blood of mice (**Figure 3C**) which was analyzed based on the protocol described above. These results obtained from our comprehensive assessment of CAR T function provide evidence that that CAR T cells harvested earlier (day 3) outperform CAR T cells harvested on day 9.

FIGURE LEGENDS:

Figure 1: Representative proliferation and differentiation profile of CAR T cells. (**A**) CART19 cell expansion following stimulation with anti-CD3/CD28 magnetic beads. (**B**) Cell size was assessed

by Coulter analysis throughout the culture. (**C**) Representative gating strategy for phenotypic analysis of T cells. (**D**) Temporal analysis of T cell differentiation.

Figure 2: Day 3 CART19 cells display enhanced effector function and proliferation relative to day 9 cells. (**A**) CART19 cells were harvested on day 3 and 9 and co-cultured at the indicated E:T ratio with CD19-expressing K562 cells (K562-19) or wild-type K562 (K562-wild type). Specific cytotoxicity was measured by ^{51}Cr release after 4 h. (**B**) CSFE-labeled CART19 cells were co-cultured with K562-19, K562-wild-type, or medium only for 120 h at a 1:1 E:T ratio. Cells were harvested at indicated timepoints. Absolute counts were assessed by flow cytometry. Relative fold changes of live T cell count normalized to T cell count at day 0 are shown. Data are plotted as mean ± standard deviation (SD). ***P < 0.001 comparing day 3 versus day 9.

Figure 3: Day 3 CART19 cells are more potent in vivo than day 9 cells. (**A**) Schematic of the xenograft model and CART19 cell treatment. Day 3 and 9 CART19 cells or control T cells (UTD) were IV-injected in mice 5−7 days after NALM6 injection. (**B**) Quantification of tumor burden by bioluminescence imaging on day 38 in mice treated with CART19 cells harvested on day 3 and day 9. Symbols represent one mouse each. Horizontal black line: mean of each group. (**C**) Absolute peripheral blood $CD45^+$ T cell counts were measured every two weeks after CART19 cell injection and at the end of the experiment by an appropriate counting method (such as TruCount) Unpaired Mann-Whitney test, two-tailed was used. **P < 0.01, ***P < 0.001, ****P < 0.0001.

DISCUSSION:

Here we describe approaches to measure the function and efficacy of CAR T cells harvested at varying intervals throughout ex vivo culture. Our methods provide comprehensive insight into assays designed to assess proliferative capacity as well as effector function in vitro. We describe how to measure CAR T cell activity following stimulation through the CAR and detail xenograft models of leukemia using CAR T cells harvested at day 3 vs day 9 of their logarithmic expansion phase.

There are inherent challenges in comparing the efficacy of CAR T cells harvested at different time-points during ex vivo expansion. As the quantity of T cells generated over a 3-day culture duration is low, there may be insufficient numbers to perform a comprehensive assessment of function. This is exacerbated in the context of patient T cells whose proliferative ability is often diminished due to extrinsic and intrinsic factors[20].

How many CAR T cells should be infused given that day 3 cells exhibit enhanced metabolic and proliferative ability compared to their day 9 counterparts that are exiting their logarithmic proliferative phase? We estimated what numbers of day 3 CAR T cells would reach 3×10^6 if they were expanded for a further 6 days in culture. We used this estimate to inform how many day 3 CAR T cells should be infused (0.5×10^6) to compare equivalently to day 9. Accordingly, we apply the “stress test” approach to compare the bioactivity, efficacy, and persistence of infused CAR T cells. Decreasing the number of infused CAR T cells from 3×10^6 to 0.5×10^6 reveals differences that would otherwise be masked by saturation of numbers. Mechanistically, tumor control relies

on the accumulation of sufficient effector cells to lyse their corresponding target cells. At high numbers of infusion, both day 3 and day 9 CAR T cells exhibit functional competence.

Another challenge in working with day 3 CAR T cells is their firm attachment to the stimulatory surface. Displacing them from the magnetic beads requires repetitive pipetting to mechanically dissociate them and enhance their recovery from culture. In contrast, day 9 cells have 1) already detached from the beads, and 2) diluted the beads to such an extent that they can be harvested with relative ease.

Another important variable in the CAR T cell manufacturing process is the choice of cell culture medium. RPMI-based medium which has been supplemented with FBS is commonly used for experimental purposes. In contrast, either X-VIVO 15 or OpTmizer, supplemented with human serum are preferred in clinical applications. While these are less characterized, they may contain components that facilitate T cell expansion in a shorter time period. Their impact on differentiation is unknown. Additionally, the addition of cytokines influences growth, survival, and phenotype. While IL-2 drives rapid proliferation and differentiation into effector cells, IL-7 and IL-15, which originate in the lymph node and have known roles in homeostatic persistence, improves expansion of T cells and promote a memory stem/central memory phenotype[22-25].

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Brentjens, R. J. et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Science Translational Medicine.* **5** (177), 177ra138 (2013).
2. Grupp, S. A. et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *The New England Journal of Medicine.* **368** (16), 1509-1518 (2013).
3. Kalos, M. et al. T Cells with Chimeric Antigen Receptors Have Potent Antitumor Effects and Can Establish Memory in Patients with Advanced Leukemia. *Science Translational Medicine.* **3** (95), 95ra73 (2011).
4. Kochenderfer, J. N., Rosenberg, S. A. Treating B-cell cancer with T cells expressing anti-CD19 chimeric antigen receptors. *Nature Reviews. Clinical Oncology.* **10** (5), 267-276 (2013).
5. Maude, S. L. et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *The New England Journal of Medicine.* **371** (16), 1507-1517 (2014).
6. Porter, D. L., Levine, B. L., Kalos, M., Bagg, A., June, C. H. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *The New England Journal of Medicine.* **365** (8), 725-733 (2011).

7. Porter, D. L. et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Science Translational Medicine.* **7** (303), 303ra139 (2015).
8. Maude, S. L., Teachey, D. T., Porter, D. L., Grupp, S. A. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Blood.* **125** (26), 4017-4023 (2015).
9. Kochenderfer, J. N. et al. Lymphoma Remissions Caused by Anti-CD19 Chimeric Antigen Receptor T Cells Are Associated With High Serum Interleukin-15 Levels. *Journal of Clinical Oncology.* **35** (16), 1803-1813 (2017).
10. Bollard, C. M., Rooney, C. M., Heslop, H. E. T-cell therapy in the treatment of post-transplant lymphoproliferative disease. *Nature Reviews. Clinical Oncology.* **9** (9), 510-519 (2012).
11. Brestrich, G. et al. Adoptive T-cell therapy of a lung transplanted patient with severe CMV disease and resistance to antiviral therapy. *American Journal of Transplantation.* **9** (7), 1679-1684 (2009).
12. Savoldo, B. et al. Treatment of solid organ transplant recipients with autologous Epstein Barr virus-specific cytotoxic T lymphocytes (CTLs). *Blood.* **108** (9), 2942-2949 (2006).
13. Berger, C. et al. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *The Journal of Clinical Investigation.* **118** (1), 294-305 (2008).
14. Gattinoni, L. et al. A human memory T cell subset with stem cell-like properties. *Nature Methods.* **17** (10), 1290-1297 (2011).
15. Hinrichs, C. S. et al. Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proceedings of the National Academy of Sciences of the United States of America.* **106** (41), 17469-17474 (2009).
16. Klebanoff, C. A. et al. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proceedings of the National Academy of Sciences of the United States of America.* **102** (27), 9571-9576 (2005).
17. Wang, X. et al. Engraftment of human central memory-derived effector CD8+ T cells in immunodeficient mice. *Blood.* **117** (6), 1888-1898 (2011).
18. Wang, X. et al. Comparison of naive and central memory derived CD8+ effector cell engraftment fitness and function following adoptive transfer. *Oncoimmunology.* **5** (1), e1072671 (2016).
19. Fraietta, J. et al. in *Nature Medicine In press* (2018).
20. Ghassemi, S. et al. Reducing Ex Vivo Culture Improves the Antileukemic Activity of Chimeric Antigen Receptor (CAR) T Cells. *Cancer Immunology Research.* **6** (9), 1100-1109 (2018).
21. Milone, M. C. et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Molecular therapy : the journal of the American Society of Gene Therapy.* **17** (8), 1453-1464 (2009).
22. Cieri, N. et al. IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors. *Blood.* **121** (4), 573-584 (2013).
23. Cui, G. et al. IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8+ T Cell Longevity. *Cell.* **161** (4), 750-761 (2015).
24. Xu, Y. et al. Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. *Blood.* **123** (24), 3750-3759 (2014).

25. Singh, N., Perazzelli, J., Grupp, S. A., Barrett, D. M. Early memory phenotypes drive T cell proliferation in patients with pediatric malignancies. *Science Translational Medicine.* **8** (320), 320ra323 (2016).

Figure

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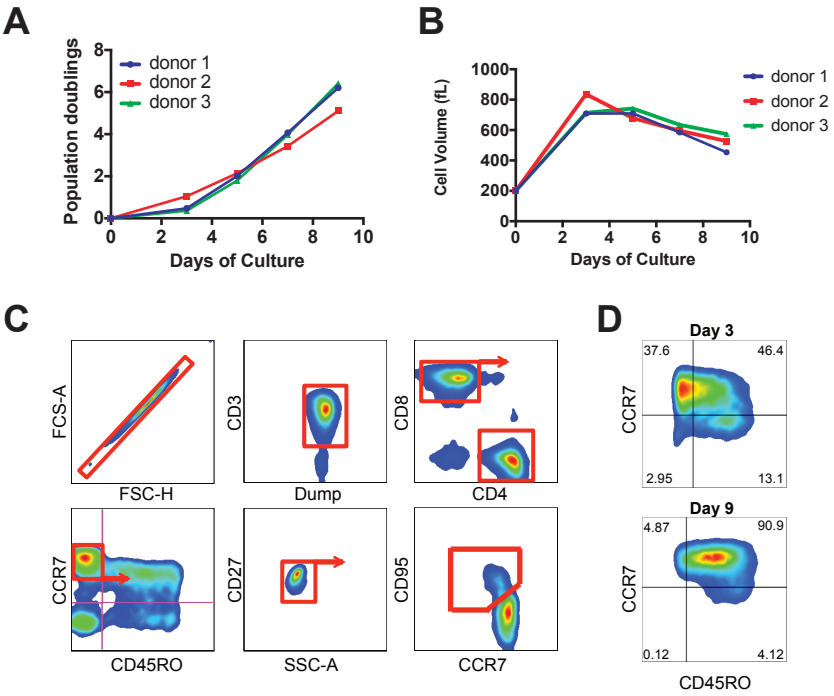

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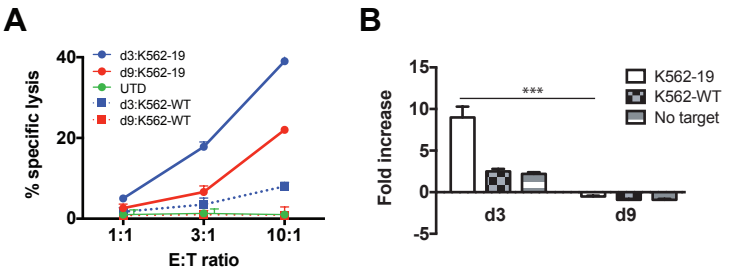
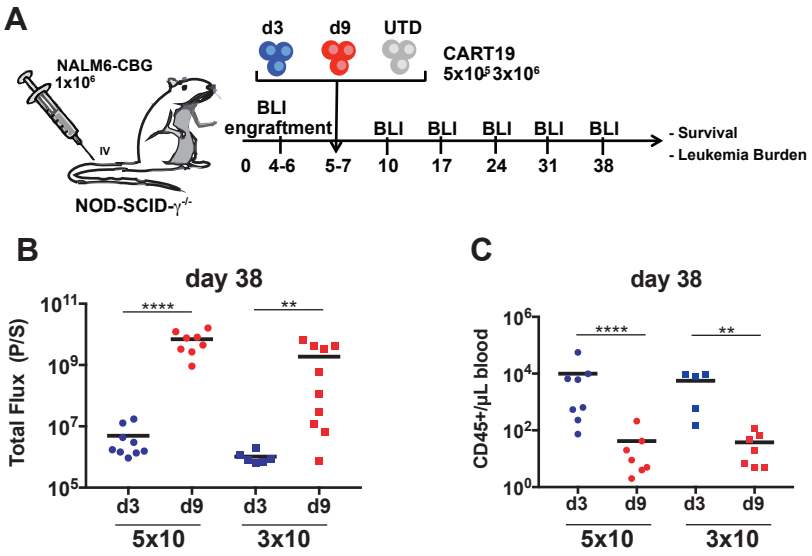


Figure



Name of Material/ Equipment	Company	Catalog Number
Anti CD3/CD28 dynabeads	Thermo Fisher	40203D
APC Mouse Anti-Human CD8	BD Biosciences	555369
APC-H7 Mouse anti-Human CD8 Antibody	BD Biosciences	560179
BD FACS Lysing Solution 10X Concentrate	BD Biosciences	349202
BD Trucount Absolute Counting Tubes	BD Biosciences	340334
Brilliant Violet 510 anti-human CD4 Antibody	BioLegend	317444
Brilliant Violet 605 anti-human CD3 Antibody	BioLegend	317322
CellTrace CFSE Cell Proliferation Kit	Life Technolohgies	C34554
CountBright Absolute Counting Beads,	Invitrogen	C36950
FITC anti-Human CD197 (CCR7) Antibody	BD Pharmingen	561271
FITC Mouse Anti-Human CD4	BD Biosciences	555346
HEPES	Gibco	15630-080
Human AB serum	Valley Biomedical	HP1022
Human IL-2 IS, premium grade	Miltenyi	130-097-744
L-glutamine	Gibco	28030-081
Liquid scintillation counter, MicroBeta trilux	Perkin Elmer	
LIVE/DEAD Fixable Violet	Molecular Probes	L34964
Multisizer Coulter Counter	Beckman Coulter	
$Na_2{}^{51}CrO_4$	Perkin Elmer	NEZ030S001MC
Pacific Blue anti-human CD14 Antibody	BioLegend	325616
Pacific Blue anti-human CD19 Antibody	BioLegend	302223
PE anti-human CD45RO Antibody	BD Biosciences	555493

PE/Cy5 anti-human CD95 (Fas) Antibody	BioLegend	305610
PE/Cy7 anti-human CD27 Antibody	Beckman Coulter	A54823
Phenol red-free medium	Gibco	10373-017
UltraPure SDS Solution, 10%	Invitrogen	15553027
Via-Probe	BD Biosciences	555815
X-VIVO 15	Gibco	04-418Q
XenoLight D-Luciferin - K+ Salt	Perkin Elmer	122799

Comments/Description

RRID:AB_398595

RRID:AB_1645481

RRID:AB_2561866

RRID:AB_2561911

RRID:AB_10561679

RRID:AB_395751

RRID:AB_830689

RRID:AB_395884

RRID:AB_493652

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Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

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Author(s):	Saba Ghassemi, Michael C. Milone

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CORRESPONDING AUTHOR

Name:	Saba Ghassemi
Department:	Pathology and Laboratory Medicine
Institution:	University of Pennsylvania
Title:	Associate Research Scientist

Signature:	Saba Ghassemi	Date:	02/27/2019

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We thank all reviewers for their thorough critiques and constructive suggestions to strengthen this manuscript. Below we provide a point-by-point response to each editor/reviewer comment.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
CAR-T vs CART vs CAR T cells? Please be consistent.

All prose have been proofread and corrected.

2. Please do not abbreviate journal titles in the References.

This has been rectified.

3. Please sort the Materials Table alphabetically by the name of the material. Please remove the TM and © symbols as well.

This has been rectified.

4. Please shorten the title to be more concise.

Title has been modified.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Dynabeads, etc.

This has been rectified in Material Table.

6. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

More details added when necessary.

7. Please provide all volumes and concentrations used throughout.

This has been rectified.

8. Much of the protocol is very generalized. Please provide specific values for specific instructions. We need these details so others can replicate the protocol.

We explained the details so that it is straightforward to replicate the protocol. Please specify any additional changes needed.

9. How was harvesting specifically done?
The word harvest has been replaced with “collected” to provide clarity.

10. What are the flow cytometry parameters used?

This will be specific to each flow cytometer machine and meaningless to add it.

11. Please provide RRIDs for the antibodies. What dilutions are used? What are their concentrations?

RRIDs for the antibodies have been included. All antibodies have to be titrated based on the specific LOT number and cell type. It is a value that must be determined.

12. How many cells are centrifuged?

This information has been added.

13. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

This information has been added.

15. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Reviewer #1:
Manuscript Summary:
Drs. Ghassemi and Milone present a technical manuscript describing their methodology for preparing engineered "CAR-T cells" intended for in vitro and in vivo functional study. The topic will be of interest to others and the manuscript is well written with sufficient detail. There are minor changes that would strengthen the manuscript prior to publication.

General points:
-in the introduction, the authors state that "CARs are synthetic receptors containing 3 distinct modules". This statement is incorrect. As defined, Chimeric antigen receptors are synthetic receptors that bind an antigen target. The precise composition is variable.

Response:
We appreciate the reviewer's comment. We have modified the main text.

-Please highlight that the phenotypic and functional characteristics of individual donors impacts the functionality and polyclonal subsets present at end-expansion. Donor to donor variability is not discussed, and should at least be mentioned, particularly as all the data displayed arises from the use of a single T cell donor.

Response:
We agree with the reviewer regarding the effect of donor to donor variability on the final product. We now included this in the text.
"The composition of the collected T cells in the premanufactured product is variable across patients and correlates with the therapeutic potential of CAR T cells.

-Please indicate the perceived etiology of the non-specific cytotoxicity seen in Figure 2A with the d3-WT and expand upon how this may affect toxicity following adoptive transfer to a human patient. Please indicate at what day the UTD (which may be untransduced control?) was harvested from culture. If d9, is there a d3 control?

Response:
In general, there is a small degree of non-specific cytotoxicity from WT T cells co-cultured with targets cells in all killing assays. The underlying reason is that nutrient-rich culture conditions (including high serum levels) induces a global stimulus triggering T cell activation and signaling. This is exacerbated in day 3 samples given their overall enhanced metabolic activity. UTD in this figure were generated at day 9. However, in years of killing assays, we see no significant differences between day 3 or day 9 (UTD).

-Please discuss the possibility of non-integrants expressed at d3, and how this may impact functionality of cells. Was CAR expression measured on the hCD45+ cells collected from the mice (Figure 3B)?

Response:
Viral-mediated integration in activated T cells occurs within 12-24 hrs.
CAR expression was measured in vitro prior to infusion. This is standard practice.

-Description of representative results indicates strongly that cells harvested with limited expansion are functionally superior. Please include the caveat "using our method" to narrow this statement to expansion under the described circumstances. Other T cell activation and expansion protocols may obtain different results.

Response:
We agree with the reviewer's point the phenotype and function of manufactured products are highly influenced by specific method of ex vivo manufacturing. We included the phrase "using this method" within the text.

Specific to methods:
-Within the description of the protocol, the media used for T cell expansion is not defined,

except on D0. Is the same media maintained throughout? Is IL-2 at the indicated concentration used throughout?

Response:
The medium is consistent throughout T cell activation and expansion unless otherwise noted.100 units/mL IL-2 is used throughout T cell expansion as mentioned in method. We now included this on the T cell activation and expansion section.

-Within "CAR-T proliferation and cytokine secretion", description of re-feeding on day 3 does not include media type and whether cytokine is added at this step.

Response:
This has been modified in the main prose.

-Luminex-based analysis of secreted cytokine is mentioned, but without data presented or a full description of the protocol or instrumentation used. Please remove, or add data and describe how these experiments are typically performed (as this is a methods paper).

Response:
This has been modified in the main prose.

-Cytotoxicity assay should include centrifugation of plate prior to collection of supernatants to transfer to LumaPlate

Response:
This has been modified in the main prose.

-When were the cells harvested from mice displayed in figure 3C? The legend indicates PB analysis every 2 weeks - is this data available to display?

Response:
The figure has been modified to include the date. We only included a representative graph due to space limitations.

Reviewer #2:
Manuscript Summary:
The authors describe methods of generation of CD19 CAR T cells using lentiviral transduction. Some additional methods to assess the function of CD19 CAR T cells in vitro and in vivo are also provided

Major Concerns:
none

Minor Concerns:
1. Perhaps the authors should also discuss the possibility of preserving CAR T expansion/persistence potential by replacing a differentiation-inducing cytokine IL-2 with something milder like IL-15 and IL-21.

Response:
We appreciate the reviewer's comment. This is discussed in the last 4 lines of the discussion.

2. Do CART cells harvested early vs late post transduction survive freeze-thaw differently? Would be interesting to look or discuss, if the authors have data

Response:
A very interesting question which we have tested previously- there is no difference in cryopreservation quality between d3 and d9 cells.

3. Legends would be helpful in Fig. 1A and B

Response:
Legends have been added accordingly.

4. A few typos, should be fixed during copyediting

Response:
We appreciate the reviewer's comment. Typographical errors have been corrected.