Journal of Visualized Experiments

# Using Human Induced Pluripotent Stem Cells for the Generation of Tumor Antigen-Specific T Cells

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE59997R1 |
| **Full Title:** | Using Human Induced Pluripotent Stem Cells for the Generation of Tumor Antigen-Specific T Cells |
| **Keywords:** | Pluripotent stem cell; induced pluripotent stem cell; Immunology; adoptive cell transfer; T cell differentiation; tumor antigen specificity |
| **Corresponding Author:** | R Dr. Vizcardo<br>National Institutes of Health<br>Bethesda, MD UNITED STATES |
| **Corresponding Author's Institution:** | National Institutes of Health |
| **Corresponding Author E-Mail:** | raul.vizcardosakoda@nih.gov |
| **Order of Authors:** | Meghan L Good |
| | R Dr. Vizcardo |
| | Takuya Maeda |
| | Naritaka Tamaoki |
| | Parisa Malekzadeh |
| | Hiroshi Kawamoto |
| | Nicholas P Restifo |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Open Access (US$4,200) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Bethesda, MD 20892, USA |

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

April 23, 2019

Dear Dr. Bajaj,

Thank you for sending us the editorial comments along with the reviewers' comments for our manuscript JoVE59997. Please find enclosed our revised version entitled: "Generation of Tumor Antigen-Specific T Cells from Human Induced Pluripotent Stem Cells", which we would like to submit for publication as a video produced by JoVE.

As requested, all comments are addressed in a separate document and the corrections are included in the updated manuscript.

Please address all correspondence to:

Raul Vizcardo, Ph.D.
National Institutes of Health
National Cancer Institute
10 Center Drive
Building 10-CRC, Rm 3-5700
Bethesda, MD 20892
Email address: vizcardo@nih.gov
Tel: (240) 760-7638

We look forward to hearing from you at your earliest convenience.

Yours sincerely,

**TITLE:**

# Using Human Induced Pluripotent Stem Cells for the Generation of Tumor Antigen-Specific T Cells

**AUTHORS AND AFFILIATIONS:**

Meghan L. Good[1,2,*], Raul Vizcardo[1,2,*], Takuya Maeda[1,2], Naritaka Tamaoki[1,2], Parisa Malekzadeh[1], Hiroshi Kawamoto[3], Nicholas P. Restifo[1,2]

[1]Surgery Branch, National Cancer Institute, NIH, Bethesda, MD, USA
[2]Center for Cell-Based Therapy, National Cancer Institute, NIH, Bethesda, MD, USA
[3]Laboratory of Immunology, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan

[*]These authors equally contributed to this work.

**Corresponding Author:**

Raul Vizcardo (vizcardo@nih.gov)

**Email Addresses of Co-authors:**

Meghan L. Good (meghan.good@nih.gov)
Takuya Maeda (takuya.maeda@nih.gov)
Naritaka Tamaoki (naritaka.tamaoki@nih.gov)
Parisa Malekzadeh (parisa.malekzadeh@nih.gov)
Hiroshi Kawamoto (kawamoto@infront.kyoto-u.ac.jp)
Nicholas P. Restifo (restifon@mail.nih.gov)

**KEYWORDS:**

pluripotent stem cell, induced pluripotent stem cell, immunology, adoptive cell transfer, T cell differentiation, tumor antigen specificity

**SUMMARY:**

This article describes a method to generate functional tumor antigen-specific induced pluripotent stem cell-derived CD8αβ$^+$ single positive T cells using OP9/DLL1 co-culture system.

**ABSTRACT:**

The generation and expansion of functional T cells in vitro can lead to a broad range of clinical applications. One such use is for the treatment of patients with advanced cancer. Adoptive T cell transfer (ACT) of highly enriched tumor antigen-specific T cells has been shown to cause durable regression of metastatic cancer in some patients. However, during expansion, these cells may become exhausted or senescent, limiting their effector function and persistence in vivo. Induced pluripotent stem cell (iPSC) technology may overcome these obstacles by leading to in vitro generation of large numbers of less differentiated tumor antigen-specific T cells. Human iPSC (hiPSC) have the capacity to differentiate into any type of somatic cell, including lymphocytes, which retain the original T cell receptor (TCR) genomic rearrangement when a T

cell is used as a starting cell. Therefore, reprogramming of human tumor antigen-specific T cells to hiPSC followed by redifferentiation to T cell lineage has the potential to produce rejuvenated tumor antigen-specific T cells. Described here is a method for generating tumor antigen-specific CD8αβ$^+$ single positive (SP) T cells from hiPSC using OP9/DLL1 co-culture system. This method is a powerful tool for in vitro T cell lineage generation and will facilitate the development of in vitro derived T cells for use in regenerative medicine and cell-based therapies.

**INTRODUCTION:**

In addition to physiological advantages, T cells have many potential therapeutic applications. The generation and expansion of T cells in vitro can be used for disease modeling and therapeutic validation as well as a source of treatment for hereditary and acquired immunodeficiency states (i.e., viral immunodeficiencies and lymphodepletion secondary to chemotherapy or transplantation) and for the eradication of cancer. This latter quality has led to the development of adoptive T cell transfer (ACT) for the treatment of patients with advanced cancer[1].

ACT consists of resecting a patient's tumor, extracting tumor-infiltrating lymphocytes (TILs), expanding TILs ex vivo, then reinfusing the expanded cells into the patient[2]. It has been shown to be an effective treatment modality for some patients with metastatic cancer. Unfortunately, not all patients respond to this therapy. Previous reports have shown that the differentiation state of transferred cells[3-9], use of large numbers of highly enriched cancer antigen-specific T cells[10], and persistence of T cells after transfer[11,12] are all correlated with more durable responses[13,14]. Therefore, when ACT fails to elicit an anti-tumor response, it may in part be due to a low yield of cancer antigen-specific T cells, inefficient ex vivo expansion leading to the exhaustion and loss of reactive clones, or lack of persistence after transfer[4]. It has been postulated that these obstacles may be overcome by the generation of large numbers of less differentiated cancer antigen-specific T cells in vitro[15,16].

Hematopoietic stem/progenitor cells (HSPCs) are a conventional source for in vitro T cell generation, although this method is limited by the small number of cells able to be recovered from a single donor[1]. Embryonic stem cells (ESCs) have also been shown to produce T cells but with low yield[17], making it inefficient for clinical applications. Furthermore, since T lineage cells experience stochastic genetic recombination of their T cell receptors (TCRs) in early developmental stages, it is not possible to use HSPCs or ESCs to generate a pure population of antigen-specific T cells without further genomic modifications like TCR gene transduction.

One approach to overcome these caveats is to reprogram TILs to human induced pluripotent stem cells (hiPSCs), which may provide a limitless source for in vitro T cell generation. It has been shown that cancer antigen-specific TILs can be reprogrammed into hiPSCs and re-differentiated to T cell lineage, which retains the same T cell receptor (TCR) gene rearrangement as the original T cell[18,19]. This detail is important for ACT because individual patient tumors have unique mutational profiles, and very few cancer antigens have been shown to be shared among patients[20]. Therefore, using cancer antigen-specific TILs as a source for in

vitro generation of hiPSC-derived T cells may provide a new strategy for the personalized treatment of patients with metastatic cancer.

Presented here in detail is a protocol for differentiating hiPSC-derived T lineage cells into functional antigen-specific CD8αβ$^+$ single positive (SP) T cells using OP9/DLL1 co-culture system. This method is a powerful tool for in vitro T cell differentiation of hiPSCs, hematopoietic progenitors, and embryonic stem cells, as well as their further applications in regenerative medicine and cell-based therapies.

**PROTOCOL:**

**1. Culturing human iPSCs (hiPSCs) on mouse embryonic fibroblasts (MEF)**

NOTE: Alternative methods for culturing hiPSCs can also be used, including but not limited to: seeding onto a 6 well plate pre-coated with gelatin, a gelatinous protein mixture, recombinant laminin 511, or any other extracellular matrix used in hiPSC expansion, and cultured using defined media specially formulated for human pluripotent stem cell culture.

1.1. Culturing MEF

1.1.1. Coat a 10 cm cell culture Petri dish with 4 mL of 0.1% gelatin and incubate for 30 min at 37 °C.

1.1.2. Thaw a vial of 4 x $10^6$ irradiated MEF quickly into 10 mL of 37 °C MEF media (DMEM + 10% FBS + 1x penicillin-streptomycin + 1x L-glutamine supplement). Centrifuge at 300 *x g* for 5 min at 4 °C. Aspirate the supernatant and resuspend the cell pellet in 9 mL of MEF media.

1.1.3. Remove the gelatin-coated dish from the incubator. Aspirate gelatin and add 7 mL of MEF media. Plate 3 mL of MEF suspension (from step 1.1.2) onto the gelatin-coated dish. Rock the dish side-to-side and front-to-back to ensure even distribution of MEF over the dish. Incubate at 37 °C for 8–36 h.

1.2. Passaging hiPSC on MEF

NOTE: The data was generated using MART-1 iPSC derived from long-term cultured melanoma TIL, which specifically recognize MART-1 peptide in the context of HLA-A*02:01, as previously described[18].

1.2.1. Passage hiPSCs when colonies are between 0.8 – 1.2 mm in diameter. Prior to passaging, check hiPSC colonies in a stereo-microscope and remove any areas of differentiation from the culture using the plastic edge of a 200 µL tip.

1.2.2. Aspirate spent media and add 10 mL hiPSC media (human ES culture media [**Table of Materials**] + 10 ng/mL human basic fibroblast growth factor [hbFGF]) supplemented with 10 µM ROCK inhibitor.

1.2.3. Hold the cell culture dish in one hand and roll a disposable cell passaging tool across the entire dish in one direction. Apply enough pressure so that the entire roller blade touches the culture dish and maintain a uniform pressure during rolling action.

1.2.4. Rotate the culture dish 90° and repeat step 1.2.3. View the plate in the microscope to visually confirm proper cutting of the colonies, which should appear checkered. Detach cut colonies by gentle mechanical flushing using a 200 µL pipette.

NOTE: Detachment of cut colonies by mechanical flushing must be done immediately after cutting colonies with the roller, because after 3 min the cut colonies will start to reattach to the dish, and it will become difficult to detach colonies of homogeneous size by flushing.

1.2.5. Transfer 350 – 600 clumps of cut colonies onto a new 10 cm dish of MEF (plated 8 – 36 h prior to hiPSC passaging) with 10 mL of fresh hiPSC media supplemented with 10 µM ROCK inhibitor. Incubate at 37 °C.

NOTE: 600 clumps represents approximately 1.0 x $10^6$ MART-1 iPSC and will yield 0.5–1.0 x $10^6$ DP cells on day 35. However, expected numbers will vary depending on the potency of the starting cell line and culture conditions.

1.2.6. The following day, aspirate spent media and add 10 mL of fresh hiPSC media. Change hiPSC media every 1–2 days depending on hiPSC growth rate.

**2. Preparation of OP9/DLL1 cells for co-culture with hiPSCs**

2.1. Culture OP9/DLL1 cells in OP9 media [α-minimum essential medium (α-MEM) + 20% fetal bovine serum (FBS) + 1x penicillin-streptomycin] at 37 °C. When OP9/DLL1 cells reach confluency, aspirate media and wash once with 5 mL of 1x magnesium, calcium, and phenol red-free phosphate buffered saline (PBS).

2.2. Aspirate PBS and add 2 mL of 0.05% Trypsin-EDTA. Incubate for 5 min at 37 °C. Then, add 4 mL of OP9 media and mechanically dissociate the cell layer by pipetting to make a single-cell suspension.

2.3. Transfer the cell suspension into a 50 mL conical tube through a 100 µm cell strainer to avoid cell clumps. Centrifuge at 300 *x g* for 5 min at 4 °C. Aspirate the supernatant and resuspend in 12 mL of OP9 media.

2.4. Add 8 mL of OP9 media to each of six new 10 cm cell culture Petri dishes. Plate 2 mL of OP9/DLL1 cell suspension from step 2.3 onto each new 10 cm dish. Rock the dish side-to-side then front-to-back to ensure even distribution of OP9/DLL1 over the dish.

2.5. Incubate at 37 °C. Repeat passage every 2 – 3 days when cells reach confluency.

NOTE: It is important to make enough frozen stock of OP9/DLL1 cells and thaw a new stock every 4–6 weeks.

**3. In vitro differentiation of hiPSCs into CD8αβ$^+$ single positive (SP) T cells**

3.1. Prepare gelatinized OP9/DLL1 dishes one week prior to co-culture with hiPSCs. To prepare 0.1% gelatin solution, add 5 mL of room temperature (RT) tissue-grade stock gelatin solution to 500 mL of PBS.

3.1.1. Coat 3 new 10 cm cell culture Petri dishes by adding 4 mL per dish of 0.1% gelatin. Incubate 30 min at 37 °C.

3.1.2. Aspirate gelatin and add 8 mL of OP9 media to each dish. Passage one confluent dish of OP9/DLL1 (as done in section 2 above) to three gelatin pre-coated dishes.

3.2. After 4 days, add 10 mL of OP9 media to each 10 cm dish of OP9/DLL1 on gelatin, for a total of 20 mL of media per dish.

3.3. After 7 – 8 days, begin the hiPSC co-culture on OP9/DLL1 confluent dishes (differentiation day 0).

3.3.1. Aspirate spent media from confluent 10 cm dish of hiPSCs on MEF. Add 10 mL of OP9 media. Cut and detach hiPSC colonies using a disposable cell passaging tool as done in steps 1.2.3 and 1.2.4.

3.3.2. Transfer 350 – 600 clumps of cut colonies onto a 10 cm pre-gelatinized OP9/DLL1 dish (step 3.1) with 10 mL of fresh OP9 media using a 200 µL pipette. Rock the culture dish side-to-side then front-to-back to ensure even distribution of colonies.

NOTE: Alternatively, pre-formed hiPSC embryoid bodies (EBs) or small clump suspension may be used. However, the use of a disposable cell passaging tool or EB formation system is preferred to produce hiPSC clumps of uniform size.

3.4. On day 1, aspirate spent media and replace with 20 mL of fresh OP9 media. hiPSC clumps co-cultured on OP9/DLL1 for 1 day will appear as small round monolayer colonies (**Figure 1**).

3.5. On day 5, aspirate 10 mL of spent media and add 10 mL of fresh OP9 media. hiPSC colonies will begin to differentiate into primitive mesoderm, which is characterized by a multilayered dark center.

3.6. On day 9, aspirate 10 mL of spent media and add 10 mL of fresh OP9 media. At this point, multilayered center structures will evolve into dome-like shapes, and a peripheral network-like area will start to become evident.

3.7. On day 13, harvest hematopoietic progenitor cells (HPCs) (**Figure 1)**. hiPSC-derived structures on day 13 are characterized by a dark central organoid surrounded by a network of dome-like areas, representative of hematopoietic zones (HZs) previously reported to enclose human embryonic stem cell-derived hematopoietic progenitors[21].

NOTE: The presence of the dome-like structures indicates a successful process even in the absence of dark centers. The inability to produce HPCs may be due to poor quality of OP9/DLL1, quality of FBS lot, confluency of iPSC clumps seeded onto OP9/DLL1 (350–600 clumps is optimal), and/or variations in potency of iPSC lines to produce hematopoietic precursors.

3.7.1. Aspirate spent media and wash 1x with 5 mL of 1x phenol red-free Hanks' balanced salt solution modified with calcium and magnesium (HBSS).

3.7.2. Aspirate HBSS and add 250 µL of 5000 Units/mL collagenase IV in 10 mL of HBSS. Incubate at 37 °C for 45 min. Aspirate HBSS with collagenase IV and wash once with 5 mL of PBS.

3.7.3. Aspirate PBS and add 5 mL of 0.25% Trypsin-EDTA. Incubate at 37 °C for 20 min. Then, add 4 mL of OP9 media and dissociate the cell layer by pipetting to make a single-cell suspension.

3.7.4. Transfer the cell suspension into a 50 mL conical tube through a 100 µm cell strainer. Centrifuge at 300 x *g* for 5 min at 4 °C. Aspirate the supernatant and resuspend in 10 mL of OP9 media.

3.7.5. Plate cell suspension onto a new gelatinized 10 cm cell-culture Petri dish (see steps 3.1.1 and 3.1.2). Incubate at 37 °C for 45 min. Then, collect non-adherent cells by gentle pipetting.

3.7.6. Transfer collected cell suspension into a 50 mL conical tube through a 100 µm cell strainer. Centrifuge at 300 x *g* for 5 min at 4 °C. Aspirate the supernatant and resuspend in 10 mL of differentiation media [OP9 media with 5 ng/mL human stem cell factor (hSCF), 5 ng/mL human Flt3 ligand (hFLT3L), and 5 ng/mL human interleukin 7 (hIL-7)].

3.7.7. Plate the cell suspension onto a new 10 cm OP9/DLL1 confluent dish.

3.8. On day 16, passage the cells.

3.8.1. Mechanically detach non-adherent cells by gentle pipetting and filter through a 100 µm cell strainer. Centrifuge at 300 *x g* for 5 min at 4 °C. Aspirate the supernatant and resuspend in 10 mL differentiation media.

3.8.2. Plate the cell suspension onto a new 10 cm OP9/DLL1 confluent dish.

3.9. Continue passaging non-adherent cells every 5–7 days thereafter by repeating step 3.8.

3.10. On day 35, enrich $CD4^+CD8^+$ double positive (DP) population and stimulate to produce $CD8\alpha\beta^+$ SP T cells (**Figure 2**).

3.10.1. Mechanically detach non-adherent cells by gentle pipetting and filter through a 100 µm cell strainer to remove cell clumps. Enrich $CD4^+$ cell population by CD4 magnetic bead isolation according to the manufacturer's protocol.

NOTE: The rationale for using CD4 magnetic beads is to remove $CD4^-CD8^-$ DN cells from the culture, as these have been demonstrated to cause direct killing of $CD4^+CD8^+$ DP cells after stimulation[22].

3.10.2. Count live CD4 enriched cells using a Neubauer hemocytometer and Trypan blue dye. Suspend in OP9 media at total concentration 0.5 x $10^6$ cells/mL. Aliquot 1 mL of the cell suspension (0.5 x $10^6$ cells) into each well of a tissue culture flat bottom 24 well plate of confluent OP9/DLL1.

3.10.3. Add 100 IU human interleukin 2 (hIL-2), 5 ng/mL hIL-7, 500 ng/mL anti-human CD3 antibody, and 2 µg/mL anti-human CD28 antibody, then culture at 37 °C.

3.10.4. On day 4 – 7 after stimulation, collect cells for molecular analysis (**Figure 3**) or co-culture with peptide-pulsed antigen presenting cells (APCs).

**4. Measuring antigen specificity of hiPSC derived $CD8\alpha\beta^+$ SP T cells**

NOTE: The type of APCs to be used for this experiment is dependent on the MHC restriction of hiPSC-derived T cells. Here, T2 cell line is used, which is a hybrid of T and B lymphoblastoid cell lines. T2 cells express HLA-A*02:01[23], which is recognized by JKF6 cells from which MART1-iPSC was derived[18]. This T2 cell line can be expanded in RPMI 1640 + 20% FBS + 1x penicillin-streptomycin and is passaged when cells reach a density of 5 x $10^5$ cells/mL.

4.1. Count live HLA-A*02:01$^+$ T-B hybrid lymphoblastoid T2 cells using a Neubauer hemocytometer and Trypan blue dye. Incubate APCs in 24 well tissue culture plate with 1 µg/mL MART-1 peptide for 2 h at 37 °C.

NOTE: Optimal peptide concentration is variable, depending on cell line and antigen specificity.

4.2. Collect APCs and wash 2x with 10 mL of PBS to remove any extra peptide.

4.3. Count APCs and suspend at 2 – 5 x $10^5$ cells/mL in OP9 media with 100 IU IL-2 and 5 ng/mL IL-7. Aliquot 100 µL of cell suspension (2-5 x $10^4$ cells) into each well of an ultra-low attachment U bottom 96 well plate or directly into a pre-coated ELISpot plate.

4.4. Sort hiPSC-derived CD8αβ$^+$ SP T cells (1 week after anti-human CD3/CD28 antibody stimulation) using a cell sorter and suspend at 1 x $10^6$ cells/mL in OP9 media with 100 IU IL-2 and 5 ng/mL IL-7. Aliquot 100 µL of cell suspension (1 x $10^5$ cells) into each well of APCs and culture for 16 – 20 h at 37 °C.

4.5. After 16 – 20 h, analyze the cytokine secretion profile by ELISpot assay per the manufacturer's protocol (**Figure 4)**.

**REPRESENTATIVE RESULTS:**

After 13 days hiPSCs co-culturing with OP9/DLL1, CD34$^+$CD43$^+$ hematopoietic progenitor cells appeared (**Figure 1**). After an additional 22 days of culture on non-gelatinized OP9/DLL1 in the presence of hSCF, hFLT3L, and hIL-7, hematopoietic progenitors differentiated into CD3$^+$CD7$^+$CD4$^+$CD8$^+$ double-positive (DP) T lineage cells, the majority of which expressed TCR specific to the MART-1 epitope (tetramer) (**Figure 2**).

It has previously been shown that CD8$^+$ SP T cells can be induced from CD4$^+$CD8$^+$ DP T cells by TCR signaling via agonist peptide or antibody-driven TCR stimulation[24,25]. Therefore, on day 35 of culture, hiPSC-derived CD4$^+$CD8$^+$ DP T cells were stimulated with anti-human CD3 and anti-human CD28 antibodies in the presence of hIL-7 and hIL-2. Four days after stimulation, the number of CD3$^+$CD8αβ$^+$ SP cells increased dramatically and remained specific for the MART-1 epitope, confirming the preservation of their inherited antigen-specificity (**Figure 3**).

To determine the functional properties of hiPSC-derived CD8αβ$^+$ SP T cells, antigen-dependent activation and secretion of interferon gamma (IFN-γ) was analyzed. After stimulation with anti-human CD3 and anti-human CD28 antibodies for 1 week, hiPSC-derived CD8αβ$^+$ SP T cells were isolated using a cell sorter and co-cultured with T2 cell line expressing HLA-A*02:01 with or without cognate MART1 peptide for 16 – 20 h. The ELISpot assay revealed that hiPSC-derived CD8αβ$^+$ SP T cells secrete higher amounts of IFN-γ compared with CD8αα$^+$ SP T cells, when cultured in the presence of MART-1 peptide. IFN-γ expression was null for T cells and APCs alone, demonstrating that human T-iPSC-derived T cells are antigen-specific and functional (**Figure 4**).

**FIGURE LEGENDS:**

**Figure 1: Generation of hiPSC-derived hematopoietic progenitor cells.** (**A**) Schematic overview of the differentiation of hiPSCs to hematopoietic lineage using OP9/DLL1 co-culture. (**B**)

Appearance of hiPSC-derived structures on days 1 (top left), 3 (top right), 7 (bottom left), and 13 (bottom right). Scale bars = 100 µm. (**C**) Flow cytometric analysis of hiPSC derived $CD34^+CD43^+$ hematopoietic progenitor cells on day 13. Data are representative of six independent experiments (n = 1 to 2).

**Figure 2: hiPSC differentiation into $Mart1^+$ $CD4^+CD8\alpha\beta^+$ DP T cells**. (**A**) Schematic overview of the differentiation of hiPSC-derived hematopoietic lineage to immature T cells using OP9/DLL1 co-culture. (**B**) Flow cytometric analysis of CD4 vs. CD8α, CD3 vs. CD8β, and MART-1 tetramer expression in hiPSC-derived T cells on day 35. Gated on lymphocytes, single cells, PI negative. Data are representative of three independent experiments (n = 3 – 8).

**Figure 3: Induction of $CD8\alpha\beta^+$ SP T cell phenotype**. Flow cytometric analysis of $CD4^-$ hiPSC-derived T cells 4 days after human anti-CD3 and human anti-CD28 antibody-driven stimulation. Gated on lymphocytes, single cells, PI negative (n = 4).

**Figure 4: Antigen specificity of hiPSC-derived $CD8\alpha\beta^+$ SP T cells.** IFN-γ secretion by ELISpot assay of hiPSC-derived $CD8\alpha\beta^+$ SP, $CD8\alpha\alpha^+$ SP, and bulk T cells after 20 h co-culture with or without T2 cells pulsed (or not) with MART-1 peptide.

**DISCUSSION:**

The co-culture of OP9 murine stromal cells is a well-established system for in vitro generation of lymphocytes (i.e., NK, B, and T cells) from HSPCs and pluripotent stem cells. Notch signaling is required to induce T lineage commitment and can be accomplished by the ectopic expression of Notch ligand DLL1 or DLL4, which have comparable efficacy for T cell generation[1]. Therefore, the OP9/DLL1 co-culture system has become a widely used method for producing T cells in vitro. Furthermore, this method is applicable for use with several types and sources of human cells, including cord blood, bone marrow HSPCs and ESCs. However, the generation of T cells from these sources is limited either by insufficient retrieval of source cells or by inefficient differentiation to T cells[1]. Additionally, a T cell product with a single TCR recombination cannot be generated from these open repertoire sources. By using regenerative medicine techniques, namely induced pluripotent stem cell (iPSC) technology, it may be possible to produce massive numbers of antigen-specific T cells for use in cell-based therapeutics[15].

hiPSCs are similar to pluripotent ESCs in their capacity for self-renewal, limitless expansion, and potential to differentiate to any type of somatic cell in the body; however, they lack the ethical concerns surrounding the use of products of embryonic origin for clinical applications. Moreover, hiPSCs can be produced from any somatic cell, allowing the development of cell products for personalized medicine. In previous reports, hiPSCs have been produced from human T cells using whole peripheral mononuclear cells, $CD3^+$ cells, or isolated cytotoxic T lymphocytes (CTLs) as a source[18,19,22,26]. When hiPSCs are generated from a T cell source (T-iPSCs), the original TCR gene rearrangement is inherited. Therefore, patient T-iPSC derived T cells may provide a model for personalized ACT treatment by targeting a patient's distinct cancer antigens.

The differentiation of human pluripotent stem cells into T lineage cells is divided into two steps: the generation of hematopoietic progenitor cells (HPCs)[27] and their further differentiation into T lineage cells[21]. Both steps can be accomplished using the OP9/DLL1 co-culture system. Importantly, the quality of OP9/DLL1 feeder cells is critical to the success of T cell differentiation. Since OP9/DLL1 cells are not an immortalized homogenous cell line, the quality of the FBS and culture conditions are critical to maintaining their expansion without losing the ability to support hiPSC differentiation. Therefore, it is recommended to pre-evaluate the lot of FBS and passage consistently when cell-to-cell cytoplasmic contact begins to occur, in order to prevent cell differentiation and senescence. One point to take into consideration is that cell-to-cell contact can appear indistinguishable from the background depending on the phase contrast and magnification of the microscope. In our experience, most OP9/DLL1 dishes will appear to be 80% confluent when ready to passage.

It has been shown that the redifferentiated T lineage cells generated from T-iPSCs by OP9/DLL1 co-culture can produce $CD8^+$ SP T cells upon stimulation[18,19]. However, regenerated $CD8^+$ SP T cells acquire the innate-like CD8αα homodimer[22,28], which is an ineffective co-receptor for TCR signaling[29]. Additionally, these regenerated $CD8^+$ SP T cells have shown strong TCR-independent cytotoxicity, making these cells unfavorable for clinical use[30]. This protocol describes a recent method involving stimulation of purified $CD4^+CD8^+$ DP cells to generate $CD8\alpha\beta^+$ SP T cells with a more conventional phenotype and improved antigen-specific cytotoxicity[22]. Although the loss of antigen specificity due to secondary TCRα allelic rearrangement occurs in the DP stage after prolonged long-term culture, this can be overcome by genome editing in T-iPSCs[31]. In our experience, hiPSC-derived DP cells start to appear on day 30 – 35 of culture, and these newly generated DP cells have not yet undergone secondary TCRα rearrangement. Therefore, most DP cells on day 35 retain antigen specificity and can be used to generate antigen-specific $CD8\alpha\beta^+$ SP T cells.

Prior to human anti-CD3 and anti-CD28 stimulation on day 35, $CD4^-CD8^-$ DN cells must be removed from the culture, as these have been demonstrated to cause direct killing of $CD4^+CD8^+$ DP cells after stimulation[22]. Using CD4 magnetic bead enrichment (step 3.10) will enrich for both DP and $CD4^+CD8^-$ intermediate single positive (ISP) cells[1], which we have been shown to have no negative effects[22]. Alternatively, fluorescence-activated cell sorting by flow cytometry can be performed to isolate DP cells. However, magnetic bead separation is preferred as it avoids the mechanical stress induced by flow cytometry.

The generation of $CD8\alpha\beta^+$ SP T cells from human pluripotent stem cells without activation-mediated agonist selection has subsequently been demonstrated by the use of 3D murine stromal cell culture[32]. However, physiological positive selection is dependent on the interaction of TCR with self-peptide-MHC complexes, which are uniquely processed and presented by thymic cortical epithelial cells[33]. Furthermore, TCR affinity for selection peptides has been shown to determine the subsequent functional capabilities of mature $CD8\alpha\beta^+$ SP T cells[34]. Currently, there is no evidence to suggest that a Notch stromal cell-based co-culture system can

provide the defined selection peptide and MHC complex required for physiological positive selection.

It has been previously reported in a murine model that T lineage cells generated from tumor antigen-specific T cell-derived hiPSCs using OP9/DLL1 alone fail to experience conventional maturation. However, iPSC-derived immature T cells generated by the OP9/DLL1 system can mature into naïve-like T cells by further physiological thymic education in a 3D culture system [28,35]. Therefore, the protocol presented here to produce iPSC-derived immature T cells generated by the OP9/DLL1 system is vital for further attempts to generate real human tumor antigen-specific post-thymic T cells capable of long-term persistence in vivo with efficiency to treat established vascularized tumors .

**ACKNOWLEDGMENTS:**

We thank Alan B. Hoofring and Erina H. He for graphical assistance. This research was supported by the Intramural Research Program of the National Cancer Institute (ZIA BC010763) and the Intramural NCI Cancer Moonshot Initiative for Cell-based Cancer Immunotherapy.

**DISCLOSURES:**

The authors have no disclosures.

**REFERENCES:**

1 Brauer, P. M., Singh, J., Xhiku, S., Zuniga-Pflucker, J. C. T Cell Genesis: In Vitro Veritas Est? *Trends in Immunology.* **37** (12), 889-901, doi:10.1016/j.it.2016.09.008 (2016).

2 Rosenberg, S. A., Restifo, N. P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science.* **348** (6230), 62-68, doi:10.1126/science.aaa4967 (2015).

3 Gattinoni, L. et al. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *Journal of Clinical Investigation.* **115** (6), 1616-1626, doi:10.1172/JCI24480 (2005).

4 Rosenberg, S. A. et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clinical Cancer Research.* **17** (13), 4550-4557, doi:10.1158/1078-0432.CCR-11-0116 (2011).

5 Crompton, J. G. et al. Lineage relationship of CD8(+) T cell subsets is revealed by progressive changes in the epigenetic landscape. *Cellular and Molecular Immunology.* **13** (4), 502-513, doi:10.1038/cmi.2015.32 (2016).

6 Henning, A. N., Klebanoff, C. A., Restifo, N. P. Silencing stemness in T cell differentiation. *Science.* **359** (6372), 163-164, doi:10.1126/science.aar5541 (2018).

7 Henning, A. N., Roychoudhuri, R., Restifo, N. P. Epigenetic control of CD8(+) T cell differentiation. *Nature Reviews Immunology.* **18** (5), 340-356, doi:10.1038/nri.2017.146 (2018).

8 Vodnala, S. K. et al. T cell stemness and dysfunction in tumors are triggered by a common mechanism. *Science.* **363** (6434), doi:10.1126/science.aau0135 (2019).

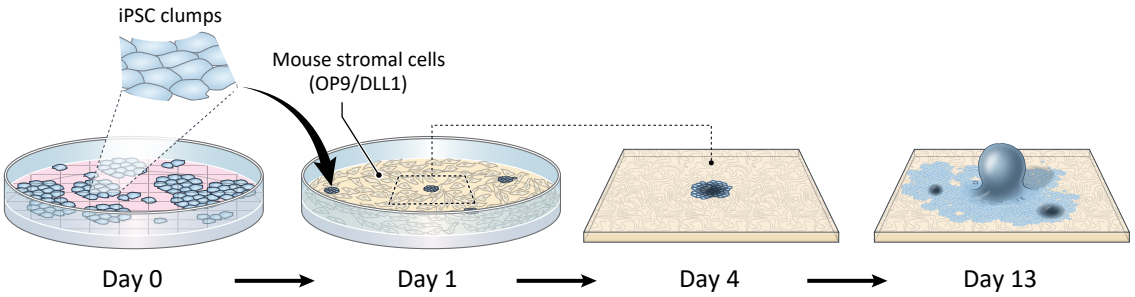
9 Restifo, N. P., Gattinoni, L. Lineage relationship of effector and memory T cells. *Current Opinion in Immunology.* **25** (5), 556-563, doi:10.1016/j.coi.2013.09.003 (2013).

10 Tran, E. et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science.* **344** (6184), 641-645, doi:10.1126/science.1251102 (2014).
11 Gattinoni, L. et al. Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nature Medicine.* **15** (7), 808-813, doi:10.1038/nm.1982 (2009).
12 Gautam, S. et al. The transcription factor c-Myb regulates CD8(+) T cell stemness and antitumor immunity. *Nature Immunology.* **20** (3), 337-349, doi:10.1038/s41590-018-0311-z (2019).
13 Klebanoff, C. A. et al. Determinants of successful CD8+ T-cell adoptive immunotherapy for large established tumors in mice. *Clinical Cancer Research.* **17** (16), 5343-5352, doi:10.1158/1078-0432.CCR-11-0503 (2011).
14 Klebanoff, C. A., Gattinoni, L., Restifo, N. P. Sorting through subsets: which T-cell populations mediate highly effective adoptive immunotherapy? *Journal of Immunotherapy.* **35** (9), 651-660, doi:10.1097/CJI.0b013e31827806e6 (2012).
15 Crompton, J. G., Clever, D., Vizcardo, R., Rao, M., Restifo, N. P. Reprogramming antitumor immunity. *Trends in Immunology.* **35** (4), 178-185, doi:10.1016/j.it.2014.02.003 (2014).
16 Crompton, J. G., Rao, M., Restifo, N. P. Memoirs of a reincarnated T cell. *Cell Stem Cell.* **12** (1), 6-8, doi:10.1016/j.stem.2012.12.009 (2013).
17 Kennedy, M. et al. T lymphocyte potential marks the emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures. *Cell Reports.* **2** (6), 1722-1735, doi:10.1016/j.celrep.2012.11.003 (2012).
18 Vizcardo, R. et al. Regeneration of human tumor antigen-specific T cells from iPSCs derived from mature CD8(+) T cells. *Cell Stem Cell.* **12** (1), 31-36, doi:10.1016/j.stem.2012.12.006 (2013).
19 Nishimura, T. et al. Generation of rejuvenated antigen-specific T cells by reprogramming to pluripotency and redifferentiation. *Cell Stem Cell.* **12** (1), 114-126, doi:10.1016/j.stem.2012.11.002 (2013).
20 Lo, W. et al. Immunologic recognition of a shared p53 mutated neoantigen in a patient with metastatic colorectal cancer. *Cancer Immunology Research.* doi:10.1158/2326-6066.CIR-18-0686 (2019).
21 Timmermans, F. et al. Generation of T cells from human embryonic stem cell-derived hematopoietic zones. *Journal of Immunology.* **182** (11), 6879-6888, doi:10.4049/jimmunol.0803670 (2009).
22 Maeda, T. et al. Regeneration of CD8alphabeta T Cells from T-cell-Derived iPSC Imparts Potent Tumor Antigen-Specific Cytotoxicity. *Cancer Research.* **76** (23), 6839-6850, doi:10.1158/0008-5472.CAN-16-1149 (2016).
23 Salter, R. D., Howell, D. N., Cresswell, P. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics.* **21** (3), 235-246 (1985).
24 Snauwaert, S. et al. In vitro generation of mature, naive antigen-specific CD8(+) T cells with a single T-cell receptor by agonist selection. *Leukemia.* **28** (4), 830-841, doi:10.1038/leu.2013.285 (2014).

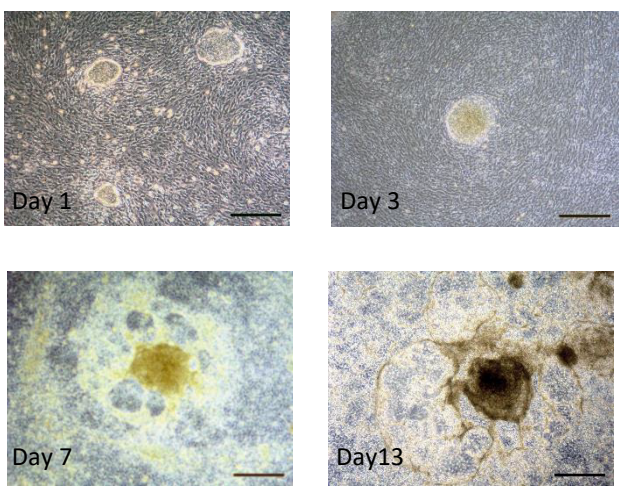
25 Takahama, Y., Suzuki, H., Katz, K. S., Grusby, M. J., Singer, A. Positive selection of CD4+ T cells by TCR ligation without aggregation even in the absence of MHC. *Nature.* **371** (6492), 67-70, doi:10.1038/371067a0 (1994).
26 Seki, T. et al. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell.* **7** (1), 11-14, doi:10.1016/j.stem.2010.06.003 (2010).
27 Vodyanik, M. A., Slukvin, II. Hematoendothelial differentiation of human embryonic stem cells. *Current Protocols in Cell Biology.* **Chapter 23,** Unit 23, 26, doi:10.1002/0471143030.cb2306s36 (2007).
28 Vizcardo, R. et al. Generation of Tumor Antigen-Specific iPSC-Derived Thymic Emigrants Using a 3D Thymic Culture System. *Cell Reports.* **22** (12), 3175-3190, doi:10.1016/j.celrep.2018.02.087 (2018).
29 McNicol, A. M. et al. CD8alpha/alpha homodimers fail to function as co-receptor for a CD8-dependent TCR. *European Journal of Immunology.* **37** (6), 1634-1641, doi:10.1002/eji.200636900 (2007).
30 Themeli, M., Riviere, I., Sadelain, M. New cell sources for T cell engineering and adoptive immunotherapy. *Cell Stem Cell.* **16** (4), 357-366, doi:10.1016/j.stem.2015.03.011 (2015).
31 Minagawa, A. et al. Enhancing T Cell Receptor Stability in Rejuvenated iPSC-Derived T Cells Improves Their Use in Cancer Immunotherapy. *Cell Stem Cell.* **23** (6), 850-858 e854, doi:10.1016/j.stem.2018.10.005 (2018).
32 Montel-Hagen, A. et al. Organoid-Induced Differentiation of Conventional T Cells from Human Pluripotent Stem Cells. *Cell Stem Cell.* **24** (3), 376-389 e378, doi:10.1016/j.stem.2018.12.011 (2019).
33 Takada, K., Kondo, K., Takahama, Y. Generation of Peptides That Promote Positive Selection in the Thymus. *Journal of Immunology.* **198** (6), 2215-2222, doi:10.4049/jimmunol.1601862 (2017).
34 Takada, K. et al. TCR affinity for thymoproteasome-dependent positively selecting peptides conditions antigen responsiveness in CD8(+) T cells. *Nature Immunology.* **16** (10), 1069-1076, doi:10.1038/ni.3237 (2015).
35 Vizcardo, R. et al. A Three-dimensional Thymic Culture System to Generate Murine Induced Pluripotent Stem Cell-derived Tumor Antigen-specific Thymic Emigrants. *JoVE*. <https://www.jove.com/video/58672/a-three-dimensional-thymic-culture-system-to-generate-murine-induced>.

# Figure 1

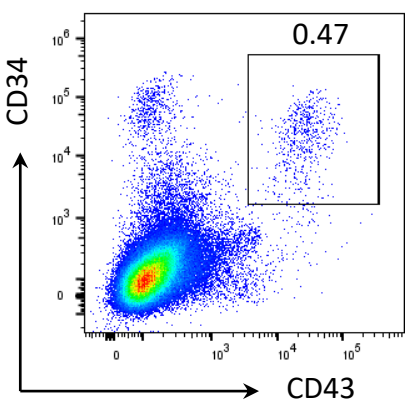
A




B

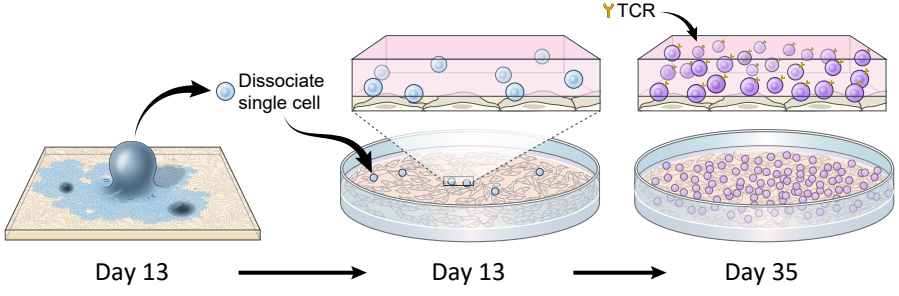



C

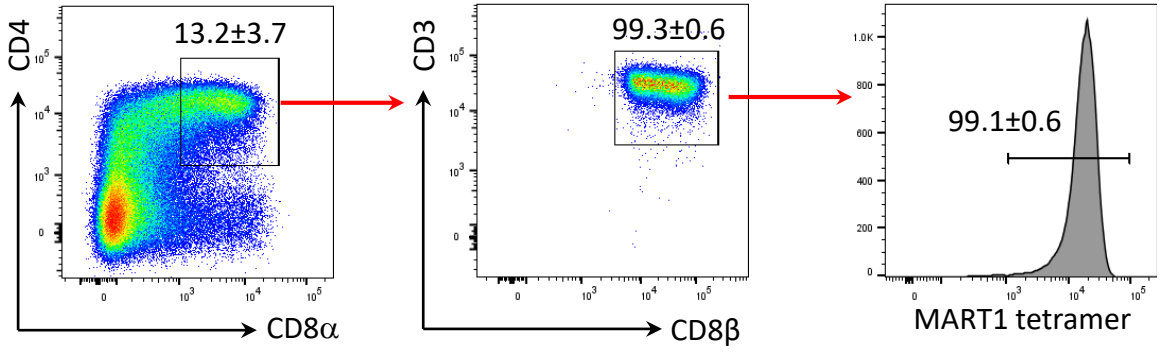


# Figure 2

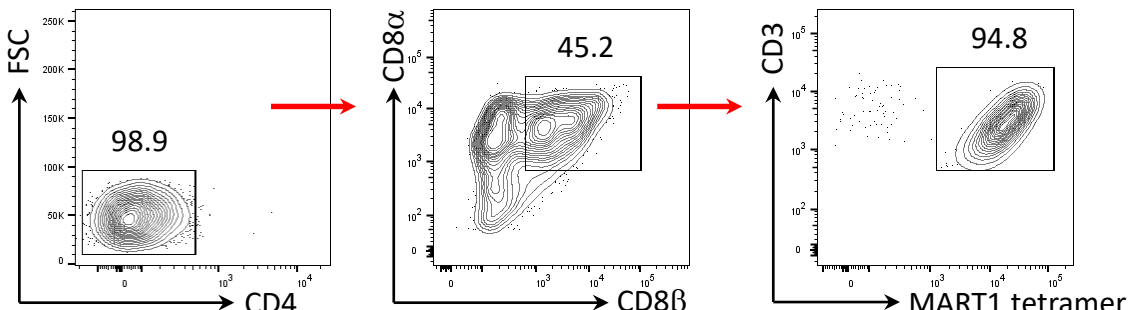
A




B



# Figure 3



# Figure 4

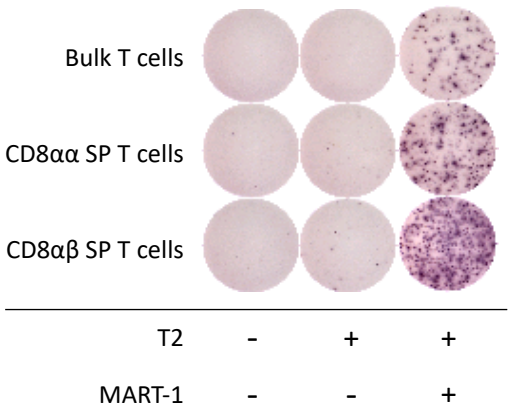


Table of Materials

Click here to access/download;Table of Materials;Copy of JoVE_Materials (F).xlsx

| Name of Material/ Equipment | Company |
|---|---|
| 10 cm dish | Corning, Inc. |
| Anti-CD3, human | BD Biosciences |
| Anti-CD34, human | BD Biosciences |
| Anti-CD4, human | Biolegend |
| Anti-CD43, human | BD Biosciences |
| Anti-CD7, human | BD Biosciences |
| Anti-CD8a, human | BD Biosciences |
| Anti-CD8b, human | BD Biosciences |
| Anti-TCRb, human | BD Biosciences |
| CD28 human monoclonal antibody (15E8), pure functional grade | Miltenyl Biotec |
| CD3 human monoclonal antibody (OKT | Miltenyl Biotec |
| CD4 Microbeads, human | Miltenyl Biotec |
| Cell strainer 100 um | Fisher Scientific |
| Fetal Bovine Serum (FBS) | Gemini |
| Flt-3 ligand | R&D Systems |
| Gelatin Solution 2% | SIGMA-Aldritch |
| GlutaMAX (100X) | Thermo Fisher Scientific |
| HBSS Mg+Ca+ Phenol-Red Free | Gibco |
| Interleukin-2 | R&D Systems |
| Interleukin-7 | R&D Systems |
| iTAG MHC Tetramer HLA-A*0201 Mart1 Tetramer -ELAGIGILTV | MBL |

| | |
|---|---|
| Mart1-hiPSC | Vizcardo et al., Cell Stem Cell 2013 |
| Melan-A, MART 1 (26-35) | InnoPep |
| MEM Non-Essential Amino Acids Solution | Gibco |
| αMEM powder | Gibco |
| Mouse Embryonic Fibroblasts (MEF) | Thermo Fisher Scientific |
| OP9/N-DLL1 | Riken Bioresource center |
| Penicillin/streptomycin | Thermo Fisher Scientific |
| Phosphate buffered saline pH 7.4 (1x) | Thermo Fisher Scientific |
| Primate ES Cell Medium | Reprocell |
| Rhok inhibitor (Y-27632 dihydrochloride) | Tocris |
| RPMI 1640 | Gibco |
| Stem Cell Factor (SCF) | R&D Systems |
| StemPro | EZPassage |
| T2-tumor | ATCC |
| Trypsin-EDTA (0.05%), phenol red | Thermo Fisher Scientific |
| Trypsin-EDTA (0.25%), phenol red | Thermo Fisher Scientific |
| U Bottom 96 well plate | Corning, Inc. |

| Catalog Number |
| --- |
| 353003 |
| Cat# 561812, RRID:AB_1089628 |
| Cat# 348791, RRID:AB_400381 |
| Cat# 344612, RRID:AB_2028479 |
| Cat# 560198, RRID:AB_1645460 |
| Cat# 555361, RRID:AB_395764 |
| Cat# 555369, RRID:AB_398595 |
| Cat# 641057, RRID:AB_1645747 |
| Cat# 555548, RRID:AB_395932 |
| 130-093-375 |
| 130-093-387 |
| 130-045-101 |
| 22-363-549 |
| 100-500 |
| 427-FL |
| G1393-100ML |
| 35050-061 |
| 14025-092 |
| 202-IL |
| 407-ML |
| Cat#TB-0009-2 |

| |
|---|
| RIKEN-IMS |
| 3146-0100 |
| 11140050 |
| 61100061 |
| C57BL/6 MEF MITC-TREATED 4M EACH; A34962 |
| Cat# RCB2927; RRID:CVCL_B220 |
| 15140-122 |
| 10010-023 |
| RCHEMD001 |
| 1254 |
| 11875093 |
| 455-MC |
| 23181-010 |
| T2 (174 x CEM.T2) (ATCC® CRL-1992™) |
| 25300-062 |
| 25200-072 |
| 3799 |

| | Comments/Description |
| --- | --- |
| L-Glutamine supplement | |

| | |
|---|---|
| OP9/DLL1 | |
| Human ESC Culture Media | |

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT

| | |
|---|---|
| Title of Article: | Generation of Tumor Antigen-Specific T Cells from Human Induced Pluripotent Stem Cells |
| Author(s): | Meghan L. Good, Raul Vizcardo, Takuya Maeda, Naritaka Tamaoki, Parisa Malekzadeh, Hiroshi Kawamoto, Nicholas P. Restifo |

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☐ The Author is **NOT** a United States government employee.

☑ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

## ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

**CORRESPONDING AUTHOR**

| | |
|---|---|
| Name: | Raul Vizcardo |
| Department: | National Cancer Institute - Surgery Branch |
| Institution: | National Institutes of Health |
| Title: | Research Fellow |
| Signature: | |
| Date: | March 14th, 2019 |

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

**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.
done

2. 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. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Matrigel, StemFit, GlutaMAX, Reprocell, etc.
done

3. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
done

4. After you have made all the recommended changes to your protocol, 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.
done

5. 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. Notes cannot usually be filmed and should be excluded from the highlighting.
done

6. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
done

7. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.
Limitations have been added to the discussion. Critical steps, modifications, and have been addressed in the protocol itself, as we feel this is most useful for the reader.

8. References: Please do not abbreviate journal titles.
Formatted for JoVE style.

9. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.
done

**Reviewers' comments:**

**Reviewer #1:**
Manuscript Summary:
The manuscript by Good et al. (Jove59997) describes a protocol to use iPSC technology and generate a potentially "rejuvenated" antigen-specific population of T cells from patient-derived TIL. The protocol is clear and concise.

Major Concerns:
none

Minor Concerns:
Authors should consider adding the following:
In critical places, a more explicit description of what needs to be observed to move the protocol forward. As a couple of examples: in section 2.5, is passage tied to a confluency state? In 3.7, harvest of progenitors, is there a quality attribute from the visual inspection that would indicate a failed process?

Thank you for the advice.
2.5: Yes, passage is tied to confluency state, which has been added to section 2.5 and is detailed in the following NOTE.
3.7: Visual inspection on day 13 can give some indication to the successful production of hematopoietic precursors. The presence of well-organized 3D structures with dark centers and surrounding dome-like structures (hematopoietic zones) are indicative of hematopoietic precursor cell (HPC) production (Timmermans et al., 2009). In our experience, it is the presence of the dome-like structures which indicates a successful process even in the absence of dark centers. The inability to produce HPCs may be due to poor quality of OP9/DLL1, the quality of FBS lot, confluency of iPSC clumps seeded onto OP9/DLL1 (350 – 600 clumps is optimal), and/or variations in potency of iPSC lines to produce hematopoietic precursors. The text has been modified to include these details.

1.2.1: Authors should include references or provide information about the derivation of the hiPSC

MART-1 iPSC were previously derived from long-term cultured TIL which specifically recognize a complex of the MART-1 peptide (Vizcardo et al., 2013). This has been added to the text.

2.2 Is Trypsin EDTA prewarmed?

In our experience, it is not necessary to prewarm Trypsin EDTA.

4.1: critical details about the peptide loading process, or reference to a publication is needed

This has been added to the text.

Figures 2 and 3: Are these data generated using TIL derived iPSC? If so, why is the entirety of the iPSC-derived population specific for MART-1?

These data were generated using MART-1 iPSC, which were derived from JKF6 cells as previously described by Vizcardo et al. JKF6 cells, which are long-term cultured TIL from a melanoma patient, specifically recognize MART-1 peptide in the context of HLA-A*02:01 (Vizcardo et al., 2013). As

published, MART-1 specific TCRab recombination was inherited in this cell line. In our experience, hiPSC-derived DP cells start to appear in day 30-35. Sequential physiological secondary TCRa rearrangement occurs in DP thymocytes until these cells either undergo successful positive selection or cell death occurs (Huang and Kanagawa, 2001). However, these newly generated MART-1 iPSC-derived DP cells in day 30-35 have not yet undergone secondary TCRα rearrangement. Therefore, at this time we still observe high tetramer specificity (Figure 2). After prolonged culture, the specificity decreases as shown by Minagawa et. al (Minagawa et al., 2018). The text has been updated to include this information.

**Reviewer #2:**

Manuscript Summary:

The paper by Good et al. describes protocol for generation of rejuvenated T cells from reprogrammed melanoma antigen-specific T cells. This protocol provides all details related to T cell differentiation and materials and equipment required for the procedure. The protocol and rationale are well-written.

Major Concerns:

1. 3.10 proposes enrichment of CD8+CD4+ double positive population using MACS sorting with CD4 antibody. However, rationale for this strategy is unclear. Fig.2B shows large number of CD4+CD8- cells in the differentiation cultures. It is expected that CD4 sorting picks up CD4+CD8- cells along with CD4+CD8+ cells. Authors should explain why CD4+ selection preferentially enriches CD4+CD8+ cells and add figure demonstrating that this enrichment strategy is working or not.

Thank you for this important point, which is now included in the protocol. The rationale for using CD4 magnetic bead enrichment is to remove CD4-CD8- DN cells from the culture, as these have been demonstrated to cause direct killing of CD4+CD8+ DP cells after stimulation (Maeda et al., 2016). It is correct to expect that this strategy will pick up immature CD4+ single positive (ISP) cells, characterized by CD4+CD8-, along with DP cells. It is known that during human T cell development, DN cells will first gain CD4 to become ISP and subsequently gain CD8 to become DP cells (Brauer et al., 2016). Using CD4 magnetic beads will enrich for both DP and ISP cells, which we have shown to have no negative effects (Maeda et al., 2016). Furthermore, although flow cytometric-based sorting can be performed to isolate DP cells, magnetic bead separation avoids the mechanical stress induced by such purification.

2. It would be helpful to explain what are CD4+CD8- cells? Single positive T cells or macrophages?

CD4+CD8- cells are ISP, an intermediate T cell lineage between DN and DP state as described above. ISP will subsequently gain CD8 to become CD4+CD8+ DP and then undergo positive and negative selection before reaching the CD4 or CD8 SP stage (Brauer et al., 2016). Since we are not using lymphoid markers such as CD45, there is a possibility of inclusion of other lineages that express CD4. However, there are no reports to suggest that non-lymphoid CD4+ cells negatively affect *in vitro* T cell differentiation.

3. Anticipated results are reasonable and useful for readers. However, additional information regarding expected numbers of total rejuvenated T cells produced in this system from 1 million of T-iPSCs should be provided. It will help readers to set up appropriate number of differentiation cultures to get desired quantities of T cells for functional analysis.

We agree with this constructive comment and have revised our protocol to include this information. In our experience, 600 clumps of cut colonies (see step 1.2.4) represents approximately 1.0 x $10^6$ MART-1 iPSC and after differentiation will yield 0.5 - 1.0 x $10^6$ DP cells on day 35. However, expected numbers

will vary depending on the potency of the starting cell line and culture conditions.

Minor Concerns:
1. "Culturing human iPSC" part should describe iPSCs that can be used for this protocol and provide appropriate reference, i.e. iPSC generated through reprogramming of tumor-specific T cells described in …?

In this protocol, we use MART-1 iPSC which were previously derived from long-term cultured TIL that specifically recognize a complex of the MART-1 peptide (Vizcardo et al., 2013). This method can also be employed for any human pluripotent stem cells (Maeda et al., 2016; Vizcardo et al., 2013). The text has been updated to reflect this point.

2. Abstract should add "human" to iPSCs definition.

Thank you, this has been added.

**Reviewer #3:**
Manuscript Summary:
The paper by Good et. Al. demonstrates the method of differentiation of iPS cells into CD8+ T cells that resemble physiological conventional CD8+ T cells in vitro by using of murine feeder cell line, that might be useful for adoptive immunotherapy. They showed in vitro IFN-g production potential of iPS-derived CD8+ T cells in recognizing the cognate cancer antigens. The authors explains that the resulting human regenerated T cells in the method represent equivalent to functionally premature T cells, as they previously showed that it needed thymus-like 3D organoid for final maturation to functionally mature T cells.

Major Concerns:
Recent paper reported by Minagawa et al.(Cell Stem Cell 2018) demonstrated elevation of RAG1 and RAG2 gene during in vitro maturation process of iPSC to CD8ab T cells, especially at CD4 and CD8ab double positive (DP) stage, that resulted in an additional TRA gene rearrangement-related antigen specificity loss by using of CDR3 sequencing in multiple T-iPSC lines. In the manuscript by Good et al., however, almost 100% of DP cells were positive for MART1 tetramer (Fig 2).It would be necessary to explain how the method by Good et al. is robust about RAG gene suppression or antigen-specificity by mentioning their result from multiple T-iPS cell line. Actually it is known that some tetramer have centricity to TCRa- or TCRb-chain for binding to peptide-HLA complex, which may cause maintenance of antigen-specificity in some TCRb-centric tetramers and loss of antigen-specificity in some TCRa-centric tetramers, even after additional TRA gene rearrangement at DP stage.

We thank the reviewer for this constructive criticism and the text has been modified accordingly. Please see the above response to Reviewer #1. In our experience, MART-1 tetramer specificity of DP cells decreases expectedly after prolonged culture (Minagawa et al., 2018), indicating that the high tetramer binding in day 35 is not due to centricity.

Minor Concerns:
About introduction and discussion, it would be better to cite recent publications about iPSC-derived T cells, such as
Minagawa A. et al. Cell Stem Cell 2018; about TCR stability of human iPS-T

Montel-Hagen A. et al Cell Stem Cell 2019; about artificial thymic organoid-based differentiation of human iPS-T

We agree with this comment and have included the above citations in the updated manuscript.

Brauer, P.M., Singh, J., Xhiku, S., and Zuniga-Pflucker, J.C. (2016). T Cell Genesis: In Vitro Veritas Est? Trends Immunol *37*, 889-901.
Huang, C., and Kanagawa, O. (2001). Ordered and coordinated rearrangement of the TCR alpha locus: role of secondary rearrangement in thymic selection. J Immunol *166*, 2597-2601.
Maeda, T., Nagano, S., Ichise, H., Kataoka, K., Yamada, D., Ogawa, S., Koseki, H., Kitawaki, T., Kadowaki, N., Takaori-Kondo, A*., et al.* (2016). Regeneration of CD8alphabeta T Cells from T-cell-Derived iPSC Imparts Potent Tumor Antigen-Specific Cytotoxicity. Cancer Res *76*, 6839-6850.
Minagawa, A., Yoshikawa, T., Yasukawa, M., Hotta, A., Kunitomo, M., Iriguchi, S., Takiguchi, M., Kassai, Y., Imai, E., Yasui, Y*., et al.* (2018). Enhancing T Cell Receptor Stability in Rejuvenated iPSC-Derived T Cells Improves Their Use in Cancer Immunotherapy. Cell Stem Cell *23*, 850-858 e854.
Timmermans, F., Velghe, I., Vanwalleghem, L., De Smedt, M., Van Coppernolle, S., Taghon, T., Moore, H.D., Leclercq, G., Langerak, A.W., Kerre, T*., et al.* (2009). Generation of T cells from human embryonic stem cell-derived hematopoietic zones. J Immunol *182*, 6879-6888.
Vizcardo, R., Masuda, K., Yamada, D., Ikawa, T., Shimizu, K., Fujii, S., Koseki, H., and Kawamoto, H. (2013). Regeneration of human tumor antigen-specific T cells from iPSCs derived from mature CD8(+) T cells. Cell Stem Cell *12*, 31-36.