Adoptive transfer of CD8+ T cells generated from induced pluripotent stem cells triggers regressions of large tumors along with immunological memory

Hidehito Saito[1], **Keisuke Okita**[2], **Alfred E. Chang**[1], and **Fumito Ito**[1,3,4,*]

[1]Department of Surgery, University of Michigan, Ann Arbor, Michigan, USA

[2]Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan

[3]Department of Surgical Oncology, Roswell Park Cancer Institute, Buffalo, New York, USA

[4]Center for Immunotherapy, Roswell Park Cancer Institute, Buffalo, New York, USA

Abstract

Current approaches to adoptive T cell therapy are limited by the difficulty of obtaining sufficient numbers of T cells against targeted antigens with useful *in vivo* characteristics. Theoretically, this limitation could be overcome by using induced pluripotent stem cells (iPSCs) that could provide an unlimited source of autologous T cells. However, the therapeutic efficacy of iPSC-derived regenerated T cells remains to be demonstrated. Here we report the first successful reprogramming of T-cell receptor (TCR) transgenic CD8+ T cells into pluripotency. As part of the work, we established a syngeneic mouse model for evaluating *in vitro* and *in vivo* antitumor reactivity of regenerated T cells from iPSCs bearing a rearranged TCR of known antigen specificity. Stably TCR retained T cell-derived iPSCs differentiated into CD4+CD8+ T cells that expressed CD3 and the desired TCR *in vitro*. Stimulation of iPSC-derived CD4+CD8+ T cells with the cognate antigen in the presence of IL-7 and IL-15 followed by expansion with IL-2, IL-7 and IL-15 generated large numbers of less-differentiated CD8+ T cells with antigen-specific potent cytokine production and cytolytic capacity. Furthermore, adoptively transferred iPSC-derived CD8+ T cells escaped immune rejection, mediated effective regression of large tumors, improved survival, and established antigen-specific immunological memory. Our findings illustrate the translational potential of iPSCs to provide an unlimited number of phenotypically defined, functional, and expandable autologous antigen-specific T cells with the characteristics needed to enable *in vivo* effectiveness.

Keywords

stem cells; reprogramming; T cells; melanoma; immunotherapy

Introduction

Adoptive transfer of *ex vivo*-expanded autologous tumor-infiltrating lymphocytes (TILs) or peripheral blood T cells genetically modified to express a T-cell receptor (TCR) or a chimeric antigen receptor (CAR) specific for an antigen expressed on tumor cells can induce durable clinical responses in significant numbers of patients with various cancers (1–3). Much progress has been made over the past decade in defining the subsets of T cells mediating effective tumor regression following adoptive T cell therapy (1,4–6). Preclinical data have demonstrated that infusion of CD62L^{high} less-differentiated T cells results in great expansion and persistence of T cells (7) and tumor destruction (4–6) compared with CD62L^{low} more-differentiated T cells. Clinical data have shown that long-term engraftment and persistence of infused T cells have been highly associated with objective tumor responses (8,9). However, TILs harvested for adoptive cell therapy are effector T cells that are functionally impaired and exhausted (10,11), and further stimulation of TILs results in terminally differentiated cells that have poor survival, and reduced proliferative capacity and antitumor efficacy.

This limitation of adoptive T cell therapy can be overcome by using induced pluripotent stem cells (iPSCs) that self-renew, maintain pluripotency (12–14), and can provide an unlimited source of autologous T cells for immunotherapy. Remarkable advances made in reprogramming technology over the past few years have facilitated the generation of human iPSCs from differentiated cells such as T cells (15–18). These advances now allow us to develop strategies for the use of reprogramming technology for autologous cell therapy in the future. Recent studies have shown that mature human T cells can be reprogrammed into iPSCs that can redifferentiate into functional T lymphocytes *in vitro* (19–22). These iPSC-derived T cells exhibit TCR gene rearrangement patterns identical to the parental T cells and harbor antigen-specific effector functions *in vitro* (19–21). Moreover, T-cell derived iPSCs genetically engineered to express a CAR differentiate to CAR-expressing T cells that display antitumor immunity in a xenograft model of lymphoma (22). These studies suggest that iPSC-derived regenerated T cells can be potentially utilized for cancer immunotherapy. However, issues including whether or not T cell-derived iPSCs can generate less-differentiated T-cell subsets that can escape immune rejection, mediate effective regression of large established tumors through their endogenous TCRs, and establish immunological memory are not known due to the lack of syngeneic mouse models.

Here, we reprogrammed TCR transgenic CD8^{+} T cells into iPSCs, and established a preclinical model to determine therapeutic efficacy of iPSC-derived regenerated T cells in a mouse model for melanoma. Our studies demonstrated that adoptive transfer of less-differentiated iPSC-derived regenerated CD8^{+} T cells mediated potent *in vivo* antitumor reactivity, and established antigen-specific immunological memory.

Materials and Methods

Mice

C57BL/6 mice and NOD/SCID mice were purchased from Harlan Laboratories and the Jackson Laboratories, respectively. Pmel-1 TCR-transgenic mice (B6.Cg Thy1[a]-

Tg(TcraTcrb)8Rest/J) were purchased from the Jackson Laboratories, and were bred in-house. All mice were 7 to 10 weeks old at the beginning of each experiment, and were housed in the Unit for Laboratory Animal Medicine at the University of Michigan in compliance with the Institutional Animal Care and Use Committee regulations.

Cell lines

The mouse embryonic stem cell (mESC) line R1 and MC38 murine colon adenocarcinoma cell line were gifts from Drs. Sue O'Shea and Weiping Zou (University of Michigan), respectively. SNL and B16F10 cells were obtained from Cell Biolabs, Inc and ATCC, respectively. OP9 and OP9-DL1 cells were kindly provided by Dr. Juan Carlos Zúñiga-Pflücker (University of Toronto, Toronto, Canada). Cells were authenticated by morphology, phenotype and growth, and routinely screened for *Mycoplasma*, and were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Generation of mouse iPSCs from Pmel-1 TCR transgenic CD8^{+} T cells

Pmel-1 CD8^{+} T cells were isolated from a single cell suspension of splenocytes using anti-CD8 beads and MACS columns (Miltenyi Biotec). The sorted 1×10^5 Pmel-1 CD8^{+} T cells were pulsed with 10μM of human (h)gp100$_{25\text{-}33}$ peptide, KVPRNQDWL (GenScript) in the presence of mitomycin-C treated 5×10^4 splenocytes from B6 mice in T-cell reprogramming medium (23). After 24 hours of culture, the solution which contained SeV vectors that individually carried each of Oct3/4, Sox2, Klf4 and c-Myc was added to wells at multiplicities of infection (MOI) 20. After 24 hours of infection, the cells were collected and transferred to a 10-cm dish that contained mitomycin C-inactivated SNL feeder cells in iPSC medium. The iPSC medium was changed every other day until the colonies were picked.

Generation of T cells from iPSCs *in vitro*

Pmel-1 iPSCs and mESCs were cultured using OP9 and OP9-DL1 stromal cell co-cultured system (24). In brief, 5×10^4 cells were plated on a 10-cm dish containing confluent OP9 cell monolayer in OP9 medium. On day 5 of culture, cells were trypsinized off, and 5×10^5 cells were transferred to a fresh culture 10-cm dish containing confluent OP9 cell monolayer in OP9 medium with the addition of hFlt3 ligand (5 ng/ml; R&D systems). On day 8 of culture and every 4 days thereafter, semiadherent stem cell-derived hematopoietic cells were collected, filtered through a 40μm nylon mesh, and were transferred onto fresh OP9-DL1 monolayers with the addition of hFlt3 ligand and mIL-7 (1 ng/ml; Peprotech). Semiadherent cells obtained from day 16 to 28 were used for further experiments.

In vitro activation of Pmel-1 iPSC-derived T cells, Pmel-1 splenocytes, and Pmel-1 thymocytes

Semiadherent Pmel-1 iPSC-derived cells on OP9-DL1 cells were harvested and filtered through a 40μm nylon mesh. CD8 expressing cells including CD4 CD8 double positive (DP) T cells and CD8 single positive (SP) T cells in Pmel-1 iPSC-derived cells, Pmel-1 splenocytes and thymocytes were isolated using anti-CD8 beads and MACS columns to eliminate OP9-DL1 cells during T cell activation. These cells (2×10^6 cells) were cultured with mIL-7 (10 ng/ml) and mIL-15 (10 ng/ml; Peprotech) for 2 days in the presence of 1μM

of hgp100 peptide and mitomycin-C treated splenocytes from B6 mice (5×10^5 cells). These activated cells were cultured with IL-7 and IL-15 or IL-7, IL-15 and IL-2 from day 3, and used for further experiments on day 6–8.

Adoptive cell transfer, vaccination and cytokine administration

Female C57BL/6 mice were injected s.c. with 3×10^5 B16F10 cells. Mice (n=5 for all groups) were treated 7–11 days later with i.v. adoptive transfer of *in vitro*-activated 3×10^6 Pmel-1 iPSC-derived T cells or splenic T cells. We injected 20,000 IU rhIL-2 intraperitoneally once on the day of vaccination and twice a day on the two following days. In some experiments mice were vaccinated with 100 μl of saline containing 100 μg of hgp100 peptide, 50 μg of CD40-specific mAb (clone FGK4.5, BioXcell), 50 μg of poly (I:C) (Invivogen) s.c., and 50 mg of imiquimod cream 5% (Aldara, Fougetra) applied on the vaccination sites after adoptive transfer. Tumor volumes were calculated by determining the length of short (*l*) and long (*L*) diameters (volume = $l^2 \times L/2$). Experimental end points were reached when tumors exceeded 20 mm in diameter or when mice became moribund and showed signs of lateral recumbency, cachexia, lack of response to noxious stimuli, or observable weight loss.

Statistics

Data sets were compared using 2-tailed unpaired Student's *t* test. Survival was analyzed with the Kaplan-Meier method using GraphPad Prism 5.0 (GraphPad Software Inc.), and groups were compared using log-rank test. $P<0.05$ was considered statistically significant. Data are presented as mean ± SEM.

Results

Generation of iPSCs from TCR transgenic CD8^{+} T cells

To establish a syngeneic mouse model to evaluate *in vitro* and *in vivo* antitumor immunity of iPSC-derived antigen-specific T cells, we chose to reprogram Pmel-1 TCR transgenic CD8^{+} T cells that were developed as a system to model treatment of melanoma using adoptive T cell therapy (25). The target antigen, *pmel-17,* is an ortholog of the melanocyte differentiation antigen gp100, which is often overexpressed in human melanomas (26). Adoptive transfer of *in vitro*-activated Pmel-1 CD8^{+} T cells expressing the anti-gp100 TCR effectively mediates the regression of large established B16 melanomas in combination with systemic administration of common γ-chain cytokine and antigen-specific vaccination with an altered peptide ligand (4,5,25,27).

Reprogramming of murine lymphocytes into iPSCs to date has been successful for B cells (28) and for natural killer T cells (29). However, mature T cells are known to be among the most difficult cells to reprogram, and reprogramming of differentiated murine T cells thus far has required a *p53* knock-out or the addition of a doxycycline-inducible gene expression system for successful iPSC generation (30,31). In the current study, we chose the Sendai virus (SeV) vector reprogramming system (15,19–21,32) to generate iPSCs from Pmel-1 CD8^{+} T cells because SeV can transduce and express foreign genes efficiently into activated

murine and human T cells (33), and have high reprogramming efficiency of human peripheral blood T cells (15).

First, Pmel-1 CD8^{+} T cells were stimulated with hgp100- peptide and IL-2 for 24 hr before SeV infection (Fig. 1A). Efficient expression of an exogenous gene in Pmel-1 CD8^{+} T cells was confirmed by following the expression of green fluorescent protein (GFP)-carrying SeV (Fig. 1B). To generate iPSCs, we used SeV that individually expressed Oct3/4, Sox2, Klf4 and c-Myc. Twenty-four hours after gene introduction, the cells were replated onto feeder layers of SNL cells with mESC medium containing the self-renewal cytokine, LIF (34). We observed the appearance of mESC-like colonies at 14–21 days after infection with the SeV vectors. However, the colonies isolated and subcultured in conventional mESC medium quickly differentiated and lost typical mESC morphology.

A previous report found that a combination of small molecule inhibitors, CHIR99021, a GSK-3 inhibitor that can activate the Wnt signaling pathway, and PD0325901, a MEK inhibitor, could promote partially reprogrammed cells to full pluripotency (35). Therefore, we investigated whether combining dual inhibition (2i) of MEK and GSK-3, LIF, and overexpression of the pluripotency transcription factors could generate iPSCs from Pmel-1 CD8^{+} T cells. Genetic transduction using the SeV system and 2i/LIF-based iPSC medium allowed us to establish two colonies with a mESC-like morphology (Fig. 1C) (an efficiency of 0.004%). These iPSCs 1 and 2 were positive for alkaline phosphatase and mESC markers, SSEA1,Oct3/4 and Nanog (Fig. 1C). To further verify expression of pluripotency genes, we performed RT-PCR analysis by primer sets that distinguish endogenous from viral transcripts (Supplementary Table S1), and found that the iPSCs expressed mESC transcripts for *Nanog, Oct3/4, Sox2* and *Ecat1* (Fig. 1D). Transgenes, *Oct3/4, Sox2, Klf4 and c-Myc*, and SeV in the iPSCs were lost after several passages (Fig. 1E).

Cytogenetic analysis showed derived iPSCs maintained normal karyotype (Fig. 2A). To evaluate the differentiation potential of the iPSCs, we first performed *in vitro* differentiation assays. The iPSCs formed embryoid bodies efficiently *in vitro*, and upregulation of marker genes for all three germ layers was detected by immunostaining (Fig. 2B). Furthermore, we tested the ability of the iPSCs to form teratomas in immune-deficient NOD/SCID mice. These iPSCs differentiated into a variety of cell types from all three germ layers (Fig. 2C). Therefore, those colonies were confirmed as typical murine iPSCs. We then examined the status of TCR gene rearrangement in those iPSCs and confirmed that they retained the same rearranged configuration as the original Pmel-1 T cells (Fig. 2D). These results demonstrated that Pmel-1 CD8^{+} T cells were successfully reprogrammed to iPSCs. We hereafter referred these established Pmel-1 CD8^{+} T cell-derived iPSCs as "Pmel-1 iPSCs."

Generation of less-differentiated CD8^{+} T cells from iPSCs for adoptive cell therapy

Directed differentiation of mESCs into T lymphocytes required the engagement of Notch receptors to the ligand DL1 expressed on the OP9-DL1 stromal cells (24). mESCs cultured on OP9-DL1 cells follow a thymocyte developmental progression from CD4 CD8 double negative (DN) stage which can be further subdivided into four sequential stages of differentiation based on CD44 and CD25 expression to CD4 CD8 DP T cells (24). To determine whether Pmel-1 iPSCs can differentiate into mature T cells *in vitro,* we cultured

Pmel-1 iPSCs on OP9 cells followed by culture on OP9-DL1 cells (Fig. 3A). On day 8 a population of cells expressing CD45 began to emerge from Pmel-1 iPSCs cultured on OP9 cells (Supplementary Fig. S1A). By day 16 of culture these CD45^{+} cells exhibited a typical thymocyte developmental progression similar to mESCs on OP9-DL1 cells, and CD4 CD8 DP T cells appeared (Supplementary Fig. S1B). On day 20 Pmel-1 iPSCs cultured on OP9-DL1 cells contained a large population of DP T cells (Fig. 3B), and the majority of DP T cells were CD8$\alpha\beta$ T cells that expressed CD5 (Supplementary Fig. S1C). No NK cells or NK-T cells were observed in the Pmel-1 iPSCs on OP9-DL1 cells (Supplementary Fig. S1D). We found very few mESC-derived DP T cells expressed CD3 or TCRβ, and only 30% of natural DP thymocytes expressed CD3 and Pmel-1 TCRβ chain Vβ13. In contrast, virtually all (>95%) of the Pmel-1 iPSC-derived DP T cells expressed CD3 and TCR Vβ13, similar to CD8 SP Pmel-1 thymocytes and splenocytes (Fig. 3B). We further characterized TCR repertoires by high throughput sequencing, and found that a majority of regenerated Pmel-1 iPSC-derived DP and CD8 SP cells bear the same TCRβ chain gene as original Pmel-1 CD8^{+} T cells (Table 1). These results suggest that iPSCs with rearranged TCR genes efficiently differentiate into TCR-expressing cells at the preselection stage which is consistent with human iPSCs bearing rearranged TCR genes (19,20).

Next, we investigated whether we could differentiate Pmel-1 iPSC-derived DP T cells into CD8 SP T cells, and thereby obtain large numbers of less-differentiated CD8^{+} T cells suitable for adoptive cell therapy (1,4–6). Signaling via TCR$\alpha\beta$ induces DP thymocytes to differentiate into mature SP T cells (36). Therefore, we stimulated Pmel-1 iPSC-derived DP T cells with their peptide epitope, hgp100$_{25\text{-}33}$ in the presence of IL-7 and IL-15, two cytokines that are responsible for cytotoxic lineage specification (37) as well as for generation of long-lived memory T cells (38,39). After 2 days of activation, DP T cells disappeared with a simultaneous increase in CD8 SP T cells (Fig. 3C). Phenotypic analysis of the CD8 SP T cells revealed that expression of IL-2 receptor α chain (CD25) and CD69 in Pmel-1 iPSC-derived CD8 SP T cells was higher than thymocytes but lower than splenocytes after 2 days of activation. Based on increased expression of CD25, we tested if the addition of IL-2 to the culture could increase the expansion of Pmel-1 iPSC-derived CD8 SP T cells. We observed a marked increase in the number of T cells after culture with IL-2, IL-7 and IL-15 from day 3 as compared with IL-7 and IL-15 (Fig. 3D). Since activation of T cells may be accompanied by differentiation to effector T cells and the loss of memory potential (1), we evaluated the phenotype of iPSC-derived CD8^{+} T cells after activation. Despite increased expansion, *in vitro*-activated iPSC-derived CD8^{+} T cells were comprised of more than 50% of CD62L^{high} less-differentiated T cells, retained expression of the memory T cell markers, CD122, chemokine (C-X-C motif) receptor 3 (CXCR3), and stem cell antigen-1 (Sca-1) (1,4), and acquired similar phenotype as *in vitro*-activated pmel-1 splenic CD8^{+} T cells (Fig. 3E).

iPSC-derived CD8^{+} T cells exhibit antigen-specific cytokine production and cytolysis

To characterize the effector function of Pmel-1 iPSC-derived CD8^{+} T cells, we examined intracellular interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) levels after activation using anti-CD3/CD28 monoclonal antibodies (mAbs), or with hgp100 peptide. Influenza nucleoprotein epitope NP$_{366\text{-}374}$ was used as an irrelevant control peptide. A high frequency

of cytokine-producing Pmel-1 iPSC-derived CD8$^+$ T cells was documented after stimulation with anti-CD3/CD28 mAbs or with the hgp100$_{25-33}$ antigen-. Importantly, cytokine production occurred in response to cognate antigen, as evidenced by the failure to detect cytokine producing T cells in response to the NP$_{366-374}$ antigen (Fig. 4A).

We next evaluated the cytolytic activity of Pmel-1 iPSC-derived T cells and Pmel-1 splenic T cells using peptide-pulsed tumor targets. As measured using an LDH release cytotoxicity assay, Pmel-1 iPSC-derived T cells showed antigen-specific cytolytic activity *in vitro* that was similar to Pmel-1 splenic T cells (Fig. 4B). Consistent with cytolytic activity, granzyme B was significantly enriched in Pmel-1 iPSC-derived T cells (Fig. 4C). Next, Pmel-1 iPSC-derived T cells and Pmel-1 splenic T cells were compared for their abilities to release cytokine against *bona fide* tumor targets, namely the poorly immunogenic B16 melanomas. MC38 colon adenocarcinoma cells were used as irrelevant targets. Despite very low levels of MHC class I molecules in B16 tumors (Fig. 4D), Pmel-1 iPSC-derived T cells were able to produce abundant IFN-γ to B16 tumors but not to MC38 tumors, which was equivalent to IFN-γ production using Pmel-1 splenic T cells (Fig. 4E). Collectively, these results indicate that Pmel-1 iPSCs are capable of generating less-differentiated antigen-specific T cells with potent effector function suitable for adoptive cell therapy.

Potent *in vivo* antitumor immunity of iPSC-derived CD8$^+$ T cells

Given the potent *in vitro* antitumor reactivity against B16 tumors, next we investigated the *in vivo* antitumor immunity of Pmel-1 iPSC-derived CD8$^+$ T cells using an established subcutaneous B16 tumor model. *In vitro*-activated Pmel-1 iPSC-derived CD8$^+$ T cells were adoptively transferred into C57BL/6 mice bearing subcutaneous B16 melanomas that had been established for 7 days. Systemic administration of IL-2 was used to enhance antitumor immunity of transferred T cells. *In vitro*-activated Pmel-1 splenic CD8$^+$ T cells were used as a comparison. B16 tumor growth was unaltered in C57BL/6 mice receiving IL-2 without adoptive cell therapy. In contrast, significant growth delays (Fig. 5A) and prolonged survival (Fig. 5B) were observed when C57BL/6 mice were adoptively transferred with Pmel-1 iPSC-derived CD8$^+$ T cells in combination with IL-2. Consistent with the *in vitro* functional studies (Fig. 4A-D), the *in vivo* antitumor immunity of Pmel-1 iPSC-derived CD8$^+$ T cells was comparable to Pmel-1 splenic CD8$^+$ T cells.

Stimulation of adoptively transferred iPSC-derived CD8$^+$ T cells through antigen-specific vaccination and IL-2 causes effective regression of large tumors and establishes immunological memory

Previous reports showed adoptive transfer of tumor-specific T cells in combination with T cell stimulation through antigen-specific vaccination and co-administration of immunostimulatory molecules and/or T cell growth and activation factors can provide to inducing maximal tumor regression (4,5,25,27). Therefore, we tested if antitumor immunity of adoptively transferred iPSC-derived CD8$^+$ T cells can be further enhanced by subcutaneous vaccination with hgp100, anti-CD40 mAb, and toll-like receptor (TLR) agonists, poly (I:C), and imiquimod cream, agonists for TLR3 and TLR7, respectively, in combination with administration of IL-2 in mice with large subcutaneous B16 melanomas established for 11 days. As shown in Fig. 6A, adoptive transfer of iPSC-derived T cells

together with vaccination with cognate antigen, hgp100, immunostimulatory molecules, TLR agonists, and IL-2 resulted in complete responses in ~ 40% of mice bearing large established tumors. These complete responses were found to be durable over an observation period of >80 days (Fig. 6B).

Given our findings of long-term responders after the treatment, we investigated whether adoptively transferred Pmel-1 iPSC-derived T cells also had the capacity to become long-lived memory T cells in surviving mice. Thirty days after adoptive transfer of Pmel-1 iPSC-derived T cells (CD90.1), splenocytes from surviving C57BL/6 mice (CD90.2) still contained transferred T cells, of which more than 20% were $CD44^{high}CD62L^{high}$ central memory T cells similar to surviving mice treated with Pmel-1 splenic T cells (Fig. 6C).

Finally, we evaluated whether treated mice developed antitumor reactivity by rechallenging survivors with implanted B16 tumors in the contralateral flank. MC38 tumors were also injected on the back to determine the antigen-specific systemic immunity by adoptive transfer of Pmel-1 iPSC-derived T cells. Age-matched, untreated non-tumor experienced mice were used as controls. As shown in Fig. 6D, significant growth delays of the B16 tumors but normal growth of the MC38 tumors were observed in surviving C57BL/6 mice that were previously treated with adoptive transfer of Pmel-1 iPSC-derived T cells or Pmel-1 splenic T cells, suggesting development of antigen-specific long-lasting systemic memory.

Discussion

Adoptive transfer of antigen-specific $CD8^+$ T cells has emerged as one of the most effective treatments for variety of cancers (1–3). A major limitation of this approach is poor survival of T cells *in vivo* following infusion. Although less-differentiated T cells would be an ideal T-cell subset for adoptive T cell therapy (1,4–6), generating large numbers of these "young" T cells is problematic. Human iPSCs hold great promise as unlimited sources of autologous T cells and can potentially generate an infinite number of naïve antigen-specific T cells for adoptive cell therapy. Two approaches have been described to generate iPSCs that can produce antigen-specific T cells (19–22). One is to reprogram T cells with known antigen specificity into iPSCs (19–21). Recent studies have shown that T cell-derived iPSCs retaining rearranged TCR genes from original T cell clone can differentiate into T cells that exhibited antigen-specific cytokine production and cytotoxicity *in vitro* (19–21). This strategy is especially relevant for TILs because nearly 80% of TILs possess autologous tumor reactivity (40), but exhibit a more differentiated phenotype and functional exhaustion (1,10,11). We have recently described that melanoma TILs can be reprogrammed to human iPSCs, albeit with low efficiency compared to peripheral blood T cells (23). Another approach to generate antigen-specific T cells from iPSCs is to genetically engineer T cell-derived iPSCs to express a CAR specific for the known tumor antigen such as CD19, which is expressed on B-cell leukemias and lymphomas, and differentiate them into CAR-expressing T cells (22). Although iPSC-derived, CAR-expressing T cells recognize the targeted antigen via a CAR not through their endogenous TCRαβ, in line with our findings, they mediate antitumor reactivity *in vivo* in a xenograft model of lymphoma (22). Of note, the latter approach is useful to target only known tumor antigens while the former approach

can be used to target both known and unknown antigens as long as antigen-specificity of the T cell is identified.

Our study has provided insight into the *in vivo* immunogenicity and antitumor reactivity of CD8^{+} T cells derived from iPSCs bearing a rearranged TCR of known antigen specificity. A central finding was that iPSC-derived regenerated CD8^{+} T cells escaped immune rejection, and mediated potent antitumor immunity *in vivo* comparable to splenic CD8^{+} T cells from TCR transgenic mice. This *in vivo* antitumor immunity of iPSC-derived CD8^{+} T cells was supported by the findings that they not only had antigen-specific cytokine-producing and cytolytic function against the cognate antigen, hgp100 but also had the ability to produce cytokine against *bona fide* tumor targets, poorly immunogenic B16 melanomas. Taken together, these results suggest that T cells not only retain antigen specificity via endogenous TCRαβ but also maintain functional integrity, proliferative capacity and effector function after reprogramming and redifferentiation processes.

The current study demonstrate for the first time that murine T cells can be reprogrammed without using gene knockout mice (30) or drug-inducible gene expression systems (31). We found the SeV reprogramming system that was shown to be highly efficient method for the generation of iPSCs from human T cells (15,19–22), could also be used for reprogramming murine T cells. The reprogramming efficiency, however, was quite low (0.004%) even with a higher MOI of 20. Furthermore, conventional mESC medium containing LIF was not sufficient to generate iPSCs from Pmel-1 T cells. We found that dual inhibition (2i) of both prodifferentiation MEK and GSK-3 pathways that was shown to support the establishment of mouse iPSCs from partially reprogrammed cells (35) was required for reprogramming of Pmel-1 T cells. The low efficiency of iPSC derivation remains one of the major limitations to apply this approach into clinical practice. Of note, we observed Pmel-1 iPSCs can be maintained in conventional mESC medium without 2i once they are established, and they maintain pluripotency and their ability to differentiate in the T lineage.

Persistence of TCR rearrangements is a distinct feature of T cell-derived iPSCs (15–18) as well as plays a pivotal role in differentiating iPSCs to CD8 SP T cells (19,20). Our findings further expand on effector function of iPSC-derived CD8^{+} T cells in comparison to naive CD8^{+} TCR transgenic T cells. We demonstrated Pmel-1 iPSC-derived DP T cells in culture with OP9-DL1 expressed high levels of CD3 and TCRVβ13, efficiently upregulated CD25 and CD69 upon stimulation via TCR, and quickly became CD8 SP T cells functionally similar to *in vitro*-activated Pmel-1 splenic CD8^{+} T cells. Although we observed a subset of mESC-derived CD8 SP T cells expressing CD3 and TCRβ (data not shown) as reported in a previous study (24), Pmel-1 iPSCs cultured on OP9-DL1 cells differentiated more efficiently than mESCs, and expressed CD3 and TCRVβ13 at the DP stage.

The early expression of prerearranged TCRα and TCRβ genes may cause undesired outcomes. Serwold et al. showed that mice derived from a reprogrammed T cell, developed spontaneous T cell lymphoma, which originated in the thymus (41). Although these mice have prerearranged TCR genes in all cells, and are different from mice receiving adoptive transfer of iPSC-derived T cells in the current studies, potential tumorigenicity of iPSC-derived T cells needs to be determined before this strategy is translated into clinical practice.

Notably, we did not find any mice that developed tumors including lymphoma after adoptive transfer of iPSC-derived T cells.

Immunogenicity of autologous iPSC-derived cells is still not well understood. Recent studies have shown that certain iPSC-derived cells such as smooth muscle cells and cardiomyocytes are immunogenic while other cell types such as retinal pigment epithelial, hepatocytes, and neuronal cells exhibit little to no immunogenicity (42–45). Although our study did not evaluate host immune response against infused iPSC-derived T cells, our findings of long-lived adoptively transferred T cells that establish immunological memory suggest iPSC-derived T cells might not cause immune rejection.

T cells with CD62L^{high} less-differentiated phenotype maintain proliferative capacity and produce effector progeny *in vivo*, resulting in a greater objective response upon adoptive transfer relative to CD62L^{low} more-differentiated T cells (1,4–6). The strategy of reprogramming and regeneration of antitumor T cells provides a more meaningful rationale for adoptive T cell therapy if we can generate T cell subsets that possess robust proliferative and engraftment capacities. A recent report showed that human T-cell derived iPSCs can differentiate to T cells with a central-memory phenotype (19). We further determined that iPSC-derived less-differentiated T cells expressing memory T-cell markers had long-term persistence and established antigen-specific immunological memory *in vivo* after adoptive transfer. Interestingly, we found potent *in vivo* antitumor reactivity of adoptive transfer of iPSC-derived T cells and IL-2 without antigen-specific vaccination which was not seen in the previous study (25). A possible explanation for this finding may reside in the different *in vitro* activation protocol of T cells where we used IL-2, IL-7 and IL-15 while others used IL-2 only (25). IL-7 and IL-15 play integral role in CD8$^+$ T-cell memory generation and maintenance (38). IL-7 is involved for the survival and homeostatic expansion of naïve CD8$^+$ T cells, and also can contribute to memory CD8$^+$ T cell homeostasis (46,47). IL-15 induces proliferation of naïve and memory CD8$^+$ T cells and is involved in the maintenance of long-lasting antigen-specific memory CD8$^+$ T cells (48–50). We found the majority of Pmel-1 iPSC-derived and splenic T cells after activation with IL-2 were CD62L^{low} more-differentiated T cells (data not shown) while ones activated with IL-2, IL-7 and IL-15 contained more than 50% of CD62L^{high} less-differentiated T cells (Fig. 3E).

In summary, our results demonstrate that adoptive transfer of iPSC-derived regenerated T cells is an effective cancer immunotherapy that improves local tumor control and overall survival in a lethal murine model of melanoma. Importantly, an establishment of antigen-specific immunological memory reveals the feasibility of generation of long-lived tumor-specific T cells via reprogramming to pluripotency and redifferentiation. Applying techniques described herein may provide an unlimited number of phenotypically defined, functional and expandable autologous antigen-specific T cells harboring characteristics necessary for *in vivo* effectiveness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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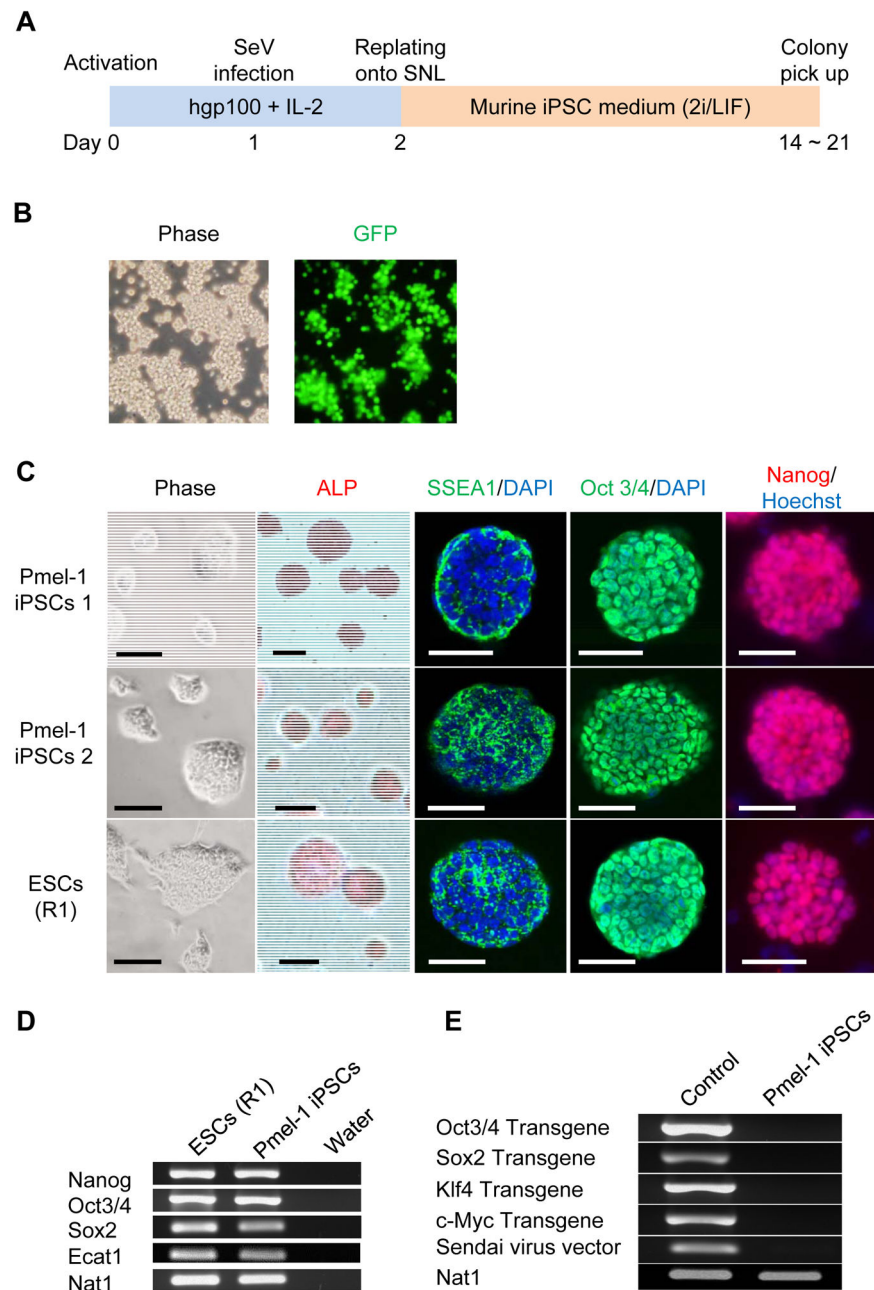
Figure 1. Generation of iPSCs from Pmel-1 TCR transgenic CD8+ T cells
(**A**) Schematic illustration showing the generation of iPSCs from Pmel-1 TCR transgenic CD8+ T cells. (**B**) Efficient GFP introduction by Sendai virus vector in Pmel-1 CD8+ T cells transfected at an MOI of 20. (**C**) Morphology, alkaline phosphatase (ALP) activity and expression of pluripotency and surface markers (SSEA1, Oct3/4 and Nanog) in Pmel-1 iPSCs 1 and 2, and mESCs. Nuclei were counterstained with DAPI or HOECHST. Scale bar: 200 μm. (**D**) RT-PCR analysis for the ESC marker genes, *Nanog, Oct3/4, Sox2,* and *Ecat1* in Pmel-1 iPSCs and mESCs. (**E**) RT-PCR analysis for the transgenes, *Oct3/4 Sox2, Klf4, c-Myc* and Sendai virus in Pmel-1 iPSCs.
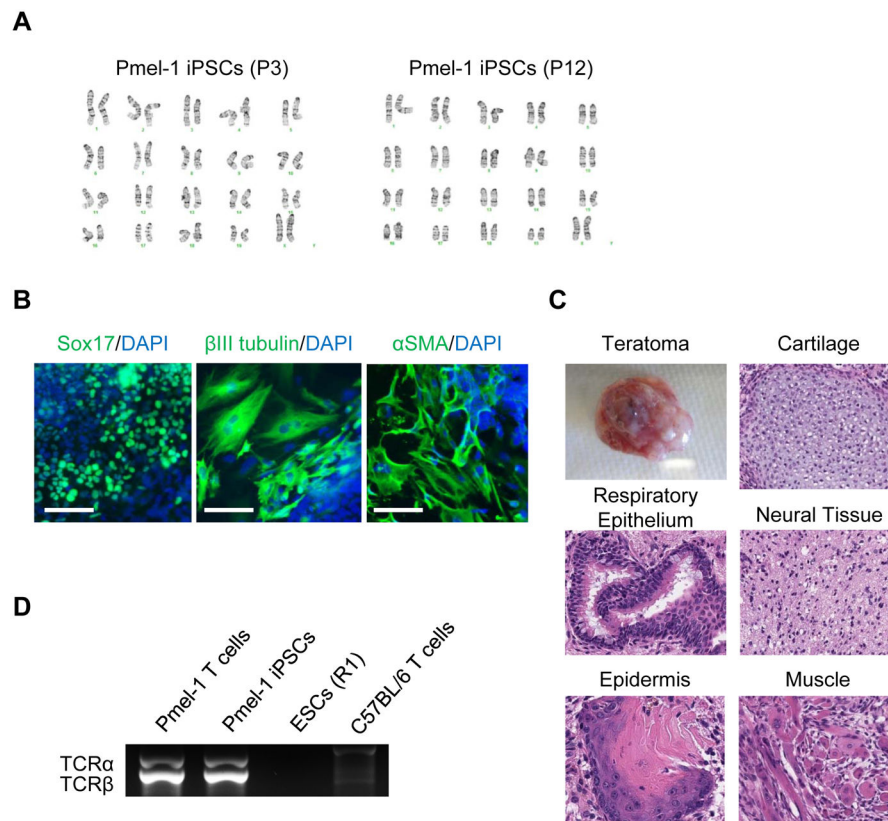
A

Pmel-1 iPSCs (P3)   Pmel-1 iPSCs (P12)

B

Sox17/DAPI   βIII tubulin/DAPI   αSMA/DAPI

C

Teratoma   Cartilage

Respiratory   Neural Tissue
Epithelium

Epidermis   Muscle

D

TCRα
TCRβ

Figure 2. Detail characterizations of Pmel-1 CD8^{+} T cell-derived iPSCs
(**A**) Normal karyogram of the Pmel-1 iPSCs at passage 3 (left) and 12 (right). (**B**) Immunostaining for Sox17 (endodermal marker), βIII tubulin (ectodermal marker), and αSMA (mesodermal marker) in Pmel-1 iPSC-derived differentiated cell. Scale bar: 100 μm (**C**) Gross morphology, hematoxylin and eosin-stained representative teratomas derived from Pmel-1 iPSCs. (**D**) PCR analysis of rearranged Pmel-1 TCR α- and β-chain genes in Pmel-1 T cells, Pmel-1 iPSCs, mESCs and C57BL/6 mouse T cells.
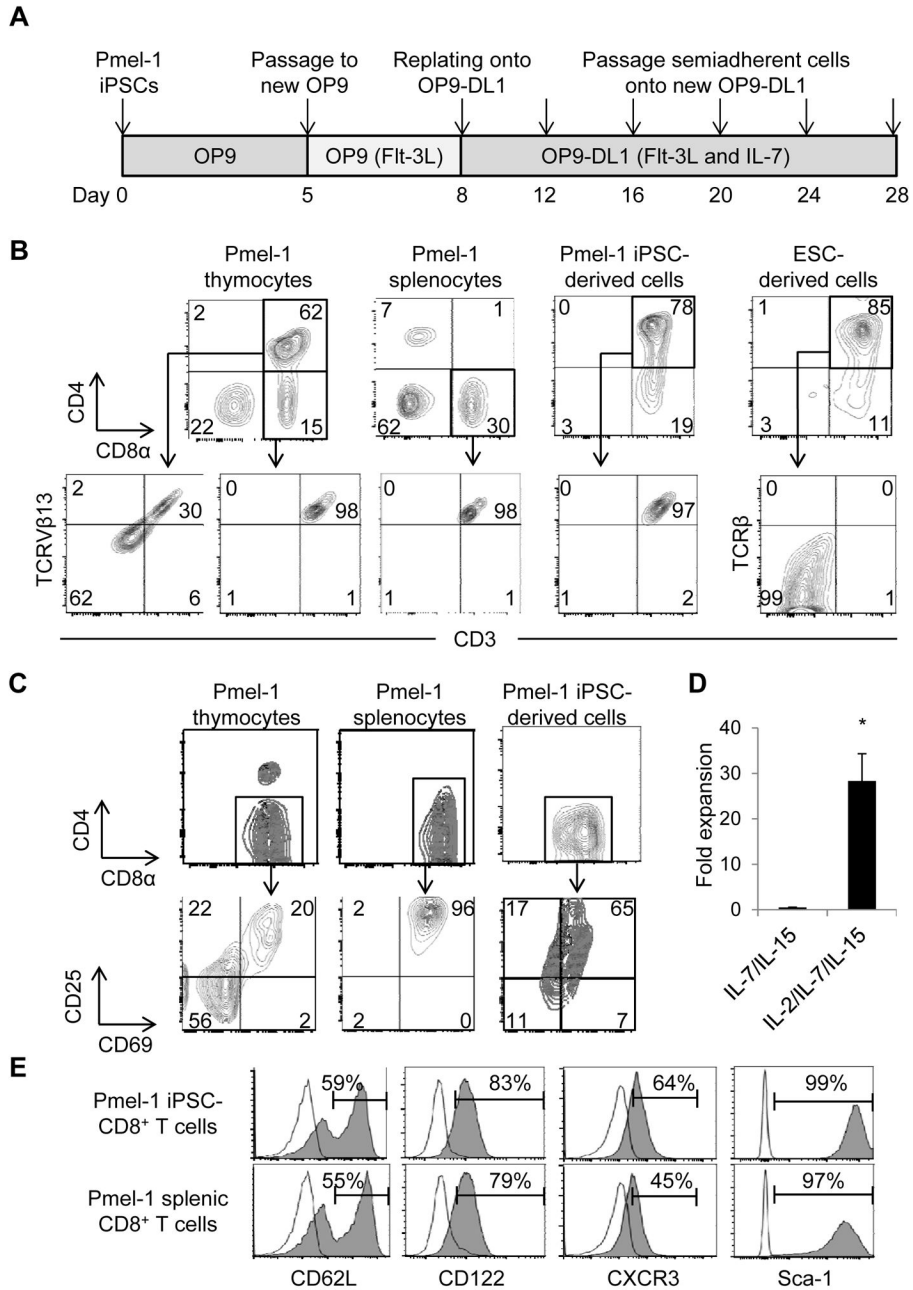
Figure 3. Generation of less-differentiated CD8+ T Cells from Pmel-1 iPSCs for adoptive cell therapy

(A) Schematic illustration showing the regeneration of T cells from Pmel-1 iPSCs. (B) Phenotypic analysis of Pmel-1 iPSC-derived cells and mESCs on OP9-DL1 cells on day 20. Pmel-1 thymocytes and splenocytes were used for comparison. Viable lymphocytes were analyzed for CD4 and CD8α surface expression (upper), and CD4 CD8 DP or CD8 SP cells were further analyzed for CD3 and TCRVβ13 or TCRβ surface expression (lower). Data are representative of 5 independent experiments. (C) Phenotypic analysis of Pmel-1 iPSC-derived DP T cells, thymocytes and splenocytes 2 days after activation with hgp100 in the presence of IL-7 and IL-15. Viable lymphocytes were gated on CD8α and analyzed for

CD25 and CD69 surface expression. Data are representative of 2 independent experiments. Numbers in (B) and (C) indicate the percentage of cells showing in each quadrant. **(D)** Fold expansion of Pmel-1 iPSC-derived T cells with IL-7 and IL-15 versus IL-2, IL-7 and IL-15 during expansion from day 2 to day 6. (*=*P* < 0.01 by 2-tailed unpaired Student's *t* test) **(E)** Phenotypic analysis of Pmel-1 iPSC-derived and splenic CD8^{+} T cells after 2-day activation with hgp100 in the presence of IL-7 and IL-15 followed by 4-day expansion with IL-2, IL-7 and IL-15. Viable lymphocytes were gated on CD8α and analyzed for CD62L, CD122, CXCR3 and Sca-1 surface expression. Open histograms indicate the isotype-control staining. Data are representative of 3 independent experiments.
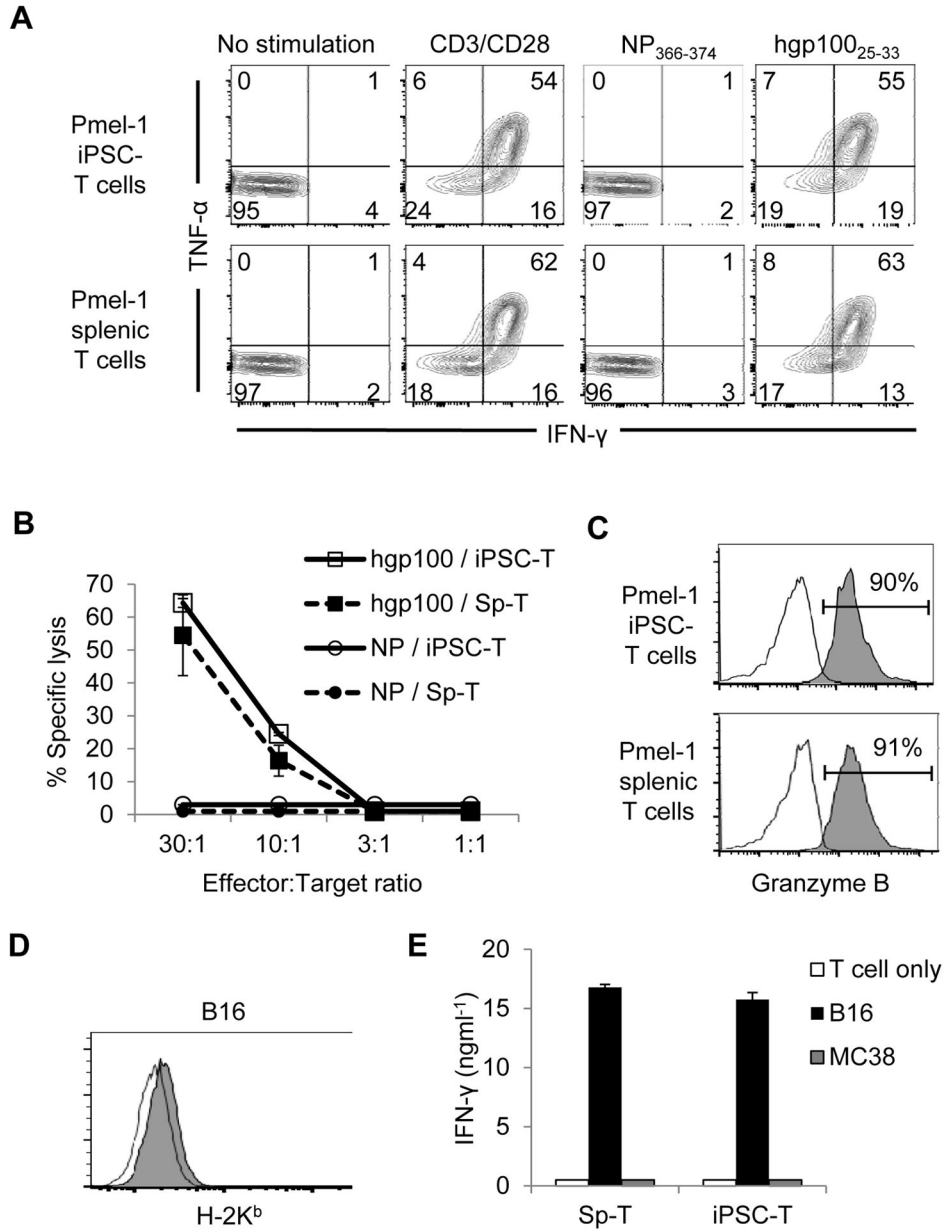
A

B

C

D

E

Figure 4. iPSC-derived CD8$^+$ T Cells exhibit antigen-specific cytokine production and cytolysis *in vitro*

(A) Intracellular production of IFN-γ and/or TNF-α by Pmel-1 iPSC-derived and splenic CD8$^+$ T cells upon stimulation with or without anti-CD3/CD28 mAb, or against the hgp100$_{25-33}$ or NP$_{366-374}$ antigens. CD8-gated populations were shown. Numbers denote percent positive cells. **(B)** Cytolytic function of Pmel-1 iPSC-derived and splenic T cells against the hgp100$_{25-33}$ or NP$_{366-374}$ antigens is shown. **(C)** Intracellular production of granzyme B induced by stimulation of Pmel-1iPSC-derived and splenic CD8$^+$ T cells with hgp100 pulsed MC38 tumor cells. **(D)** MHC-I (H-2Kb) expression on B16 melanoma tumors. Open histograms in (C) and (D) indicate the isotype-control staining. **(E)** Production of IFN-γ by Pmel-1 iPSC-derived and splenic T cells against B16 melanomas and MC38

colon adenocarcinomas. Data shown in (A) – (E) are representative of 2 independent experiments.
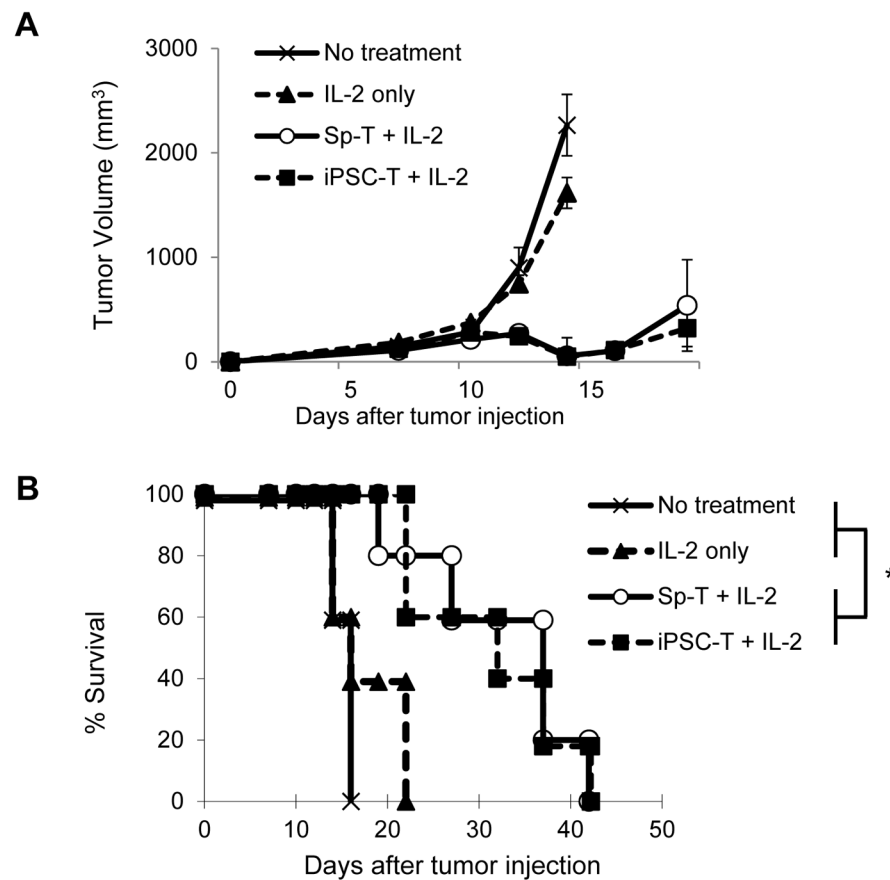
A

B

Figure 5. Potent *in vivo* antitumor immunity of iPSC-derived less-differentiated CD8^{+} T cells
(**A** and **B**) Tumor growth curves (**A**) and survival curves (**B**) in C57BL/6 mice bearing B16 melanomas established for 7 days in different treatment groups. Sp-T: Pmel-1 splenic CD8^{+} T cells. iPSC-T: Pmel-1 iPSC-derived CD8^{+} T cells. Tumor volume results are the mean of measurements from 5 mice per group. (* = *P* < 0.0001 using log-rank (Mantel-Cox) test.) Data shown are representative of 2 independent experiments.
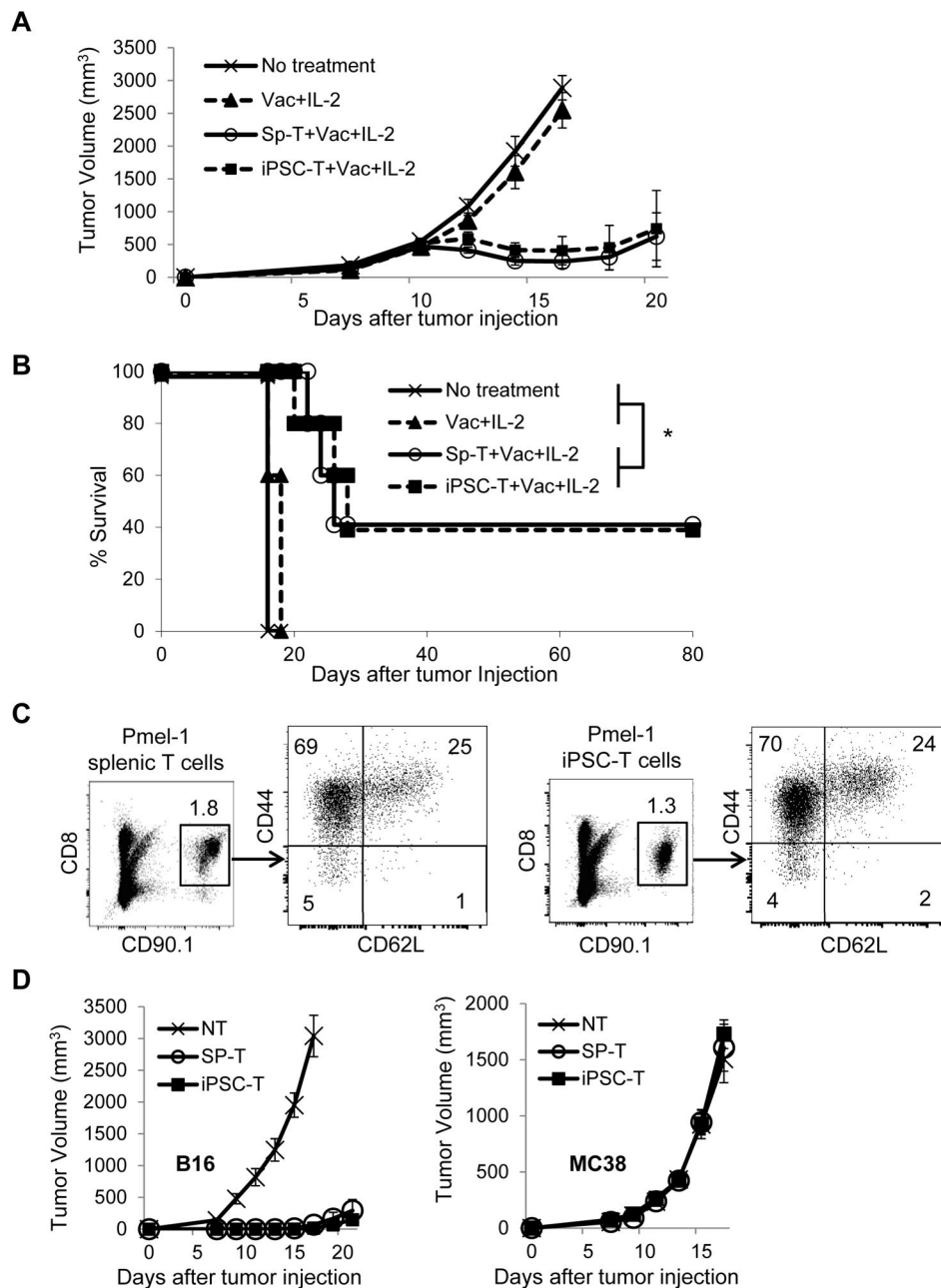
Figure 6. Stimulation of adoptively transferred iPSC-derived CD8⁺ T cells through antigen-specific vaccination and IL-2 causes effective regression of large tumors and establishes immunological memory

(**A** and **B**) Tumor growth curves (**A**) and survival curves (**B**) in C57BL/6 mice bearing B16 melanomas established for 11 days in different treatment groups. Vac: vaccination with the gp100 antigen, anti-CD40 mAb, poly (I:C), and imiquimod cream. Tumor volume results are the mean of measurements from 5 mice per group. (*=*P*< 0.0001 using log-rank (Mantel-Cox) test.) Data shown are representative of 2 independent experiments. (**C**) Phenotypic analysis of spleen from surviving C57BL/6 (CD90.2) mice that were treated with adoptive transfer of Pmel-1 iPSC-derived or splenic T cells (CD90.1) in combination with vaccination

and IL-2. Numbers denote percent CD8+CD90.1+ cells or indicate the percentage of cells showing in each quadrant. **(D)** Surviving mice (n=4) after adoptive transfer of Pmel-1 iPSC-derived or splenic T cells, vaccination and IL-2 were rechallenged with B16 cells into the contralateral flank and MC38 cells on back on day 80. Tumor growth curves are depicted in which T=0 corresponds to the time of injection of secondary tumors. As a control, tumor growth was monitored following inoculation of the same tumor cell dose into non-tumor (NT) experienced naive C57BL/6 mice (n=5).

Table 1

The sequence of rearranged TCRβ chain genes and frequency in Pmel-1 iPSC-derived DP, CD8 SP on OP9-DL1 cells and splenic CD8^{+} T cells.

Cell type	Productive rearrangement	Productive frequency	Frequency	Amino acid
Pmel-1 iPSC-CD8^{+} T cells	TCRBV14-01*01 TCRBD01-01*01 TCRBJ01-06*01	97.8%	93.2%	CASSFHRDYNSPLYF
Pmel-1 splenic CD8^{+} T cells	TCRBV14-01*01 TCRBD01-01*01 TCRBJ01-06*01	97.8%	95.0%	CASSFHRDYNSPLYF

Cell type	Nucleotide
Pmel-1 iPSC- CD8^{+} T cells	CTCAAGATCCAGTCTGCAAAGCAGGGCGACACAGCCACCTATC TCTGTGCCAGCAGTTTCCACAGGGACTATAATTCGCCCCTCTAC
Pmel-1 splenic CD8^{+} T cells	CTCAAGATCCAGTCTGCAAAGCAGGGCGACACAGCCACCTATC TCTGTGCCAGCAGTTTCCACAGGGACTATAATTCGCCCCTCTAC