Cell Stem Cell
Brief Report

Regeneration of Human Tumor Antigen-Specific T Cells from iPSCs Derived from Mature CD8$^+$ T Cells

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SUMMARY

Antigen-specific T cells represent a potential therapeutic avenue for a variety of conditions, but current approaches for generating such cells for therapeutic purposes are limited. In this study, we established iPSCs from mature cytotoxic T cells specific for the melanoma epitope MART-1. When cocultured with OP9/DLL1 cells, these iPSCs efficiently generated TCRβ$^+$CD4$^+$CD8$^+$ double positive (DP) cells expressing a T cell receptor (TCR) specific for the MART-1 epitope. Stimulation of these DP cells with anti-CD3 antibody generated a large number of CD8$^+$ T cells, and more than 90% of the resulting cells were specific for the original MART-1 epitope. Stimulation of the CD8$^+$ T cells with MART-1 antigen-presenting cells led to the secretion of IFNγ, demonstrating their specific reactivity. The present study therefore illustrates an approach for cloning and expanding functional antigen-specific CD8$^+$ T cells that might be applicable in cell-based therapy of cancer.

Induced pluripotent stem cells (iPSCs) can be produced from various types of somatic cells by reprogramming via the Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Grskovic et al., 2011). iPSCs are reported to be very similar to embryonic stem cells (ESCs) in many respects, including gene expression pattern and pluripotent characteristics. Because iPSCs are not subject to the same ethical concerns as ESCs, they have great potential as a major cell source for the production of various types of cells or organs in regenerative medicine.

One of the possible applications of iPSCs is to use them as a cell source for producing lymphocytes for cell-based therapy against cancer. Immunotherapy approaches to treat cancer have already been widely applied in clinical settings. Most of the approaches employed aim to activate tumor-specific cytotoxic T lymphocytes (CTLs), which are phenotypically characterized as CD3$^+$CD4$^-$CD8$^+$ (CD8 single positive: CD8SP) cells (Sensi and Anichini, 2006). Although the resulting activated CTLs exhibit some efficacy in killing tumor cells, in most cases this effect is not sufficiently substantial to cure the patient. A major limiting factor in this type of approach is the short life span of activated CTLs, which are inactivated quite rapidly by antigen-induced cell death (Mescher et al., 2007; Willimsky and Blankenstein, 2005).

It seemed to us that the application of iPSC technology to clone and expand tumor antigen-specific T cells could potentially address this issue. Previous studies have reported successful reprogramming of mature T cells and B cells using nuclear transfer, leading to the production of cloned mice that provided formal proof that the nucleus of a mature somatic cell can be completely reprogrammed (Hochedlinger and Jaenisch, 2002; Watarai et al., 2010b). Thus far, reprogramming of mature lymphocytes into iPSCs has been successfully accomplished for murine B cells (Hanna et al., 2008; Wada et al., 2011), for murine T cells (Watarai et al., 2010a), and for human T cells (Loh et al., 2010; Seki et al., 2010). Concurrently, we have developed methods for differentiating lymphocytes from iPSCs that were derived from a lymphocyte. In mice, we have already demonstrated success with this approach using mature NKT cells (Watarai et al., 2010a).

The previous studies that succeeded in producing iPSCs from human T cells used whole peripheral mononuclear cells or CD3$^+$ cells as a source (Loh et al., 2010; Seki et al., 2010). Therefore, it remains to be shown that isolated CTLs can be reprogrammed to form iPSCs. Initially, therefore, we investigated the reprogramming of CD8$^+$ T cells isolated from human cord blood and adult peripheral blood by a cell sorter (Figure S1A available online). Whole CD3$^+$ cells, as well as non-T cells, i.e., lineage marker negative (Lin$^-$) CD34$^+$ hematopoietic progenitor

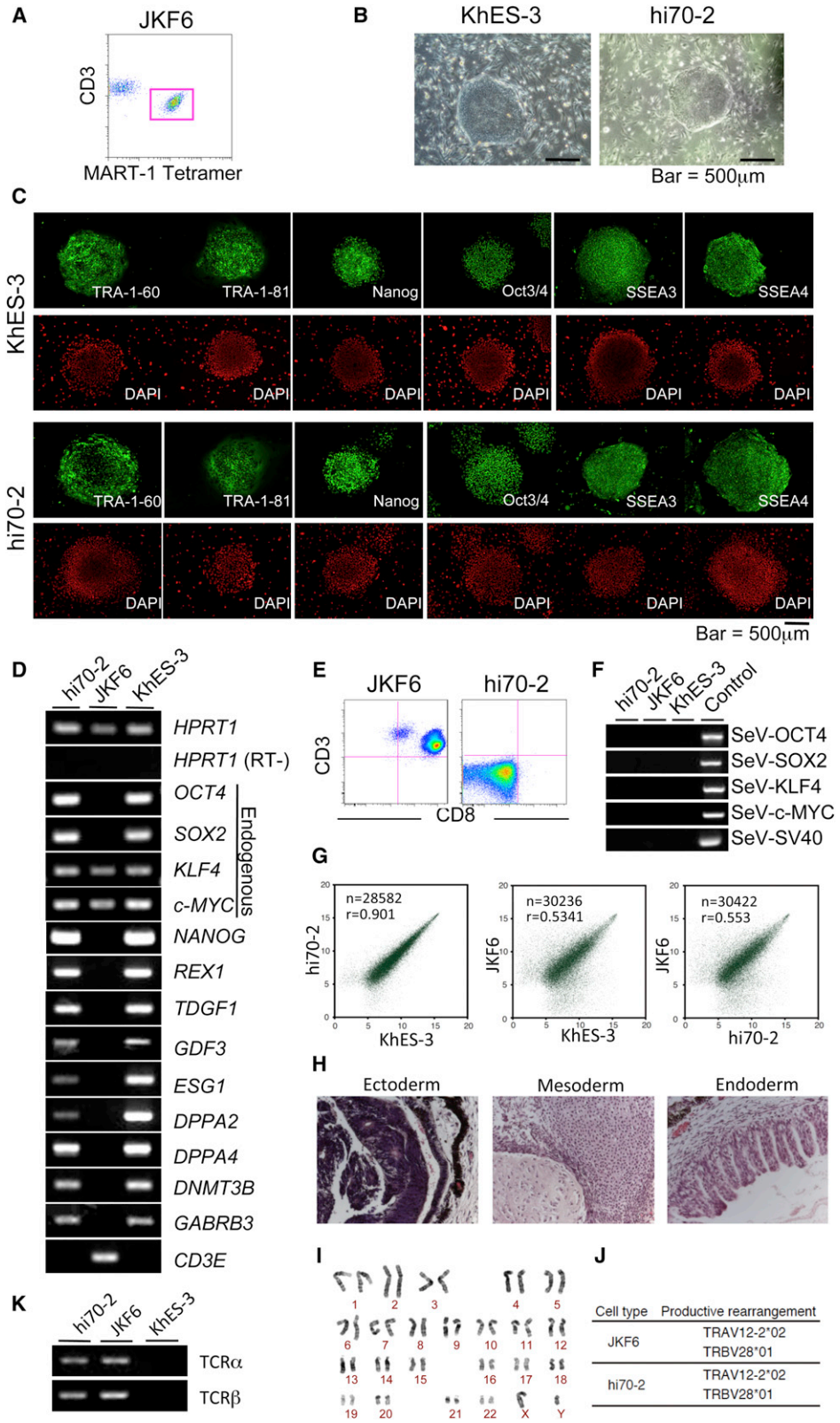

Figure 1. Generation of iPSCs from MART-1-Specific CD8$^+$ T Cell Line JKF6

(A) Flow cytometric profile of JKF6 cells. Cells were stained in two colors with anti-CD3 and MART-1-tetramer.

(B and C) Colony shape analysis (B) and immunofluorescence analysis (C) for the expression of TRA-1-60, TRA-1-81, NANOG, OCT3/4, SSEA3, or SSEA4 in iPSCs derived from MART-1 epitope-specific T cells (hi70-2) or hESCs (KhES-3). hESCs were used as a control.

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cells (Figure S1B), were also used as cell sources. The isolated T cells were also activated by receiving stimulation from anti-CD3 and anti-CD28 monoclonal antibodies. On day 1, cells were infected with Sendai virus (Fusaki et al., 2009) carrying the four Yamanaka factors (Klf4, Sox2, Oct4, and c-Myc) and SV40 (large T Antigen) (Park et al., 2008). The resulting colonies were picked up between day 21 and day 35 for expansion (Figure S1C). Eventually the colonies generated from T cells showed a morphology that is almost identical to that of typical human ESC (hESC) colonies (Figure S1D). The iPSCs expressed characteristic ESC markers such as SSEA4, TRA-1-60, and TRA-1-81. We designated the iPSCs derived from $CD34^+$ cord blood cells, whole $CD3^+$ T cells, and $CD8^+$ T cells as hCB-iPSCs, hT-iPSCs, and hCD8-iPSCs, respectively.

To confirm that the hT-iPSCs and hCD8-iPSCs are derived from T cells, we analyzed the rearrangement status of TCRβ chain gene by determining the sequence of TCRVβ region. In all cases, rearranged constructs were detected (Figure S1E), indicating that the cells were derived from T cells. We also examined whether hCD8-iPSCs are able to produce T lineage cells by coculturing them with OP9 and then OP9/DLL1 stromal cells (Figure S2A) (Timmermans et al., 2009; Vodyanik and Slukvin, 2007), with comparison to hESCs and hCB-iPSCs as controls. Approximately on day 40, $CD4^+CD8^+$ double positive (DP) cells were generated in all three groups (Figure S2B). Although the flow cytometric profiles of CD4 versus CD8 expression were almost indistinguishable among the three groups, the proportion of $TCR\beta^+CD3^+$ cells in DP cells from hCD8-iPSCs was much higher than in the other groups (Figure S2C). These results indicate that iPSCs bearing rearranged TCR genes are able to efficiently give rise to TCR-expressing cells at the preselection stage.

We then applied these findings to generate iPSCs from antigen-specific T cells. Because our overall goal is to regenerate T cells that can be used for cell-based therapy against cancer, as a cell source we selected CTLs specific for the melanoma epitope MART-1. JKF6 cells are long-term cultured tumor infiltrated lymphocytes that were originally derived from a melanoma patient and have been maintained at the Surgery Branch of the National Cancer Institute (Yang et al., 2011). JKF6 cells specifically recognize a complex of the MART-1-peptide and the HLA-A*02:01 molecule, which can be detected using MART-1-tetramer by flow cytometry (Figure 1A).

MART-1-specific T cells were transduced with the Yamanaka factors using the same approach as shown in Figure S1C. Approximately 20 colonies were generated from 5×10^5 JKF cells, and we picked up 10 colonies out of them. Among them, two were analyzed for the rearrangement status of TCRβ chain gene, and both were found to carry the rearranged configuration of the gene that was the same as the one found in JKF cells. Hereafter we focus on one of these two clones, clone hi70-2, for further experiments. hi70-2 cells formed a colony exhibiting hESC-like morphology (Figure 1B). hi70-2 cells were positive for TRA-1-60, TRA-1-81, OCT3/4, NANOG, SSEA3, and SSEA4 by immunofluorescence staining (Figure 1C). RT-PCR analysis showed that hi70-2 cells are positive for endogenous *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*, as well as other ESC markers, while negative for the T cell marker *CD3ε* (Figure 1D). We also confirmed the loss of the T cell markers CD3 and CD8 by flow cytometry (Figure 1E). SeV-derived *OCT3/4*, *SOX2*, *KLF4*, *c-MYC*, or *SV40* was not expressed in these cells (Figure 1F). Global gene expression analysis by microarray showed more similarity between hi70-2 cells and hESCs than the original CTLs (Figure 1G). We further showed that hi70-2 cells are able to form teratomas with three germ layers upon subcutaneous injection into nude mice (Figure 1H). Karyotype analysis showed that the cells have no chromosomal abnormalities (Figure 1I). We thus concluded that hi70-2 cells represent human iPSCs. We finally examined the rearrangement status of TCRα and β chain genes of hi70-2 cells and found that they retain the same rearranged configuration as original JKF6 cells (Figures 1J and 1K). These results demonstrated that MART-1-specific T cells were successfully reprogrammed to iPSCs. Hereafter the hi70-2 cells are designated as "MART-1-iPSCs."

To examine whether MART-1-iPSCs are able to differentiate into mature T cells, we cultured them with OP9 and subsequently with OP9/DLL1 stromal cells as described in Figure S2A. As controls, hESCs, hCB-iPSCs, and hCD8-iPSCs were also cultured. In all four groups, $CD34^+CD43^+$ cells, representing very early hematopoietic progenitors (Vodyanik et al., 2006), were generated on day 13 (Figure 2A). At this time point, $CD34^+CD43^+$ cells from all four groups had comparable capacity to produce myeloid and erythroid cells in colony-forming assays (Figure 2B).

On day 40, $CD4^+CD8^+$ DP cells were generated in all four groups (data not shown). The majority of the DP cells generated from MART-1-iPSCs expressed TCR specific to the MART-1 epitope, although about 30% of $CD3^+$ DP cells were negative or only weakly positive for MART-1-tetramer staining (Figure 2C).

In a physiological context, MART-1-specific T cells could be positively selected by thymic cortical epithelial cells that express a complex of HLA-A*02:01 and positively-selecting peptides distinct from the MART-1 epitope. However, with current technology it is difficult to reproduce positive selection based on

(D) The expression of hESC marker genes in hi70-2, JKF6, or KhES-3 cells.
(E) Flow cytometric profile of JKF6 cells and hi70-2 cells for the expression of CD3 versus CD8.
(F) Expression profiles of SeV-derived OCT3/4, SOX2, KLF4, c-MYC, and SV40 in hi70-2, JKF6, or KhES-3 cells. SeV-transfected cells (2 days after infection) were used as a control.
(G) Comparison of global gene expression of hi70-2 cells against that of khES3 or JKF6 cells. Pearson's correlation coefficient (r) and number of probes whose signals were present (n) are shown in each scatter plot.
(H) Representative three germ layers (mesoderm, ectoderm, and endoderm) developed in hi70-2 cells. Histological sections of teratomas, formed 4 weeks after subcutaneous injection of hi70-2 cells to nude mice, were stained with hematoxylin and eosin.
(I) Karyotype analysis of hi70-2 cells.
(J) The sequence of rearranged TCRα or TCRβ chain gene in hi70-2 or JKF6 cells.
(K) PCR analysis of rearranged TCRα or TCRβ chain gene in hi70-2, JKF6, or KhES-3 cells.
See also Figure S1 and Table S1.

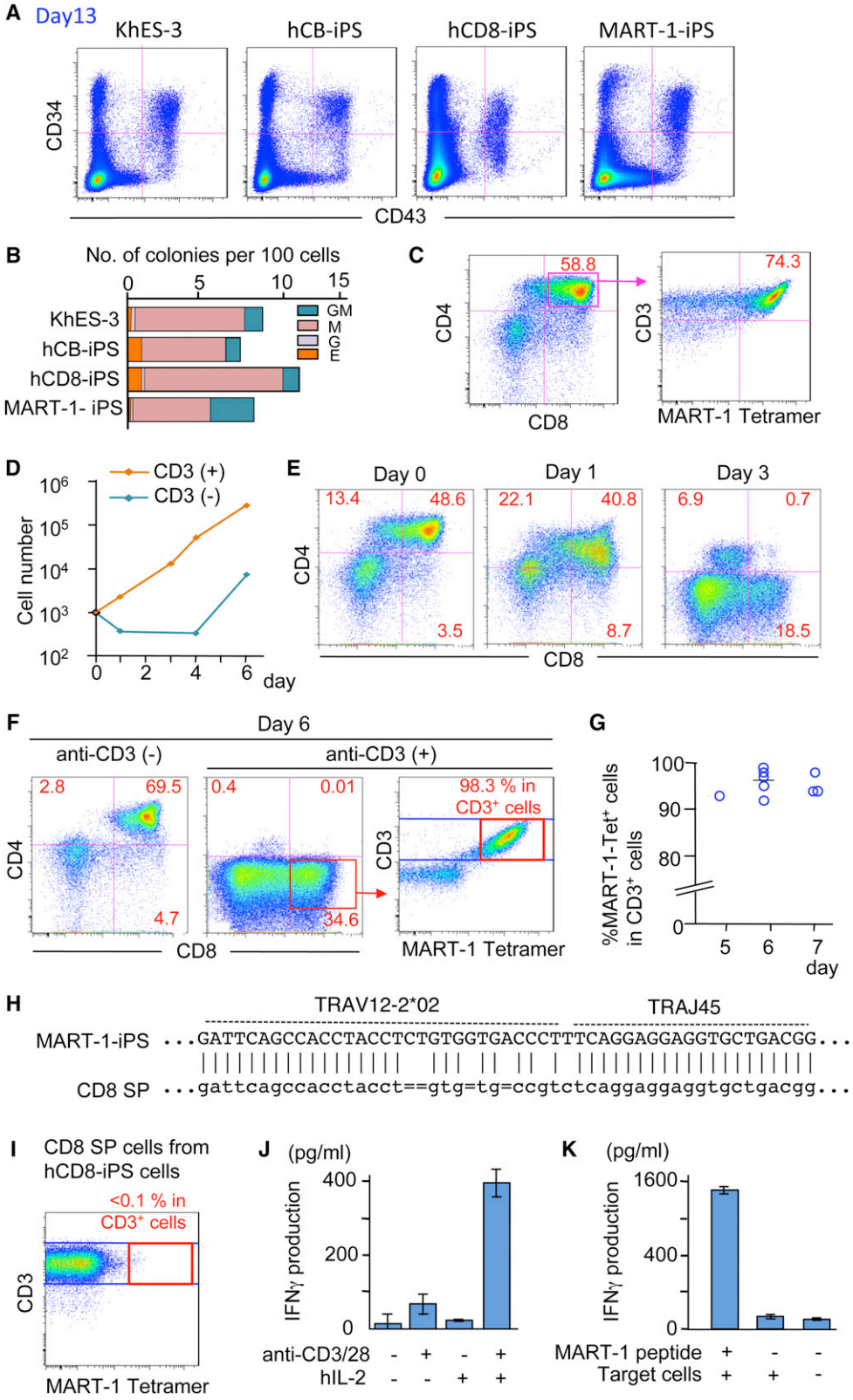

Figure 2. Regeneration of Antigen-Specific CD8SP Mature T Cells in an In Vitro Culture from hT-iPSCs

(A) Generation of hematopoietic progenitor cells from iPSCs of different origins. A cluster of human iPSC colonies was cut into small clumps and plated on a monolayer of OP9 stromal cells. On day 13, cells were harvested by tripsinization and stained for the expression of CD34 versus CD43.

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the MHC-TCR ligation in a monolayer coculture system. For this approach, we felt that a more straightforward induction would suffice, particularly as most of the DP cells generated express a TCR that is specific for the MART-1 epitope. SP cells can be induced from DP cells with the simple addition of anti-CD3 antibody even in the absence of TCR-MHC ligation (Takahama et al., 1994). Thus, we added anti-CD3 mAb to the culture on day 35, when DP cells are already abundant. The number of $CD8^+CD3^+$ MART-1^+ cells increased by 300-fold over the 6 days following TCR stimulation (Figure 2D). After stimulation, the DP cells started to decrease on day 1 and were barely detectable by day 3, when populations of $CD4^+$ and $CD8^+$ T cells became evident (Figure 2E). This time course suggests that the generation of $CD4^+$ and $CD8^+$ T cells is not due to the expansion of pre-existing mature T cells but instead to the induction of maturation from DP into SP T cells. On day 6, the $CD4^+$ T cells disappeared and the number of $CD8^+$ T cells increased further (Figure 2F, middle panel). In the control nonstimulated samples, the cells remained virtually unchanged (Figure 2F, left panel). Importantly, the resulting $CD8^+$ T cells were almost exclusively specific for the MART-1 epitope (Figure 2F, right panel). We obtained similar findings in nine independent experiments (Figure 2G). Sequence analysis of TCRα chain mRNA in the regenerated CD8SP T cells in one of these experiments confirmed that a large majority of regenerated CD8SP cells bear the same TCRα chain gene as original MART-1-iPSCs (Figure 2H). By contrast, CD8SP cells induced from hCD8-iPSCs express virtually no MART-1-tetramer$^+$ cells (Figure 2I).

We also examined whether the generated CD8SP cells are functionally mature. MART-$1^+CD3^+CD8^+$ T cells were isolated using a cell sorter and stimulated with beads coated with a mixture of anti-CD3 mAb and anti-CD28 mAb. IFNγ production by the $CD8^+$ T cells was detected following this CD3/CD28 stimulation, and production of IFNγ was synergistically enhanced by the addition of hIL-2 (Figure 2J). To examine whether the $CD8^+$ T cells can be activated in an antigen-specific manner, they were cocultured with human EBV-lymphoblastoid cell line expressing HLA-A*02:01 with or without MART-1-peptide for 24 hr. The $CD8^+$ T cells produced a substantial amount of IFNγ in the presence of a specific peptide (Figure 2K). Thus, this iPSC-based approach appears to be effective in regenerating the functional antigen-specific CTLs.

It is important to note that the proportion of MART-1-specific T cells in the $CD3^+$ cells population was around 70%–80% in DP cells, but it became more than 95% in the $CD8^+$ T cells. It is known that DP cells rearrange their TCRα chain genes several times unless they get positively or negatively selected (Petrie et al., 1993). Thus, it is likely that the MART-1-tetramer negative/low cells in DP cells (Figure 2C) represent cells in which the inherited Vα-Jα gene has been deleted through further rearrangement. Why, then, do MART-1-tetramer$^+$ cells eventually predominate in the $CD8^+$ T cells? One explanation may come from differential stability of pairing of TCRα and β chains; the cells that have formed a new TCR by replacing the original TCRα chain may not be able to compete with T cells bearing the inherited TCR.

To summarize, we have succeeded in the expansion of antigen-specific T cells by making iPSCs from T cells and differentiating them back into T cells. Further studies are needed to determine whether these regenerated mature T cells can kill cancer cells in vitro or in vivo. It may also be possible to develop a strategy that avoids the need to produce mature T cells in vitro. One idea is to produce T cell progenitors from antigen-specific iPSCs and transfer them to patients, anticipating that the transferred T cell progenitors would migrate into the thymus and produce a large number of naive CTLs that are specific for the tumor antigen. Along these lines, we have developed a coculture system using murine stromal cells that can be used to expand T cell progenitors induced from human hematopoietic stem cells (Meek et al., 2010). We have also developed a feeder-free culture system that makes it possible to expand mouse early T cell progenitors in an almost limitless fashion (Ikawa et al., 2010). If this type of approach is effective, a large number of naive CTLs bearing tumor-antigen-specific TCR could be generated in vivo in a patient. Thus, by immunizing the patient with the tumor antigen, we could expect to see a strong and long-lasting immune response to the tumor.

(B) Sorted $CD34^+CD43^+$ cells were subjected to CFU-C assay. Average numbers and proportions of colony type of triplicate cultures are shown. GM, G, M, and E stand for colony containing granulocytes and monocytes, granulocytes, monocytes, and erythroid cells, respectively.
(C) Cells generated from MART-1-iPSCs on day 40 of culture. The proportion of MART-1-tetramer$^+$ cells in $CD3^+$DP cells in six independent experiments was 72.5 ± 11.1 (mean ± SD).
(D) Number of MART-1-specific $CD8^+$ T cells in the culture with or without anti-CD3 mAb treatment.
(E) Time course of CD8SP cell generation in an in vitro culture system. CD4 versus CD8 profiles of cells before and after TCR stimulation (day 1 and day 3) are shown.
(F) Efficient generation of antigen-specific mature T cells in vitro from T-iPSCs. Left and middle panels show CD4 versus CD8 profiles of cells generated on day 6 without or with TCR stimulation, respectively. Right panel shows the CD3 versus MART-1-tetramer staining of $CD8^+$ T cells gated in a rectangle in the middle panel.
(G) Proportion of MART-1-specific T cells in the generated $CD8^+$ T cells. A total of nine independent experiments were performed. $CD8^+$ T cells were induced by TCR stimulation in the same manner as in (D), and generated cells were recovered on indicated days. The proportion of MART-1-specific T cells was determined in a similar manner as that shown in (F). A horizontal bar in the panel represents an average (96.5%) of five values on day 6. An average of all nine values was 95.5%.
(H) Sequence data of TCRα chain gene in CD8SP cells generated from MART-1-iPSCs in comparison with that in the original MART-1-iPSCs.
(I) Flow cytometric profile of CD8SP cells generated from hCD8-iPSCs.
(J) Production of IFNγ by $CD8^+$ T cells upon TCR stimulation. $CD8^+$ T cells were isolated by a cell sorter and stimulated by adding human T-Activator CD3/CD28 Dynabeads, in the absence or presence of hIL-2. After 16 hr, the concentration of IFNγ in medium was measured by ELISA.
(K) Production of IFNγ by $CD8^+$ T cells upon antigen-specific stimulation. IFNγ secretion was measured by ELISA from the supernatant after 24 hr coculture of 1×10^5 CD8 SP cells derived from MART-1-iPSCs with 1×10^4 HLA-A*02:01-positive EBV-lymphoblastoid cells (CIRA0201) pulsed or not with MART-1-peptide (EAAGIGILTV). Mean ± SE of triplicates is shown.
See also Figure S2.

ACCESSION NUMBERS

The array data shown in this paper are available in the RCAI RefDIC database as follows: hi70-2 sample RSM15825, KhES-3 sample RSM14046, and JKF6 sample RSM14328.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes two figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.12.006.

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