



# Generation of Rejuvenated Antigen-Specific T Cells by Reprogramming to Pluripotency and Redifferentiation

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# SUMMARY

Adoptive immunotherapy with functional T cells is potentially an effective therapeutic strategy for combating many types of cancer and viral infection. However, exhaustion of antigen-specific T cells represents a major challenge to this type of approach. In an effort to overcome this problem, we reprogrammed clonally expanded antigen-specific CD8<sup>+</sup> T cells from an HIV-1-infected patient to pluripotency. The T cell-derived induced pluripotent stem cells were then redifferentiated into CD8<sup>+</sup> T cells that had a high proliferative capacity and elongated telomeres. These "rejuvenated" cells possessed antigen-specific killing activity and exhibited T cell receptor gene-rearrangement patterns identical to those of the original T cell clone from the patient. We also found that this method can be effective for generating specific T cells for other pathology-associated antigens. Thus, this type of approach may have broad applications in the field of adoptive immunotherapy.

# INTRODUCTION

T cells play a central role in acquired immunity and the configuration of systemic immunity against pathogens. In particular, cytotoxic T lymphocytes (CTLs) are major components of this systemic response to microorganisms, viral infections, and neoplasms (Greenberg, 1991; Zhang and Bevan, 2011). T cells initiate their proliferative and effector functions upon human leukocyte antigen (HLA)-restricted recognition of specific antigen peptides via T cell receptors (TCRs). This is greatly beneficial in enabling the selective recognition and eradication of target cells, and also in long-term immunological surveillance by long-lived memory T cells (Butler et al., 2011; Jameson and Masopust, 2009; MacLeod et al., 2010). However, viruses in chronic infection or cancers often hamper or escape the T cell immunity by decreasing the expression of molecules required for T cell recognition or by inhibiting antigen presentation (Virgin et al., 2009). In addition, continuous exposure to chronically expressed viral antigens or cancer/self-antigens can drive T cells into an "exhausted" state. This is characterized by loss of effector functions and the potential for long-term survival and proliferation, ultimately leading to the depletion of antigen-responding T cell pools (Klebanoff et al., 2006; Wherry, 2011).

The infusion of ex vivo-expanded autologous antigen-specific T cells is being developed clinically for T cell immunotherapy. However, up to now, highly expanded T cells have not proven to be particularly effective (June, 2007). This is in part explained by losses of function that occur during the ex vivo manipulation of patient autologous T cells. In another instance, genetic modification of antigen receptors is an ambitious but only partially successful way to add desired antigen specificity to nonspecific T cells (Morgan et al., 2006; Porter et al., 2011). The therapeutic effect also strongly depends on the extent of functional loss that occurs during the ex vivo manipulation of T cells and on the stability of exogenous antigen receptor expression specific to target molecules in the presence of the endogenous TCR genes (Bendle et al., 2010; Brenner and Okur, 2009).

For the purpose of overcoming these obstacles, the therapeutic potential of induced pluripotent stem cells (iPSCs) is being

explored. Embryonic stem cells (ESCs) or iPSCs have the capacity for self-renewal while maintaining pluripotency (Takahashi et al., 2007) and could potentially form the basis for the unlimited induction of antigen-specific juvenile T cells. However, there are challenges to this approach as well, given that methods for the differentiation and immunological education of ESCs and iPSCs, or indeed that of intermediate hematopoietic stem and/or progenitor cells, into fully matured functional human T cells are not well established (Timmermans et al., 2009). Reprogramming the nuclei of lymphocytes was historically performed for studying whether terminally differentiated or fully matured somatic cells could revert to a pluripotent state. The first demonstration of lymphocyte reprogramming employed somatic cell nuclear transfer in murine B and T cells, proving that terminally differentiated somatic cells were reprogrammable (Hochedlinger and Jaenisch, 2002). Reprogramming murine B cells into pluripotent stem cells by iPSC technology also provided definite proof for fate reversibility in fully matured somatic cells (Hanna et al., 2008). From another point of view, nuclear reprogramming of lymphocytes is seen as having applications for regenerative medicine different than those for scientific research. The irreversible rearrangement of genes encoding immunoglobulins and TCRs was recognized solely as a genetic marker in somatic cell nuclear transfer and iPSC research. However, the preserved rearrangements in genomic DNA can also provide a blueprint of "educated" weapons for attacking cancers and pathogens in adoptive immunotherapy. Although several groups have reported the generation of T cell-derived iPSCs (T-iPSCs), their clinical applications have yet to be thoroughly explored (Brown et al., 2010; Loh et al., 2010; Seki et al., 2010; Staerk et al., 2010).

In the present study, we chose a T cell clone specific to an HIV type 1 (HIV-1) epitope of known structure to act as a generic representation of <u>iPSC-</u>mediated T cell regeneration. We successfully induced <u>iPSCs</u> from antigen-specific T cells and redifferentiated them into functional T cells. This may act as proof of concept for the application of "rejuvenated" T cells in treating various diseases. Crucial to this concept was that T-<u>iPSCs</u> retained the assembled "endogenous" TCR genes even after being subjected to nuclear reprogramming. Furthermore, redifferentiated T cells showed the same pattern of TCR gene arrangement as that in the original T cells. These features may therefore serve as the foundation for the reproduction of unlimited numbers of T cells that express desired TCRs conferring to antigen specificity.

# RESULTS

# Reprogramming an Antigen-Specific Cytotoxic T Cell Clone into Pluripotency

To establish T cell-derived **iPSCs**, we magnetically separated the CD3<sup>+</sup> T cell population from peripheral blood mononuclear cells (PBMCs) of healthy volunteers. The isolated CD3<sup>+</sup> T cells were stimulated with human CD3 and CD28 antibody-coated microbeads ( $\alpha$ -CD3/28 beads) in the presence of interleukin-2 (IL-2). We then transduced the activated CD3<sup>+</sup> T cells with separate retroviral vectors that individually code for *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*. Human ESC-like colonies were obtained within 25 days of culture (Figure S1A available online).

We also isolated PBMCs from an HLA-A24-positive patient with a chronic HIV-1 infection. CD8<sup>+</sup> CTL clones specific for an

antigenic peptide (amino acids [aa] 138-145) from the HIV-1 Nef protein (Nef-138-8(WT); RYPLTFGW) (Altfeld et al., 2006) were established. One of the clones, H25-#4, was stimulated using a-CD3/28 beads in the presence of IL-2 and then transduced simultaneously with six retroviral vectors encoding OCT3/4, SOX2, KLF4, c-MYC, NANOG, and LIN28A. However, we could not reprogram H25-#4 into pluripotency, possibly due to the cells being in a low infectious and exhausted state, or due to insufficient expression of the reprogramming factors. In response, we attempted to increase transduction efficiency and transgene expression by using two Sendai virus (SeV) vectors. One of them encodes tetracistronic factors (OCT3/4, SOX2, KLF4, and c-MYC) (Nishimura et al., 2011) with the miR-302 target sequence (SeVp[KOSM302L]; K.N., M.O., and M.N., data not shown), and another encodes SV40 large T antigen (SeV18[T]) (Fusaki et al., 2009). After transduction of phytohemagglutinin (PHA)-activated H25-#4 cells with the SeV vectors in the presence of IL-7 and IL-15, sufficient numbers of human ESC-like colonies appeared within 40 days of culture (Figure 1A). Use of this SeV system and optimization of transduction conditions greatly improved the reprogramming efficiency. It enabled us to reprogram several CD8<sup>+</sup> or CD4<sup>+</sup> T cell clones specific to pp65 antigen in cytomegalovirus (CMV), glutamic acid decarboxylase (GAD) antigen in type 1 diabetes, and  $\alpha$ -GalCer (Table 1).

The resultant CD3<sup>+</sup> T cell- and H25-#4-derived ESC-like colonies (TkT3V1-7 and H254SeVT-3, respectively) exhibited alkaline phosphatase (AP) activity and expressed the pluripotent cell markers SSEA-4, Tra-1-60, and Tra-1-81 (Figures S1B-S1E and 1B-1E). H254SeVT-3 expressed HLA-A24 (Figure 1F). Both TkT3V1-7 and H254SeVT-3 also expressed human ESCrelated genes (Figures S1F and 1G). The expression of exogenous reprogramming factors from the integrated provirus (TkT3V1-7) was halted (Figure S1F), and nonintegrated SeV genomic RNA was successfully removed from the cytosol by RNAi or by self-degradation caused by temperature-sensitive mutations (H254SeVT-3) (Figure 1H). Comparison of geneexpression profiles revealed that the gene-expression patterns in the ESC-like cells were similar to those in human ESCs, but differed significantly from those in peripheral blood (PB) T cells (Figure S1G). Scant methylation of the OCT3/4 and NANOG promoter regions was confirmed using bisulfite PCR, thus indicating successful reprogramming (Freberg et al., 2007) (Figures S1H and 1I). In addition, when injected into nonobese diabetic severe combined immunodeficient (NOD-Scid) mice, those cells formed teratomas containing characteristic tissues derived from all three germ layers, which is indicative of pluripotency (Brivanlou et al., 2003) (Figures S3 and 2A). Therefore, those colonies were confirmed as typical human iPSCs.

# T-iPSCs Carry Preassembled TCR Genes from the Original T Cell

Almost all TCRs are composed of heterodimerically associated  $\alpha$  and  $\beta$  chains. *TCRA* or *TCRB* gene (encoding  $\alpha$  chain or  $\beta$  chain, respectively) rearrangements are involved in normal  $\alpha\beta$  T cell development in the thymus. These rearrangements enabled us to determine retrospectively whether the **iPSCs** were derived from an  $\alpha\beta$  T cell. The BIOMED-2 consortium designed multiplex-PCR primers for analyzing *TCRB* gene assemblies (van Dongen et al., 2003), and we designed the primers for detecting *TCRA* 



# Figure 1. Generation of Human iPSCs from a CTL Clone

(A) Schematic illustration showing the generation of T-IPSCs from H25-#4 T cells using SeV vectors encoding polycistronic OCT3/4, SOX2, KLF4, and c-MYC, or SV40 large T antigen. The "tapering" indicates the gradual replacement of culture medium with human IPSC medium.

(B–F) AP activity (B) and expression of pluripotency markers (SSEA-4, C; Tra-1-60, D; and Tra-1-81, E) and HLA-A24 (F) in H254SeVT-3 cells. Nuclei were counterstained with DAPI. The scale bar represents 200 µm.

(G) Quantitative PCR for pluripotency genes in H25-#4, KhES3, TkT3V1-7, and H254SeVT-3 cells. Individual PCR reactions were normalized against 18S ribosomal RNA (rRNA).

Table 1. Generation of Human T-iPSCs from Various Patient-Derived T Cell Specimens					
Antigen	T Cell Source	Initial Cell Number	No. of ESC-like Colonies	No. of Colonies Picked up for Establishing T- <mark>iPSC</mark> Clones	Date (MM/YYYY)
HIV-1 Nef	monoclonal T cell clone	4 × 10 <sup>5</sup>	7	7	05/2011
CMV pp65	polyclonal tetramer-sorted cells	${\sim}$ 5,000	15	15	07/2011
GAD	monoclonal T cell clone	1 × 10 <sup>6</sup>	>100	not picked up	08/2012
		5 × 10 <sup>5</sup>	>100	19	08/2012
α-GalCer	FACS-sorted V $\alpha$ 24 <sup>+</sup> cells	1 × 10 <sup>6</sup>	>100	not picked up	08/2012
		5 × 10 <sup>5</sup>	>100	7	08/2012

Sample cells were transduced with *OCT3/4*, *SOX2*, *KLF4*, *c-MYC*, and SV40 large T-antigen by using two Sendai virus (SeV) vectors (SeVp [KOSM302L] and SeV18[T]). After around 40 days, the number of embryonic stem cell (ESC)-like colonies were counted on the basis of morphology and alkaline phosphatase (AP) activity. All established T cell-derived induced pluripotent stem cell (T-**iPSC**) lines were free from residual SeV vectors (one example in the case of the HIV-1 Nef-specific T-**iPSC**) clone is shown in Figure 1H). CMV, cytomegalovirus; GAD, glutamic acid decarboxylase; FACS, fluorescence-activated cell sorting.

gene assemblies (Figure S2). *TCRB* and *TCRA* gene assemblies were identified as single bands representing each allele in TkT3V1-7 and H254SeVT-3 (Figures S1H, S1I, 1J, and 1K).

We next confirmed the presence of an antigen-recognition site on the TCR that consisted of three complementarity-determining regions (CDR1, CDR2, and CDR3). CDR3 is the most diversifiable among the three because it spans the V(D)J-junction region, where several random nucleotides (N or P nucleotides) are inserted (Alt and Baltimore, 1982; Lafaille et al., 1989). We determined the CDR3 sequences of the assembled TCRA and TCRB genes in TkT3V1-7 and H254SeVT-3 and identified a set of productive TCRA and TCRB gene rearrangements (i.e., in-frame junction with no stop codon) (Table S1 and Table 2). Furthermore, the sequences of CDR3 from H254SeVT-3 and H25-#4 were completely identical at both TCRA and TCRB gene loci. These results indicated that the iPSCs established were derived from a single T cell and that the antigen specificity encoded in the genomic DNA of the T cell was conserved during reprogramming.

# Redifferentiation of T-**iPSCs** into CD8 Single-Positive T Cells Expressing the Desired TCR

Following the application of specific in vitro differentiation protocols, *iPSCs* can give rise to mesoderm-derived cell types, especially hematopoietic stem and/or progenitor cells (Takayama et al., 2008; Vodyanik et al., 2005) (Figure 2B). This was applied to assess the capacity of T-*iPSCs* for hematopoietic differentiation by coculturing on C3H10T1/2 feeder cells in the presence of VEGF, SCF, and FLT-3L for the generation of CD34<sup>+</sup> hematopoietic stem and/or progenitor cells. On day 14 of culture, the cells were transferred onto Delta-like 1-expressing OP9 (OP9-DL1) feeder cells (Timmermans et al., 2009) and were cocultured in the presence of FLT-3L and IL-7 (Ikawa et al., 2010) (Figure 2B). After 21–28 days of culture, the hematopoietic cells differentiated into CD45<sup>+</sup>, CD38<sup>+</sup>, CD7<sup>+</sup>, CD45RA<sup>+</sup>, CD3<sup>+</sup>, and TCR $\alpha\beta^+$ T lineage cells (Figure S4). As was the case with TCR $\alpha\beta$  transgenic mice (Borgulya et al., 1992) and chimeric mice derived from ESCs produced through nuclear transplantation of T cells (Serwold et al., 2007), aberrant expression of TCR $\alpha\beta$  was observed at the CD4/CD8 double-negative (DN) stage. Although some of these T lineage cells differentiated into the CD4/CD8 double-positive (DP) stage and the more mature CD4 or CD8 single-positive (SP) stages (Figure 2C), we could not characterize the small number of SP cells in more detail.

During thymocyte development, the CD4/CD8 DN and DP stages correspond respectively to the *TCRB*-encoded  $\beta$  chain and TCRA-encoded  $\alpha$  chain assembly stages (von Boehmer, 2004). In the TCRB locus, the negative-feedback regulation of gene assembly and the capacity to deter further rearrangement are very strict (Khor and Sleckman, 2002). In the TCRA locus, by contrast, negative-feedback regulation is relatively loose, and further gene assembly of the preassembled gene, a phenomenon known as "receptor revision," tends to occur (Huang and Kanagawa, 2001; Krangel, 2009). In experiments using TCRα transgenic mice, the reactivation of Rag1 and Rag2, genes related to recombination machinery, occurred in CD4/CD8 DP-stage thymocytes, and gene assembly of endogenous Tcra was observed (Padovan et al., 1993; Petrie et al., 1993). Such further gene assembly would be exceedingly undesirable for our purposes, because it would probably convert the tropism of the TCR and render the redifferentiated T cells incapable of attacking the previously targeted antigen. To determine whether such receptor revision could occur in redifferentiating T lineage cells, we collected CD1a<sup>-</sup> DN- and CD1a<sup>+</sup> DP-stage cells from among the CD45<sup>+</sup>, CD3<sup>+</sup>, TCR $\alpha\beta^+$ , and CD5<sup>+</sup> T lineage cells and then analyzed the gene rearrangement of TCR messenger RNAs (mRNAs) (Figures S5A-S5C). Nucleotide sequences of TCRB mRNAs in the T lineage cells were identical to those in

(K) Multiplex PCR analysis for detection of TCRA gene rearrangements (V-J $\alpha$  assemblies).

See Figures S1, S2, and S3 for additional data.

<sup>(</sup>H) Detection of the remnants of SeV genomic RNAs by RT-PCR. Each column represents the template cDNA synthesized from H254SeVT-3 cells, SeVp [KOSM302L] virus solution, and SeV18[T] virus solution. cDNAs from virus solution were the positive controls.

<sup>(</sup>I) Bisulfite sequencing analyses of the OCT3/4 and NANOG promoter regions in H25-#4 and H254SeVT-3 cells. White and black circles represent unmethylated and methylated (Me) CpG dinucleotides, respectively.

<sup>(</sup>J) Multiplex PCR analysis to detect *TCRB* gene rearrangements in the H254SeVT-3 genome. Tubes A and B contain Vβ-(D)Jβ assemblies; Tube C contains D-Jβ assemblies.



# Figure 2. Redifferentiation of T-iPSCs into T Cells

(A) Representative hematoxylin- and eosin-stained sections of a teratoma formed in a NOD/ShiJic-*scid* mouse testis. H254SeVT-3 differentiated into cell lineages derived from endoderm (goblet cells in gut-like epithelial), mesoderm (smooth myocytes in muscle tissue), and ectoderm (retina cells in pigmented epithelial). The scale bar represents 100  $\mu$ m.

the T-**iPSCs** at both the DN and DP stages. By contrast, some *TCRA* mRNAs at the DN and DP stages were identical to those in the T lineage cells, but others differed, and differing sequences were observed more frequently at the DP stage than the DN stage (Table S2). *RAG1* and *RAG2* expression were observed at both the DN and the DP stages, though stronger expression was observed at the DP stage (Figure S5D).

To create mature CD8 SP cells from T-iPSC-derived T lineage cells without receptor revision, we focused on TCR signaling. Turka et al. (1991) reported that TCR signaling via peptide-major histocompatibility complex (MHC) complexes during positive selection ends expression of RAG genes and prevents further assembly of TCR genes. They also showed that mimicking TCR signaling using CD3 antibodies had the same effect. Therefore, we tried to stimulate the TCRs of redifferentiating T lineage cells before the completion of the DN-to-DP transition (Figure 2B). For this experiment, we cultured T lineage-committed cells on OP9-DL1, stimulated them with  $\alpha$ -CD3/28 beads or PHA (we defined this as the first stimulation) and then cocultured them with irradiated HLA-A24<sup>-</sup> PMBCs in the presence of IL-7 and IL-15, which are required for the generation of memory phenotype CD8<sup>+</sup> T cells (Kaneko et al., 2009; Prlic et al., 2002; Tan et al., 2002). After 14 days, CD8 SP cells appeared (Figure 2D). These were deemed to be derivatives of H254SeV-3 based on their expression of HLA-A24 (Figure 2E). These CD8 SP cells did not express the immature thymocyte marker CD1a, but they were positive for CD56, which is expressed on CD8<sup>+</sup> T cells cultured in vitro (Lu and Negrin, 1994). In addition, these cells expressed CD7 and some CD2, but not CD5. On the one hand, they did not express PD-1, a marker of exhausted T cells (Figure 2E). On the other hand, some of them expressed the memory T cell markers CCR7, CD27, and CD28 simultaneously, thus representing a central memory T cell phenotype (Figures 2F and 2G) (Romero et al., 2007).

To test whether the redifferentiated CD8 SP cells would recognize the same epitope on the same HLA, the entire population of redifferentiated T cells was mixed with the A24/Nef-138-8(WT) tetramer and subjected to flow-cytometric analysis (Kawana-Tachikawa et al., 2002). Most of the CD8 SP cells were stained positively by the A24/Nef-138-8(WT) tetramer, but not by the control tetramer, which represents HIV-1 envelope-derived peptides (RYLRDQQLL; Figure 3A and data not shown). We then collected the A24/Nef-138-8(WT) tetramer-reactive CD8+ cells and expanded them once again using α-CD3/28 beads or PHA stimulation (defined as the second stimulation; Figure 3A). Finally, after several independent redifferentiation experiments, we obtained A24/Nef-138-8(WT) tetramer-reactive CD8 SP cells (reT-1, reT-2.1, reT-2.2, and reT-3). As expected, sequence analysis of TCRA and TCRB mRNAs in the redifferentiated CD8 SP cells revealed that the TCR gene rearrangement pattern was identical to that in the H25-#4 original T cell clone (Figure 3B and Table 1).

To determine whether the redifferentiated CD8 SP cells were of the T cell lineage, we used quantitative PCR to compare gene-expression profiles among redifferentiated CD8 SP cells, PB CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and the H25-#4 original T cell clone. As shown in Figure 3C, the expression patterns of CD3, CD4, and CD8 were similar among PB CD8<sup>+</sup> T cells, redifferentiated CD8 SP cells, and the H25-#4 original T cell clone. However, the pattern differed from those in PB CD4<sup>+</sup> T cells (Figure 3C). Cytotoxic "signature" genes such as granzyme B (GZMB), perforin (PRF1), interferon- $\gamma$  (IFN- $\gamma$ ; IFNG), and FAS ligand (FASLG) were expressed in PB CD8<sup>+</sup> T cells. These genes were also expressed relatively strongly in redifferentiated CD8 SP cells and in the H25-#4 original T cell clone; that is, in already-primed T cells (Figure 3D). The expression patterns of several factors involved in transcription or signal transduction and of cell-surface molecules were similar among PB CD8<sup>+</sup> T cells, redifferentiated CD8 SP cells, and the H25-#4 original T cell clone (Figure 3E). To exclude the possibility that the redifferentiated CD8 SP cells had acquired natural killer (NK)-like properties during their coculture with OP9-DL1 or PBMCs, we used a complementary DNA (cDNA) microarray to analyze global gene-expression profiles in redifferentiated CD8 cells, the H25-#4 original T cell clone, and PB NK cells. Correlation and cluster analyses of the gene-expression profile of the redifferentiated CD8 SP cells showed it to be similar to that of the H25-#4 original T cell clone but different from that of NK cells (Figures 3F and 3G). These data strongly suggest that T-iPSCs are able to redifferentiate into CD8+ T cells that exhibit the same antigen specificity as that of the original T cell.

# Generation of Highly Proliferative T Cells through TiPSCs

Fewer than  $10^5$  T lineage cells were obtained from  $\sim 3 \times 10^5$ T-iPSCs after coculture with C3H10T1/2 and OP9-DL1 cells. However, they could be expanded to  $>10^8$  cells with the first stimulation (data not shown). After separating A24/Nef-138-8(WT) tetramer-reactive CD8<sup>+</sup> cells, we assessed the expansion rate induced by the second stimulation and also assessed the establishment of reT-1, reT-2.2, and reT-3. We found that these cells expanded from 100-fold to 1,000-fold within 2 weeks in the presence of IL-7 and IL-15, whereas the H25-#4 original T cell clone expanded only about 20-fold (Figure 4A). Even after 100to-1,000-fold expansions, some cells still expressed central memory T cell markers such as CCR7, CD27, and CD28 (Figure S6). Perhaps with passage through the iPSC state, wherein telomerase activity is guite high (Marion et al., 2009; Takahashi et al., 2007), re-elongation of shortened telomeres in the H25-#4 original T cell clone gives the redifferentiated T cells high replicative potential (Monteiro et al., 1996; Weng et al., 1998). In fact, the redifferentiated T cells carried longer telomeres than the original T cell clone (Figure 4B), an overall process that we call

<sup>(</sup>B) Schematic illustration of redifferentiation from T-iPSCs into T cells.

<sup>(</sup>C) Flow-cytometric analysis of the phenotypes of differentiating T lineage cells at 37 days after starting redifferentiation.

<sup>(</sup>D and E) Flow-cytometric analysis of the phenotypes of T cells at 60 days after starting redifferentiation. Fluorescence-activated cell sorting (FACS) analyses revealed CD8 single-positive maturation (D) and expression of several T cell markers (E).

<sup>(</sup>F and G) Memory phenotypes of redifferentiated CD8<sup>+</sup> T cells. There existed memory-phenotyped cells such as all positive for CCR7 (F), CD27, and CD28 (G). Data are representative of at least three independent experiments. See Figures S3, S4, and S5 and Table S2 for additional data.

#### Table 2. TCR Gene Rearrangements in H25-4, H254SeVT-3, or Redifferentiated CD8<sup>+</sup> T Cells Genome or Cell mRNA Productivity Rearrangement Sequence of Junctional Region Vα Jα 3′Vα P(N) 5′Jα TCRA H25-4 TRAV8-3\*01 TRAJ10\*01 TGTGCTGTGGGT Т TCACGGGAGGAGGAAACAAACTC productive genome ACCTTTT unproductive<sup>a</sup> TRAV13-1\*01 TRAJ29\*01 TGTGCAGCAA TCC TCAGGAAACACACCTCTTGTCTTT Т TCACGGGAGGAGGAAACAAACTC H254SeVT-3 productive TRAV8-3\*01 TRAJ10\*01 TGTGCTGTGGGT genome ACCTTTT TCC TCAGGAAACACACCTCTTGTCTTT TRAV13-1\*01 TRAJ29\*01 TGTGCAGCAA unproductive<sup>a</sup> Т reT-1 mRNA productive TRAV8-3\*01 TRAJ10\*01 TGTGCTGTGGGT TCACGGGAGGAGGAAACAAACTC ACCTTTT TRAV13-1\*01 TRAJ29\*01 TCC TCAGGAAACACACCTCTTGTCTTT unproductive<sup>a</sup> TGTGCAGCAA reT-2.1 mRNA productive TRAV8-3\*01 TRAJ10\*01 TGTGCTGTGGGT Т TCACGGGAGGAGGAAACAAACTC ACCTTTT unproductive<sup>a</sup> TRAV13-1\*01 TRAJ29\*01 TGTGCAGCAA TCC TCAGGAAACACACCTCTTGTCTTT reT-3 mRNA productive TRAV8-3\*01 TRAJ10\*01 TGTGCTGTGGGT Т TCACGGGAGGAGGAAACAAACTC ACCTTTT unproductive<sup>a</sup> TRAV13-1\*01 TRAJ29\*01 TGTGCAGCAA TCC TCAGGAAACACACCTCTTGTCTTT Vβ Dβ Jβ 3'Vβ N1-Dβ-N2 5'Jβ TCRB H25-4 genome productive TRBV7-9\*01 TRBD1\*01 TRBJ2-5\*01 TGTGCCAGCAGCTTA CGGGACAGGGTGCCG GAGACCCAGTACTTC TRBJ2-7\*01 TACAAAGCTGTAACATTGTG GGGACAACT CTACGAGCAGTACTTCGGGCCG unproductive germline TRBD1\*01 H254SeVT-3 productive TRBV7-9\*01 TRBD1\*01 TRBJ2-5\*01 TGTGCCAGCAGCTTA CGGGACAGGGTGCCG GAGACCCAGTACTTC genome TRBD1\*01 TRBJ2-7\*01 TACAAAGCTGTAACATTGTG GGGACAACT CTACGAGCAGTACTTCGGGCCG unproductive germline reT-1 mRNA productive TRBV7-9\*01 TRBD1\*01 TRBJ2-5\*01 TGTGCCAGCAGCTTA CGGGACAGGGTGCCG GAGACCCAGTACTTC TRBJ2-5\*01 reT-2.1 mRNA TRBV7-9\*01 TRBD1\*01 TGTGCCAGCAGCTTA CGGGACAGGGTGCCG GAGACCCAGTACTTC productive reT-3 TRBV7-9\*01 TRBD1\*01 TRBJ2-5\*01 TGTGCCAGCAGCTTA CGGGACAGGGTGCCG GAGACCCAGTACTTC mRNA productive

PCR-amplified samples (H25-4: not shown; H254SeVT-3: shown in Figures 1J and 1K; reT-1, reT-2.1, and reT-3: shown in Figure 3B) were sequenced, then V, D, and J segment usages and junctional sequences in CDR3 were identified. Following reprogramming and redifferentiation, there were no alterations in gene rearrangement in either allele at the *TCRA* and *TCRB* gene loci. See Table S1 for additional data on another T-iPSC clone (TkT3V1-7).

<sup>a</sup>Out-of-frame junction (at CDR3).



### Figure 3. Characterizations of Redifferentiated T Cells as T Cells

(A) Recognition of A24/Nef-138-8(WT) tetramer at 50–60 days after starting redifferentiation, analyzed by flow cytometry (upper panel). Tetramer-positive cells were sorted by FACS or magnetically selected, then cultured for an additional 14 days, after which the expanded T cells were reanalyzed for tetramer (lower panel).

(B) TCR mRNAs were identified in a SMART-mediated cDNA library for reT-1, reT-2.1, and reT-3 cells. GAPDH is an internal control for PCRs.

(C–E) Quantitative PCR to compare the expression of major cell surface molecules (C), cell lytic molecules (D), and transcription factors and signal-transduction molecules (E) among PB CD4<sup>+</sup>, PB CD8<sup>+</sup>, reT-2.1, and H25-#4 cells. Individual PCR reactions were normalized against 18S rRNA.

(F and G) Global gene expression was analyzed using a cDNA microarray. Heat maps show the correlation coefficients between samples (F) and differential expression (>3-fold) of genes relative to NK cells (G). Red and green colorations indicate increased and decreased expression, respectively.

# Cell Stem Cell Rejuvenation of T Cells through Reprogramming



"rejuvenation." Throughout the experiments, neither autonomous cell expansion nor aberrant cell survival without cytokines as leukemia cells was observed (data not shown). Taken together, these data indicate that by passing through the TiPSC state, cloned cytotoxic T cells can become "rejuvenated" to central memory-like T cells with excellent potential for proliferation and survival.

# Redifferentiated CD8<sup>+</sup> T Cells Exhibit Antigen-Specific T Cell Functionality

To determine whether redifferentiated CD8<sup>+</sup> T cells exerted cytotoxic effects upon recognition of specific peptides in the context of an MHC, we performed functional assays using HLA-A24-positive B-LCL cells as antigen-presenting cells. Gag-28-9(WT) (KYKLKHIVW) is an antigenic peptide (aa 28–36) from the HIV-1 Gag protein (Altfeld et al., 2006), whereas Nef-138-8(2F) (RFPLTFGW) is a Tyr-to-Phe-substituted single-

# Figure 4. Redifferentiated T Cells Show T Cell Functionality and the Same Antigen Specificity as the Original CTL Clone

(A) Expansion ratios for reT-1, reT-2.2, and reT-3 cells elicited by PHA, IL-7, and IL-15 stimulation for 2 weeks. H25-#4 is the original clone. S19-#7 and T26-#26 were other Nef-138-8(WT)-specific CTL clones derived from different patients.

(B) Relative telomere length determined using flow-FISH. Data are presented as mean  $\pm$  SEM.

(C) Intracellular production of granzyme B (left panel) and CD107a mobilization (right panel) induced by stimulation of reT-2.1 cells with  $\alpha$ -CD3/CD28 beads or Nef-138-8(WT). Shaded plot: stimulated cells, isotype antibody; gray line: unstimulated cells, granzyme B or CD107a antibody; black line: stimulated cells, granzyme B or CD107a antibody.

(D) IFN- $\gamma$  production in the presence of Nef-138-8(WT) measured using ELISPOT. Data are presented as mean  $\pm$  SD. N.D., not determined. (E) Standard <sup>51</sup>Cr release assay performed using

the indicated concentrations of Nef-138-8(WT). Effector:target = 5:1.

See Figure S6 for additional data.

residue mutant form of Nef-138-8(WT). Both peptides were presented on HLA-A24 cells.

One of the major mechanisms by which **CTLS** induce cytotoxity is the secretion of cytolytic molecules triggered by TCR signaling. Intracellular staining revealed that the cytolytic molecule granzyme B was produced and stored in the granules of redifferentiated CD8<sup>+</sup> T cells (Figure 4C, left column). CD107a, also known as lysosomal-associated membrane protein 1 (LAMP1), is a granulocyte membrane protein that transiently appears at the cell surface and is coupled to degranulation (secretion of cytolytic molecules) of the stimulated **CTLS**, after which CD107a re-

turns to the cytoplasm (Rubio et al., 2003). CD107a molecules on the cell surface were captured by a fluorochrome-conjugated antibody when redifferentiated CD8<sup>+</sup> T cells were stimulated with  $\alpha$ -CD3/28 beads or Nef-138-8(WT) peptide, but not in the absence of the beads or Gag-28-9(WT) peptide (Figure 4C, right column). In the second experiment, we used the enzyme-linked immunosorbent spot (ELISPOT) assay to assess cytokine productivity per cell and confirmed that redifferentiated CD8<sup>+</sup> T cells produced significant levels of IFN- $\gamma$  in response to stimulation by its specific antigen, Nef-138-8(WT) (Figure 4D). In a separate experiment, we used a <sup>51</sup>Cr release assay to investigate cytolytic capacity and found that redifferentiated CD8<sup>+</sup> T cells lysed <sup>51</sup>Cr-incorporated B-LCLs only when Nef-138-8(WT) was presented on B-LCLs (Figure 4E).

These results are highly indicative that redifferentiated CD8<sup>+</sup> T cells can release cytotoxic molecules and kill antigen-expressing target cells in an antigen-specific manner. Moreover, monoclonal TCRs mediate highly precise cell targeting that should broaden the therapeutic window for antigen-specific T cell therapy by avoiding the troublesome mispairing TCRs that can occur with the commonly used exogenous TCR transfer technique for inducing antigen-specific T cells from hematopoietic stem cells or peripheral mature T cells (Bendle et al., 2010; Brenner and Okur, 2009).

# DISCUSSION

Using a HIV-1-epitope-specific **CTL** clone as a model, we demonstrated here that the reprogramming into pluripotency of a T cell clone and the subsequent redifferentiation to mature functional CD8<sup>+</sup> T cells are possible. These redifferentiated CD8<sup>+</sup> T cells are highly proliferative naive cells with elongated telomeres, and they exert T cell functions in the same HIV-1-epitope-specific manner, permitting the inference that this process of reprogramming and redifferentiation can rejuvenate mature antigen-specific T cells.

Generation of iPSCs from T cells was initially difficult. On the basis of reports by Seki et al. (2010), we also found that SeV is suitable for the reprogramming of aged and exhausted fibroblasts, as well as of T cells. We also found that coexpression of SV40 large T antigen acted synergistically with the classic Yamanaka factors in enhancing the reprogramming efficiency of T cells. Therefore, SV40 large-T antigen introduction using the SeV vector system was also included in the protocol. Worth noting is that c-MYC is a known oncogene, and when it is inserted into the genomic DNA by the retroviral vector, it may become a risk for tumorigenesis in the generation of iPSCs. The same concern does not apply to SeV vector systems, given that the genomic RNA could be removed from the cytosol after reprogramming. Therefore, the utilization of SeV vectors both improved reprogramming efficiency and shielded redifferentiating cells from oncogene- or provirus-mediated tumorigenesis (Kohn et al., 2003).

In the redifferentiation experiments, mimicking TCR signaling led to CD8-linage specification without reassembly of TCRA genes. Preassembled TCR genes are a distinctive feature of T-iPSCs not found on other pluripotent stem cells. TCR $\alpha\beta$  is aberrantly expressed on redifferentiating CD4/CD8 DN cells, and the TCR signaling evoked results in the cessation of RAG expression. Serwold and colleagues reported that aberrantly early expression of TCR from preassembled Tcra and Tcrb following TCR signaling in murine thymocytes drives later lymphomagenesis (Serwold et al., 2010). They cautioned that T-iPSCs might confer risk for TCR-mediated lymphomagenesis. Therefore, the redifferentiation method will need to be further optimized and confirmed for clinical safety before application in practical treatments. This may be achieved by the use of an inducible suicide-gene system for eliminating unwanted tumors after injections (Hara et al., 2008; Veldwijk et al., 2004).

Immunological assays found that the redifferentiated CD8<sup>+</sup> T cells exerted T cell functions such as cytolytic activity, IFN- $\gamma$  secretion, and degranulation in a normal manner when stimulated with their specific antigens. The most striking difference was in their proliferation capacity and elongated telomeres, which correlates with the central-memory T cell phenotype. Stem cell-like memory T cells (T<sub>SCM</sub>) were recently identified as

a subpopulation of T cells that has the capacity for self-renewal and that is multipotent and able to generate central memory, effector memory, and effector T cells (Gattinoni et al., 2011; Turtle et al., 2009). In a humanized mouse model, T<sub>SCM</sub> cells reconstituted the T cell population more efficiently than other known memory subsets while mediating a superior antitumor response. It was found that inhibition of GSK3 $\beta$  enhances the generation of T<sub>SCM</sub> in culture. Combining T-iPSC-mediated T cell rejuvenation with GSK3 $\beta$  inhibition may therefore enable efficient generation of T<sub>SCM</sub> cells and permit highly effective immunotherapy along with the reconstitution of a normal T cell immune system.

Although these data suggest that rejuvenated T cells enjoy an advantage over the original T cell clone, it remains unclear whether these HIV-epitope-specific rejuvenated T cells are effective in improving the overall status of HIV infection. This is because the role of CD8<sup>+</sup> T cells in HIV infection appears to vary depending on the disease stage (Appay et al., 2000; Borrow et al., 1994; Brodie et al., 1999; Day et al., 2006; Koup et al., 1994). Evasion of the immune response through CTL escape is another important factor in HIV pathogenesis, and the escaped virus is a substantial hurdle for HIV therapies (Phillips et al., 1991). Therefore, this system may work best instead against tumors such as a melanoma, for which certain antigenic epitopes are known, or against viral infections other than HIV, for which the roles of CD8<sup>+</sup> cytotoxic T cells are more established. Nonetheless, the system described in our study will make it possible to preserve and to supply highly proliferative, functional CD8<sup>+</sup> T cells specific to a variety of HIV epitopes without worrying about exhaustion. It may also act as a valuable tool in better understanding the role of adoptive immunity in HIV infection.

Here, we have presented a proof of concept of CD8<sup>+</sup> T cell rejuvenation. The concept is not limited only to CD8<sup>+</sup> cytotoxic T cells. It may also be applied to CD4<sup>+</sup> helper or regulatory T cells to control desired or undesired immune reactions in the context of malignancies, chronic viral infections, autoimmune diseases, or transplantation-related immune disorders, if optimization of redifferentiation conditions can be achieved. Biological and technical challenges lie ahead, but the data presented in this work open new avenues toward antigen-specific T cell therapies that will supply unlimited numbers of rejuvenated T cells and will regenerate patients' immune systems.

#### **EXPERIMENTAL PROCEDURES**

#### Generation of Antigen-Specific CTL Clones

Nef138-8(WT)-specific **CTL** lines were induced from PBMCs of a patient chronically infected with HIV-1 who is positive for HLA-A24, as described (Kawana-Tachikawa et al., 2002). Each **CTL** line was expanded from a single-cell sorted tetramer<sup>+</sup> T cell, and the cells in every **CTL** line were confirmed for expression of only one kind of TCR $\alpha\beta$ . For more details of **CTL-**clone establishment, see the Supplemental Experimental Procedures.

### Generation of T-iPSCs

Human <code>iPSCs</code> were established from PB T cells or a <code>CTL</code> clone as described (Takayama et al., 2010), slightly modifying the culture conditions. In brief, T cells were stimulated by  $\alpha$ -CD3/CD28 antibody-coated beads (Miltenyi Biotec) or by 5  $\mu$ g/ml PHA-L (Sigma-Aldrich). The activated cells were transduced with reprogramming factors via retroviral or SeV vectors and were cultured in RH10 medium (RPMI-1640 supplemented with 10% human AB Serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin), which was

gradually replaced with human iPSC medium (Dulbecco's modified Eagle's medium/F12 FAM supplemented with 20% knockout serum replacer, 2 mM L-glutamine, 1% nonessential amino acids, 10  $\mu$ M 2-mercaptoethanol, and 5 ng/ml basic fibroblast growth factor [bFGF]). The established iPSC clones were transfected with small interfering RNA L527 (Nishimura et al., 2011) using Lipofectamine RNAi Max (Invitrogen) for removal of SeV vectors from the cytoplasm.

### Analysis of TCR Gene Rearrangement in Genomic DNA

Genomic DNA was extracted from approximately 5 × 10<sup>6</sup> cells using QIAamp DNA kits (QIAGEN) according to the manufacturer's instructions. For *TCRB* gene rearrangement analysis, PCR was performed according to BIOMED-2 protocols (van Dongen et al., 2003). For *TCRA* gene rearrangement analysis, PCR was performed using the primers shown in Figure S2 and LA Taq HS (TaKaRa). The PCR protocol entailed three amplification cycles (30 s at 95°C, 45 s at 68°C, and 6 min at 72°C); 15 amplification cycles (30 s at 95°C, 45 s at 62°C, and 6 min at 72°C); and 12 amplification cycles (31 s at 95°C, 30 s at 62°C, and 6 min at 72°C). The dominant band within the expected size range was purified using a QIAquick gel-extraction kit (QIAGEN) and was then sequenced. V, D, and J segment usages were identified by comparison to the ImMunoGeneTics (IMGT) database (http://www.imgt.org/) and by using an online tool (IMGT/V-QUEST) (Lefranc, 2003). Gene-segment nomenclature follows IMGT usage.

#### Analysis of TCR Gene Rearrangement in mRNA

A method based on the "switch mechanism at the 5'-end of the reverse transcript (SMART)" (Du et al., 2006) was used to synthesize double-stranded cDNAs (Super SMART cDNA synthesis kit; BD Clontech). Reverse transcription was conducted with the 3' SMART CDS primer, SMART II A oligonucleotides (Super SMART cDNA synthesis kit), and PrimeScript Reverse Transcriptase (TaKaRa) for 90 min at 42°C. Double-stranded cDNA was then synthesize and was amplified with 5' PCR Primer II A (Super SMART cDNA synthesis kit), and reagents were provided in an Advantage 2 PCR Kit (BD Clontech). The PCR protocol entailed 20 cycles of 5 s at 95°C, 5 s at 65°C, and 3 min at 68°C. The amplified double-stranded cDNA was used as templates in TCRA- or TCRB-specific amplification reactions. With forward primer (2<sup>nd</sup>\_5'-SMART) and reverse primer (3'-TRAC for *TCRA*), 25 cycles of amplification were performed (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C). PCR products were cloned into pGEM-T Easy Vector (Promega) and were sequenced.

#### T Cell Differentiation from T-iPSCs

To differentiate human iPSCs into hematopoietic cells, we slightly modified a previously described protocol (Takayama et al., 2008). Small clumps of iPSCs (<100 cells) were transferred onto irradiated C3H10T1/2 cells and cocultured in EB medium (Iscove's modified Dulbecco's medium supplemented with 15% fetal bovine serum [FBS] and a cocktail of 10  $\mu$ g/ml human insulin, 5.5 µg/ml human transferrin, 5 ng/ml sodium selenite, 2 mM L-glutamine, 0.45 mM  $\alpha\text{-monothioglycerol,}$  and 50  $\mu\text{g/ml}$  ascorbic acid) in the presence of VEGF. SCF. and FLT-3L. Hematopoietic cells contained in *iPSC* sacs were collected and were transferred onto irradiated OP9-DL1 cells (provided by RIKEN BRC through the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology [MEXT]) (Watarai et al., 2010). The hematopoietic cells underwent T lineage differentiation on OP9-DL1 cells during coculture in OP9 medium (aMEM supplemented with 15% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin) in the presence of FLT-3L and IL-7. The T lineage cells were then harvested, mixed with irradiated HLA-A24<sup>-</sup> PBMCs, and cocultured in RH10 medium in the presence of IL-7 and IL-15.

#### **Intracellular Staining**

For intracellular staining of granzyme B, T cells were stimulated by  $\alpha$ -CD3/28 beads or peptide-loaded HLA-A24<sup>+</sup> B-LCLs. After 2 hr, brefeldin A (5  $\mu$ g/ml; Invitrogen) was added, with incubation for 4 hours more. Cells were then harvested and fixed in Fixation/Permeabilization solution (BD Biosciences). Intracellular staining was performed as per the manufacturer's protocol using Perm/Wash buffer (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated granzyme B antibody (BD Biosciences). For capturing CD107a

transiently expressed on cell surfaces, T cells were incubated with  $\alpha$ -CD3/28 beads or peptide-loaded HLA-A24<sup>+</sup> B-LCLs and were cultured with FITCconjugated CD107a antibody (BioLegend) for 6 hr. Harvested cells were fixed and stained as described above. Data were acquired on FACSAria II equipment (BD Biosciences) and analyzed using FlowJo software (Tree Star).

#### Measurement of Telomere Length by Flow-FISH

Telomere length was measured using a Telomere PNA Kit/FITC (DAKO) as previously described (Neuber et al., 2003).

### ELISPOT and <sup>51</sup>Cr Release Assays

The antigen-specific responses of T cells were measured using an ELISPOT assay for IFN- $\gamma$  and a standard <sup>51</sup>Cr release assay as described (Kawana-Tachikawa et al., 2002; Tsunetsugu-Yokota et al., 2003). HLA-A24<sup>+</sup> B-LCLs were used as antigen-presenting cells.

#### **Statistics**

All data are presented as mean  $\pm$  SD. All statistics were performed using Excel (Microsoft) and Prism (GraphPad software) programs, applying two-tailed Student's t test. Values of p < 0.05 were considered significant. For additional details, see the Supplemental Experimental Procedures.

#### **ACCESSION NUMBERS**

The Gene Expression Omnibus accession number for microarray data reported in this paper is GSE43136.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx. doi.org/10.1016/j.stem.2012.11.002.

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