

Regeneration of CD8 $\alpha\beta$ T Cells from T-cell-Derived iPSC Imparts Potent Tumor Antigen-Specific Cytotoxicity

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Abstract

Although adoptive transfer of cytotoxic T lymphocytes (CTL) offer a promising cancer therapeutic direction, the generation of antigen-specific CTL from patients has faced difficulty in efficient expansion in *ex vivo* culture. To resolve this issue, several groups have proposed that induced pluripotent stem cell technology be applied for the expansion of antigen-specific CTL, which retain expression of the same T-cell receptor as original CTL. However, in these previous studies, the regenerated CTL are mostly of the CD8 $\alpha\alpha$ ⁺ innate type and have less antigen-specific cytotoxic activity than primary CTL. Here we report that, by stimulating

purified iPSC-derived CD4/CD8 double-positive cells with anti-CD3 antibody, T cells expressing CD8 $\alpha\beta$ were generated and exhibited improved antigen-specific cytotoxicity compared with CD8 $\alpha\alpha$ ⁺ CTL. Failure of CD8 $\alpha\beta$ T-cell production using the previous method was found to be due to killing of double-positive cells by the double-negative cells in the mixed cultures. We found that WT1 tumor antigen-specific CTL regenerated by this method prolonged the survival of mice bearing WT1-expressing leukemic cells. Implementation of our methods may offer a useful clinical tool. *Cancer Res*; 76(23): 6839–50. ©2016 AACR.

Introduction

During the last several years, cancer immunotherapy using a variety of strategies has progressed remarkably, for example, blockade of inhibitory signals in the immune system (1–3), adoptive transfer of cytotoxic T lymphocytes (CTL; refs. 4–6), transfer of T-cell receptor (TCR) genes (7–9), etc. Although these achievements may suggest that CTLs present in cancer patients have the potential to cure them, researchers and clinicians have been facing a major obstacle in getting enough antigen-specific CTLs for therapy. Some groups have reported that tumor infiltrating lymphocytes (TIL) or tumor antigen specific CTLs can be efficiently expanded (10–13), but in general it is not so easy. This problem is mainly attributable to the nature of CTLs; they easily become exhausted or die during cultivation.

To solve this problem, we came to the idea of utilizing induced pluripotent stem cell (iPSC) technology for the cloning and

expansion of CTLs. When iPSCs are established from antigen-specific T cells (T-iPSC), they should inherit rearranged TCR genes, and thus all T cells regenerated from T-iPSCs should express the same TCR. Because iPSC expansion *in vitro* is almost unlimited, it should be possible to obtain as many fresh CTLs as needed. In keeping with this idea, we have recently succeeded in regenerating MART1-specific CTLs from iPSCs originally derived from CTLs of a melanoma patient (14). Other groups also have regenerated viral antigen-specific T cells (15, 16), T cells expressing an invariant TCRs (17–19), and T cells that were genetically engineered to express a so-called chimeric antigen receptor (CAR; ref. 20).

None of the previous studies, however, showed whether these regenerated CTLs are as good as primary CTLs in terms of antigen-specific cytotoxic activity. Indeed, in the study using CAR-T-iPSCs, the authors stated that regenerated CTLs are phenotypically similar to $\gamma\delta$ T cells (20).

Authentic CTLs are CD4[−] CD8 α ⁺ CD8 β ⁺ (CD8 $\alpha\beta$ heterodimer⁺). CD8 $\alpha\alpha$ type T cells represent a minor population in most lymphoid tissues but are one of the major populations in the mucosal tissues, where they are regarded as "innate type" T cells. Of note, the CD8 $\alpha\alpha$ homodimer does not function well as a TCR coreceptor, because it does not efficiently bind to MHC class I molecules (21). Although regenerated CD8 T cells in the previous studies all seemed to be of the innate type, in this study we describe a novel yet simple method to generate CD8 $\alpha\beta$ T cells and show that they are active against leukemia.

Materials and Methods

Study approval

This study was approved by the Institutional Review Board of the Graduate School of Medicine, Kyoto University

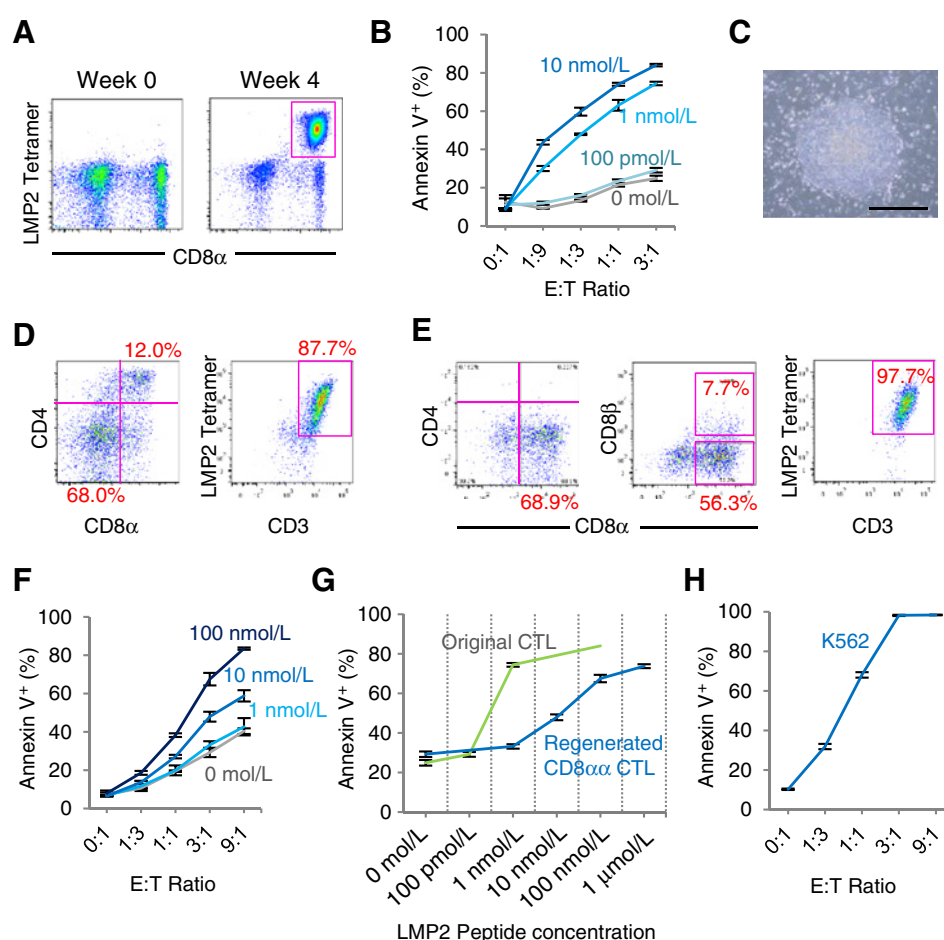
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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Figure 1.**

Regeneration of CD8αα innate type T cells by the conventional method. **A**, Flow cytometric profiles of PBMC of a healthy volunteer before and after stimulation with LMP2 peptide pulsed LCLs, which were used as antigen-presenting cells. LMP2-specific CTLs were detected as the CD8α⁺ LMP2-tetramer⁺ fraction (boxed). **B**, *In vitro* cytotoxic assay of LMP2-specific CTLs from **A** against HLA-A*24:02⁺ THP1 cells pulsed with LMP2 peptide at different concentrations. E:T ratio, effector-to-target ratio. **C**, Photomicrograph of T-iPSCs; LMP2#1-1 established from the LMP2-specific CTLs. Scale bar, 500 μm. **D**, Flow cytometric profiles of cells generated from T-iPSCs; LMP2#1-1 at day 40 of culture. **E**, Flow cytometric profiles of cells generated from those shown in **D** after stimulation with CD3 Ab for 6 days. **F**, *In vitro* cytotoxic assay of regenerated CD8αα T cells against THP1 targets pulsed with LMP2 peptide at different concentrations. Representative of three independent experiments. **G**, Comparison of the cytotoxic activity between the original CTLs and the regenerated CD8αα T cells against THP1 targets at different concentrations of LMP2 peptide. The E:T ratio was fixed at 3:1. **H**, *In vitro* cytotoxic assay of regenerated CD8αα T cells against K562 cells. Representative of three independent experiments.

(approval number: G761) and abided by the tenets of the Declaration of Helsinki. All specimens from healthy individuals and patients were collected after written informed consent was obtained.

Cell lines

Op9, OP9/DLL1, and 409B2 were purchased from RIKEN BRC. THP1 was purchased from ATCC. HL60, HEL, and KG1a were maintained in our department. OUN1 and MEG01 were gifts from Dr. Masaki Yasukawa (Ehime University, Ehime, Japan). K562 was a gift from Dr. Kiyotaka Kuzushima (Aichi Cancer Center, Aichi, Japan). C1R-A*24:02 was a gift from Dr. Masafumi Takiguchi (Kumamoto University, Kumamoto, Japan). Upon receiving the cell lines, frozen stocks were prepared within one to five passages and new stocks were thawed frequently to keep the original condition. The cell lines were passaged for less than 3

months after receipt or resuscitation. They were also authenticated by morphology, growth rate, and surface phenotype, especially expression of HLA class I.

Differentiation of T-iPSCs to CD8αβ single positive cells

T-iPSCs were differentiated to CD4/8 double-positive (DP) cells using the OP9 and OP9/DLL1 stromal cell coculture systems as described (14, 22, 23), with slight modification. In brief, iPSC colonies were dissociated using trypsin (0.25%) and collagenase IV (1 mg/mL) and mechanically disrupted into small clumps by pipetting. About 600 iPSC clumps were collected and plated on gelatin precoated OP9 dishes filled with OP9 medium, that is, α-MEM (Invitrogen) with 20% FCS. On day 13, colonies were treated with collagenase Type IV (50 U/mL) and trypsin-EDTA (0.05%). Cells were plated in a OP9/DLL1 semiconfluent dish on OP9 medium containing hIL-7 (5 ng/mL), hFlt-3L (5 ng/mL), and

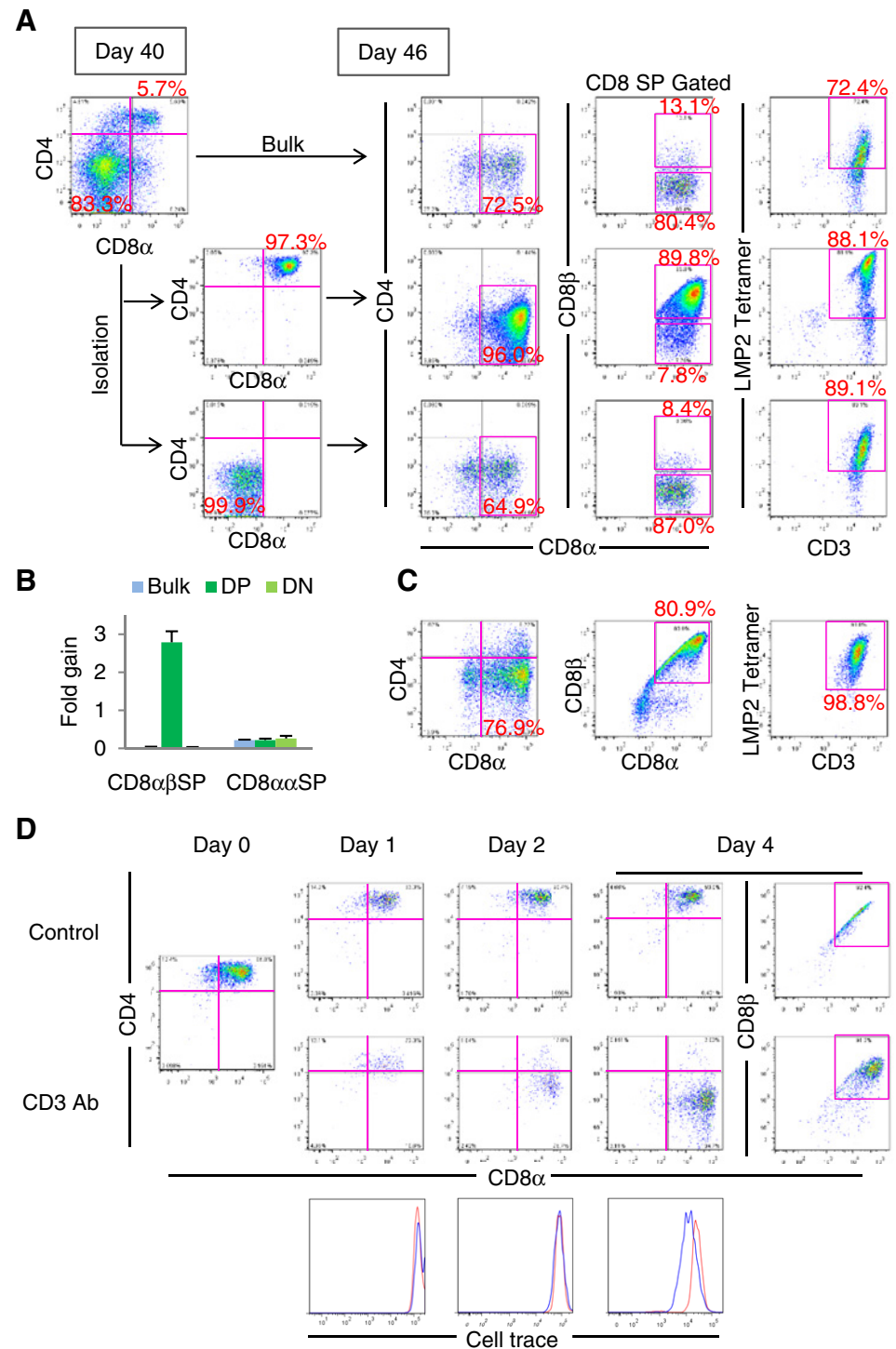
hSCF (5 ng/mL). On day 15, semiadherent cells were collected and passage into a new dish layered with OP9/DLL1 cells. From this point, passage was done every 7 days. On day 40, floating cells were collected and CD4/8 DP cells were enriched by using CD4 microbeads (Milteny Biotec). DP cells were stimulated with CD3 Ab (15 ng/mL; OKT-3, eBioscience) in the presence of hIL-2 (100 U/mL) and hIL-7 (5 ng/mL). CD8 SP cells were stimulated one to

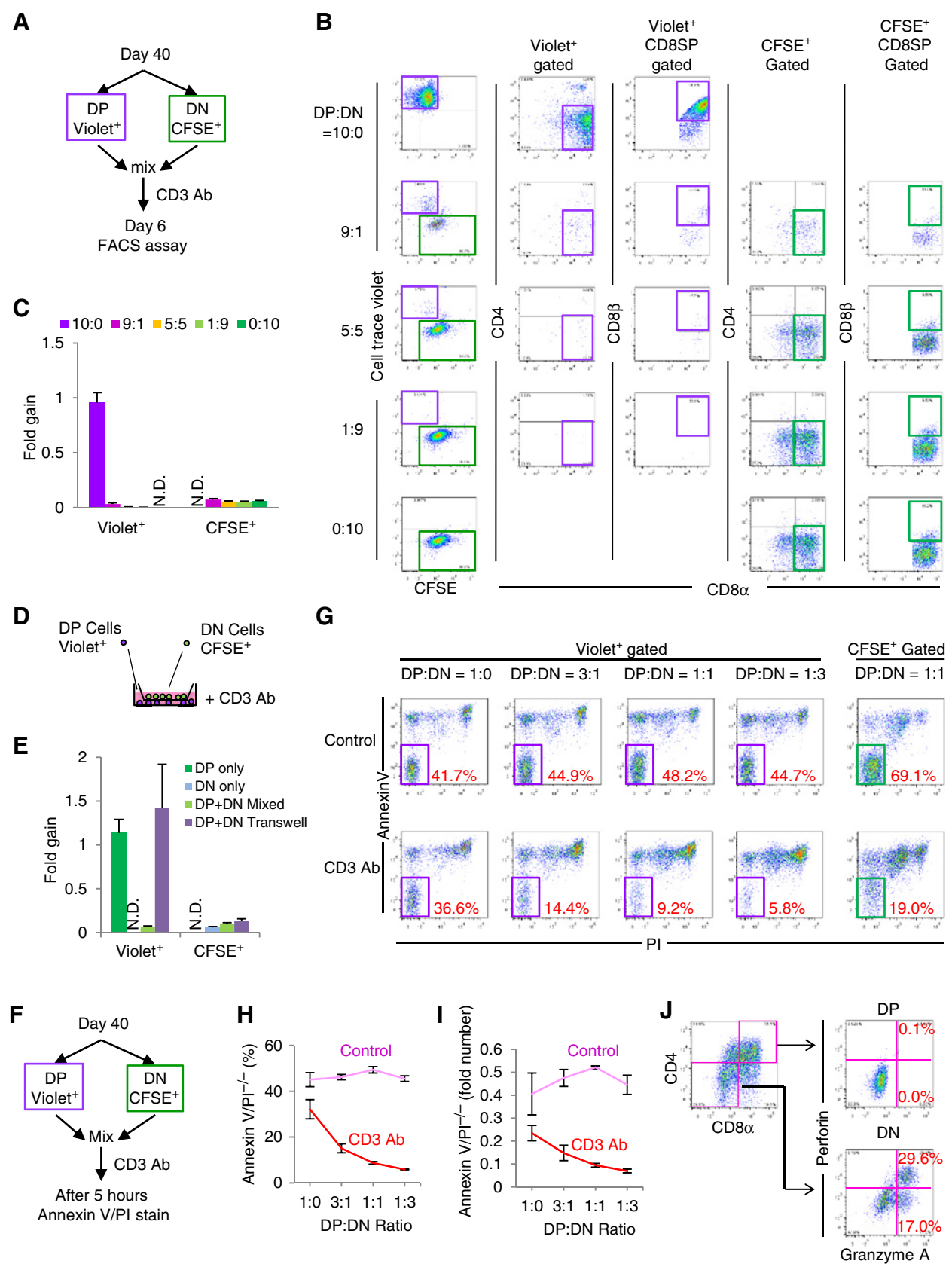
five times by HLA-A*24:02⁺ lymphoblastoid cell line (LCL) pulsed with the peptide in the presence of hIL-7 (5 ng/mL) and hIL-21 (10 ng/mL).

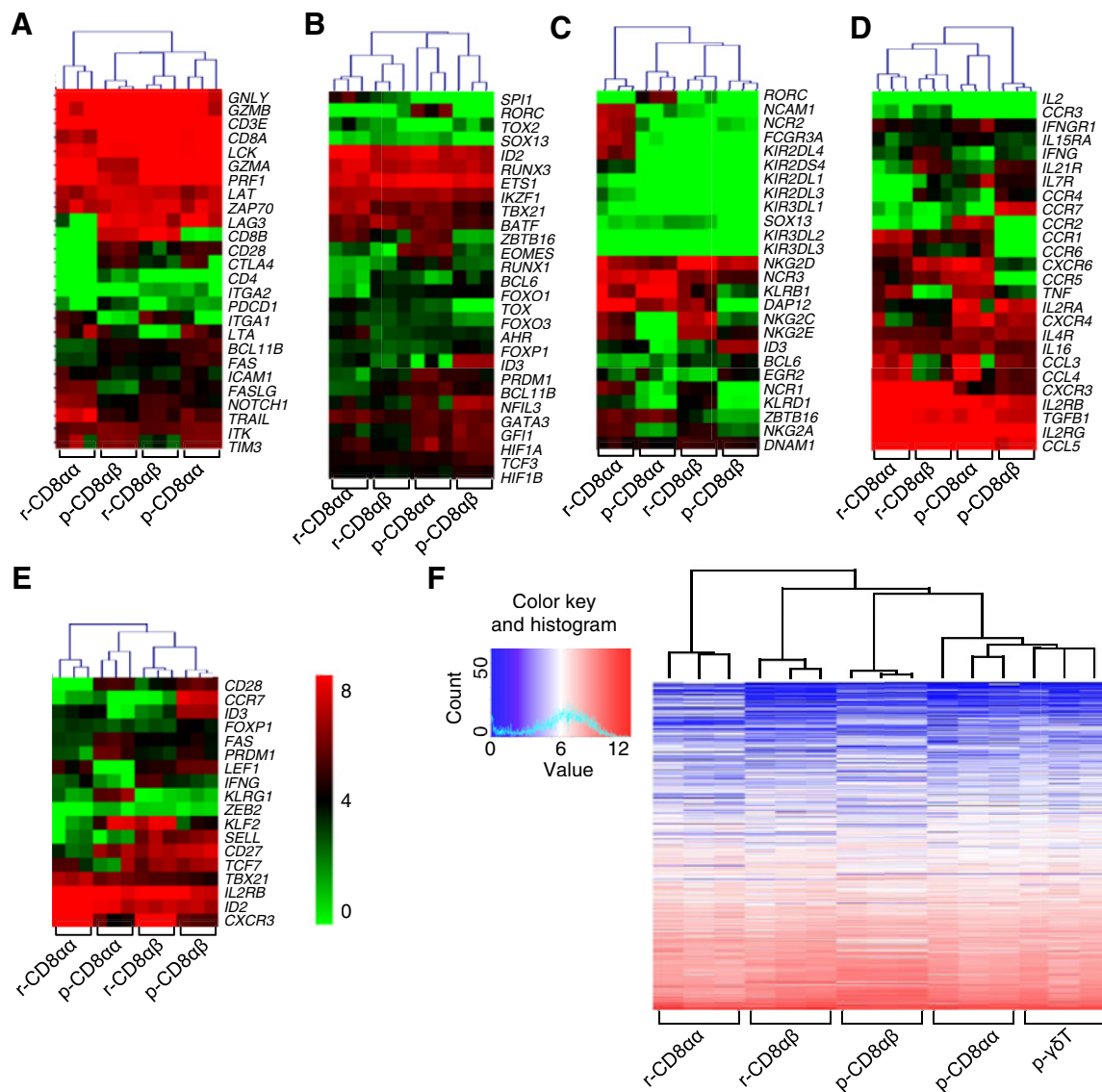
Coculture of immature DP and double-negative cells

Induced DP and double-negative (DN) cells (day 40) from T-iPSCs LMP2#1-1 were isolated and labeled with Cell Trace

Figure 2. Induction of CD8 $\alpha\beta$ SP cells from DP cells. **A**, Flow cytometric profiles of generated immature T cells on day 40 (top left), purified CD4/8 DP cells (middle left), purified CD4/8 DN cells (bottom left), and activated T cells derived from total cells (top right), purified CD4/8 DP cells (middle right) or purified CD4/8 DN cells (bottom right) after stimulation with CD3 Ab for 6 days. **B**, Fold gain of regenerated CD8 $\alpha\beta$ SP cells and CD8 $\alpha\alpha$ SP cells from each population as described in **A**. Representative of three independent experiments. **C**, Flow cytometric profiles of regenerated T cells derived from CD4/8 DP cells stimulated by LCL pulsed with LMP2 peptide. **D**, Time course analysis from DP cells to CD8 $\alpha\beta$ SP cells by CD3 Ab stimulation. The bottom row shows the dilution of Cell Trace Violet. Red lines and blue lines depict cells without and with CD3 Ab, respectively.





**Figure 4.**

Gene expression profiles of regenerated CD8αβ SP cells. **A–E**, Heat map comparing expression of the indicated mRNA transcripts detected by RNA sequencing of regenerated CD8αβ T cells (r-CD8αβ), regenerated CD8αα T cells (r-CD8αα), peripheral blood CD8αβ T cells (p-CD8αβ), and peripheral blood CD8αα T cells (p-CD8αα). The FPKM values were log₂ transformed. The heat maps represent different ontology groups, including genes encoding for cytotoxicity (**A**), transcription factors (**B**), NK-associated (**C**), cytokines and chemokines (**D**), and T-cell maturation markers (**E**). **F**, Unsupervised hierarchical clustering of the indicated transcriptomes generated by RNA sequencing. Bars above the heat maps indicate similarities between the different samples presented in each of the lanes. p-γδT, peripheral blood γδ T cells.

Figure 3.

DN cells kill DP cells upon stimulation. **A**, Scheme of the DP and DN coculture experiment for **B** and **C**. Violet⁺ purified CD4/8 DP cells and CFSE⁺ DN cells were mixed at various ratios and cultured with CD3 Ab. **B**, Flow cytometric profiles of generated cells from DP and DN cells as depicted in **A**. DP-derived cells and DN-derived cells were detected as Cell Trace Violet and CFSE positive cells, respectively. **C**, Fold gain of CD8αβ SP cells from each population described in **B**. Representative of three independent experiments. N.D., not done. **D**, Scheme of the transwell coculture experiment for examining the effect of soluble factors. Violet⁺ purified CD4/8 DP cells and CFSE⁺ DN cells were cultured in the lower and upper compartments, respectively. **E**, Fold gain of CD8αβ SP cells derived from DP cells (Violet⁺) and DN cells (CFSE⁺) in each culture condition. Isolated DP cells, isolated DN cells, DP and DN cells mixed, and DP cells and DN cells cocultured using transwell as depicted in **D** were stimulated with CD3 Ab for 6 days. Representative of three independent experiments. **F**, Scheme of the cytotoxic assay of DN cells against DP cells for **G**. Violet⁺ purified CD4/8 DP cells and CFSE⁺ DN cells were mixed at various ratios, cultured with CD3 Ab for 5 hours, and stained with Annexin V and PI. **G**, Flow cytometric profiles of DP (Violet⁺) or DN (CFSE⁺) cells as shown in **F**. The percentages of Annexin V/PI⁺ live cells are highlighted by the boxes. **H** and **I**, Cytotoxic assay of DN cells against DP cells. Plot shows percentage (**H**) and fold number (**I**) of Annexin V/PI⁺ live cells among Violet⁺ cells as shown in **G**. Representative of three independent experiments. **J**, Flow cytometric analysis of intracellular granzyme A and perforin expression in DP and DN cells at day 40 of culture without TCR stimulation.

Violet and CFSE, respectively. A total of 10^5 cells of the DP and DN cell mixture at the indicated ratios were cultured with CD3 Ab (15 ng/mL). After 6 days, cells were analyzed by flow cytometer.

Transwell coculture of immature DP and DN cells

The transwell system was used for the detection of soluble mediator effects. Labeled DP and DN cells (5×10^4 cells each) as above were seeded in the lower and upper compartments, respectively, of a 0.4 μ m pore size transwell plate (Coaster), and cultured in the presence of CD3 Ab (15 ng/mL). In control wells, DP or DN cells (5×10^4 cells) or their mixture (5×10^4 cells each) were cultured with CD3 Ab. After 6 days, cells were analyzed by flow cytometer.

Cytotoxic assay of DN cells against DP cells

Labeled DP and DN cells as above were cocultured at the indicated ratio with or without CD3 Ab for 5 hours. Annexin V (BioLegend) and PI double-negative cells were considered as alive.

RNA sequencing and data analysis

Regenerated LMP2-specific CD8 $\alpha\alpha$ T cells and CD8 $\alpha\beta$ T cells were isolated by FACS Aria III (BD Bioscience). Isolated peripheral blood CD8 $\alpha\alpha$ T cells (TCR $\alpha\beta^+$ CD4 $^-$ CD8 α^+ CD8 β^-), CD8 $\alpha\beta$ T cells (TCR $\alpha\beta^+$ CD4 $^-$ CD8 α^+ CD8 β^+), and $\gamma\delta$ T cells (TCR $\gamma\delta^+$ TCR $\alpha\beta^-$) were stimulated with CD3/28 stimulator (Milteny Biotec) for 7 days according to the manufacturer's instructions in the presence of hIL-7 (10 ng/mL) and hIL-15 (20 ng/mL). Total

RNA was isolated using an RNeasy Plus Mini Kit (QIAGEN). Libraries for RNA sequence were prepared using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs). Sequence alignment was performed using our in-house pipelines as previously described (24). Fragments per kilobase of exon per million fragments mapped (FPKM) was calculated for Refseq genes and then log-transformed. The hierarchical clustering of gene expression data was performed using Pearson's dissimilarity as distance measure and centroid method for linkage. For this analysis, we removed genes with no or low expression (mean FPKM < 2), then identified the most variant 5% of genes ($n = 527$). Multi Experiment Viewer software was used to generate heat maps and hierarchical clustering. GEO accession number is GSE 81975.

Statistical analysis

All data with error bars are presented as mean $\pm$ SD. Difference was assessed using paired *t*-test using Prism (GraphPad software). Values of $P < 0.05$ were considered significant.

Results

CD8 T cells induced by the conventional method are of the innate type, expressing a CD8 $\alpha\alpha$ homodimer

Latent membrane protein 2 (LMP2) is an Epstein-Barr virus (EBV)-encoded antigen that is considered as a good CTL target in EBV-related tumors (25, 26). LMP2-specific CTLs, defined by LMP2 tetramer staining, from a healthy volunteer were

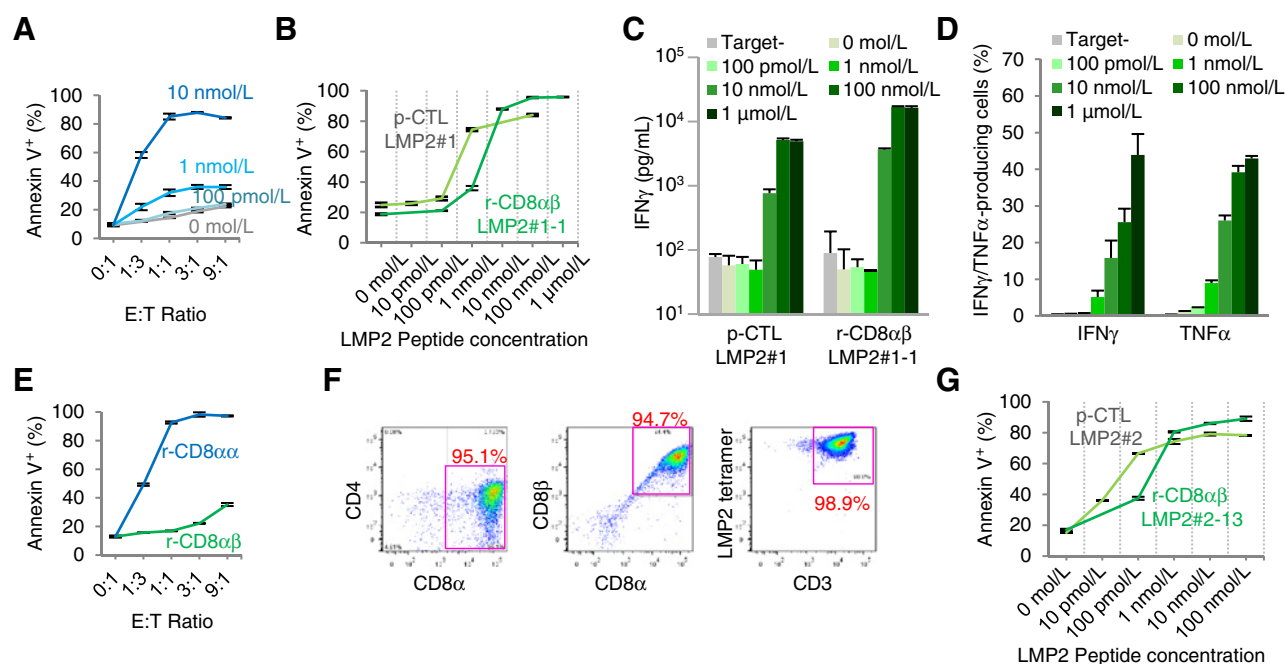


Figure 5.

Cytotoxic activity of regenerated CD8 $\alpha\beta$ SP cells. **A**, *In vitro* cytotoxic assay of regenerated CD8 $\alpha\beta$ T cells, r-CD8 $\alpha\beta$ LMP2#1-1, against THP1 cells pulsed with LMP2 peptide at different concentrations. Representative of three independent experiments. **B**, Comparison of the cytotoxicity between the primary CTLs, p-CTL LMP2#1, and r-CD8 $\alpha\beta$ LMP2#1-1, against THP1 cells pulsed with different concentrations of LMP2 peptide. The effector-to-target (E:T) ratio was fixed at 3:1. **C**, Comparison of IFN γ production between p-CTL LMP2#1 and r-CD8 $\alpha\beta$ LMP2#1-1 against THP1 cells pulsed with different concentrations of LMP2 peptide. **D**, Intracellular cytokine staining of r-CD8 $\alpha\beta$ LMP2#1-1 against THP1 with different concentration of LMP2 peptide. Representative of three independent experiments. **E**, NK-like cytotoxicity of regenerated CD8 $\alpha\alpha$ T cells (r-CD8 $\alpha\alpha$) and r-CD8 $\alpha\beta$ against K562 cells. Representative of three independent experiments. **F**, Flow cytometric profiles of regenerated T cells derived from T-iPSCs, LMP2#2-13. **G**, Cytotoxic assay of p-CTL LMP2#2 and r-CD8 $\alpha\beta$ LMP2#2-13 against THP1 cells pulsed with different concentrations of LMP2 peptide. The E:T ratio was fixed at 3:1.

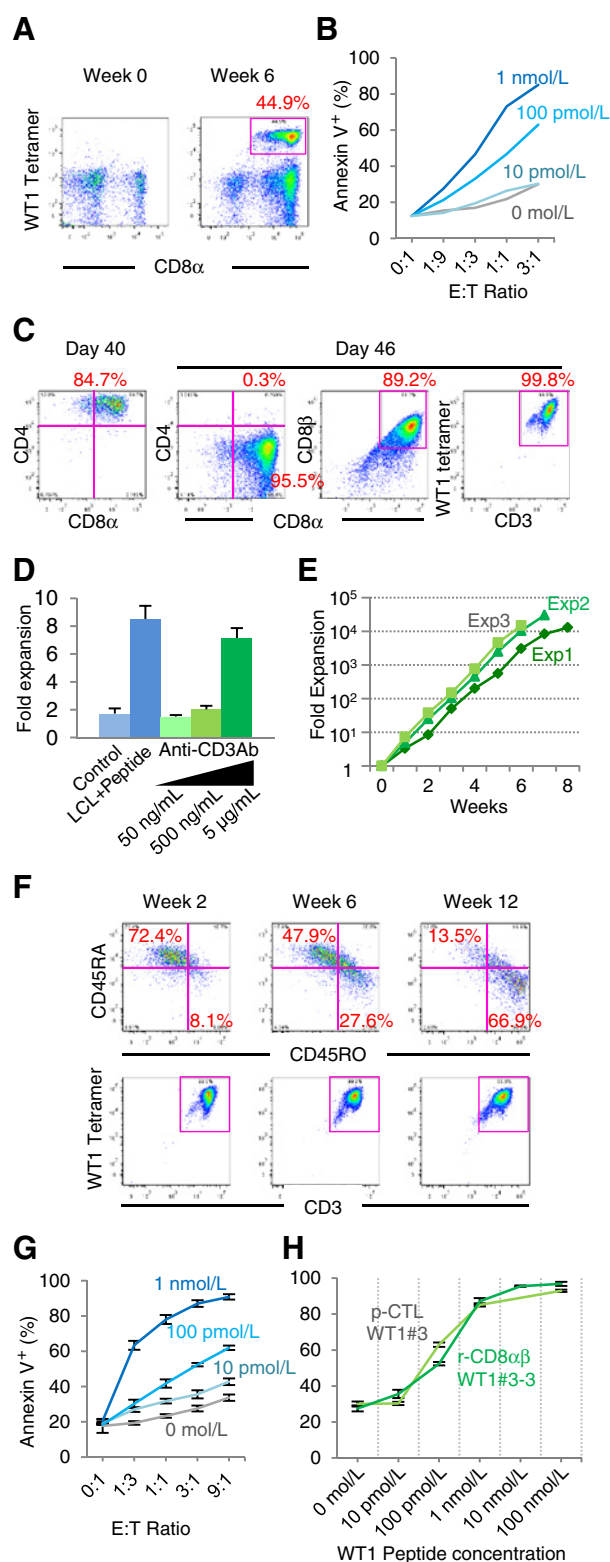


Figure 6. Regeneration of functional WT1 specific CTLs. **A**, Flow cytometric profiles of PBMC from a healthy volunteer before and after stimulation with WT1 peptide pulsed LCLs. Cells were stained with CD8 α Ab and WT1-tetramer. **B**, *In vitro* cytotoxic assay of WT1-specific CTLs #3 against CIR-A*24:02 cells pulsed with

expanded by stimulation with antigen-presenting cells loaded with LMP2 peptide (Fig. 1A). After several weeks of expansion, these antigen-specific CTLs efficiently killed leukemic cells loaded with LMP2 peptide at 1 nmol/L concentration (Fig. 1B).

For establishment of iPSC cells from CTLs, we used SV40 Large T antigen (27, 28) in addition to Yamanaka factors to enhance the efficiency of reprogramming (Supplementary Fig. S1). For transduction of CTLs, we used Sendi virus vector, which has been shown to be safe because transferred genes as well as viral genes are not integrated into genome (29, 30). By transducing these CTLs with Yamanaka factors and SV40, two clones of iPSC lines, LMP2#1-1 and LMP2#2-13, were established. Sendai virus vectors containing Yamanaka factors and SV40 were not detected in either of these clones, and expression profiles of various stem cell genes were indistinguishable from those of control iPSCs (Supplementary Fig. S2A and S2B). Hereafter, we will focus on the clone LMP2#1-1 (Fig. 1C). Expression of stem cell genes in the LMP2#1-1 cell line was confirmed by cytochemistry, it had a normal karyotype, and was pluripotent, as judged by teratoma formation (Supplementary Fig. S2C–S2E).

We then induced T cells from LMP2-T-iPSCs using the conventional method (14). As has been reported, DP and DN cells expressed TCR on Day 40 (Fig. 1D). Upon stimulation with CD3 Ab, CD8 T cells were generated after 6 days and were exclusively of the CD8 α type and LMP2-tetramer positive (Fig. 1E). However, as much as 100 nmol/L peptide was required to induce efficient cytotoxic activity (Fig. 1F), making these cells ~100-fold less competent than primary CTLs (Fig. 1G). However, the regenerated CTLs were able to efficiently kill K562 (Fig. 1H), indicating that they have high Natural Killer (NK)-like cytotoxicity.

Isolated DP cells give rise to CD8 α T cells upon stimulation

We searched then for conditions in which CD8 α T cells could be induced. We found that when purified DP cells were stimulated with CD3 Ab, CD8SP cells were generated and they were mostly of CD8 α type (Fig. 2A). By contrast, purified DN cells gave rise to CD8 α T cells upon activation (Fig. 2A and B). The CD8 α T cells could also be efficiently generated from purified DP cells stimulated by autologous LCLs loaded with LMP2 peptide (Fig. 2C), indicating that the absence of DN cells, rather than the stimulation method, is critical for the induction of CD8 α T cells.

To exclude the possibility that a preexisting small number of CD8 α cells were preferentially expanded, purified DP cells were labeled with Cell Trace Violet, and then stimulated

WT1 peptide at different concentrations. **C**, Flow cytometric profiles of cells regenerated from T-iPSCs, WT1#3-3. The far left panel shows the profile of purified DP cells at day 40 and right three panels show the profiles of regenerated T cells after 6 days' stimulation with CD3 Ab. **D**, Fold expansion of r-CD8 α β WT1#3-3 after stimulation by autologous LCL pulsed with WT1 peptide or by immobilized CD3 Ab in 7 days. Representative of three independent experiments. **E**, Fold expansion of r-CD8 α β WT1#3-3 by repeated stimulation. CD8 α β T cells were stimulated every week by coculturing with LCLs pulsed with WT1 peptide (100 nmol/L). Plot shows the data of three independent experiments. Exp, experiment. **F**, Flow cytometric profiles of the phenotype of CD8 α β T cells repeatedly stimulated with LCLs. **G**, *In vitro* cytotoxic assay of r-CD8 α β WT1#3-3 against CIR-A*24:02 cells pulsed with WT1 peptide at different concentrations. Representative of three independent experiments. **H**, Comparison of cytotoxicity between p-CTL WT1#3 and r-CD8 α β WT1#3-3 against CIR-A*24:02 cells loaded with different concentrations of WT1 peptide. The effector-to-target (E:T) ratio was fixed at 3:1.

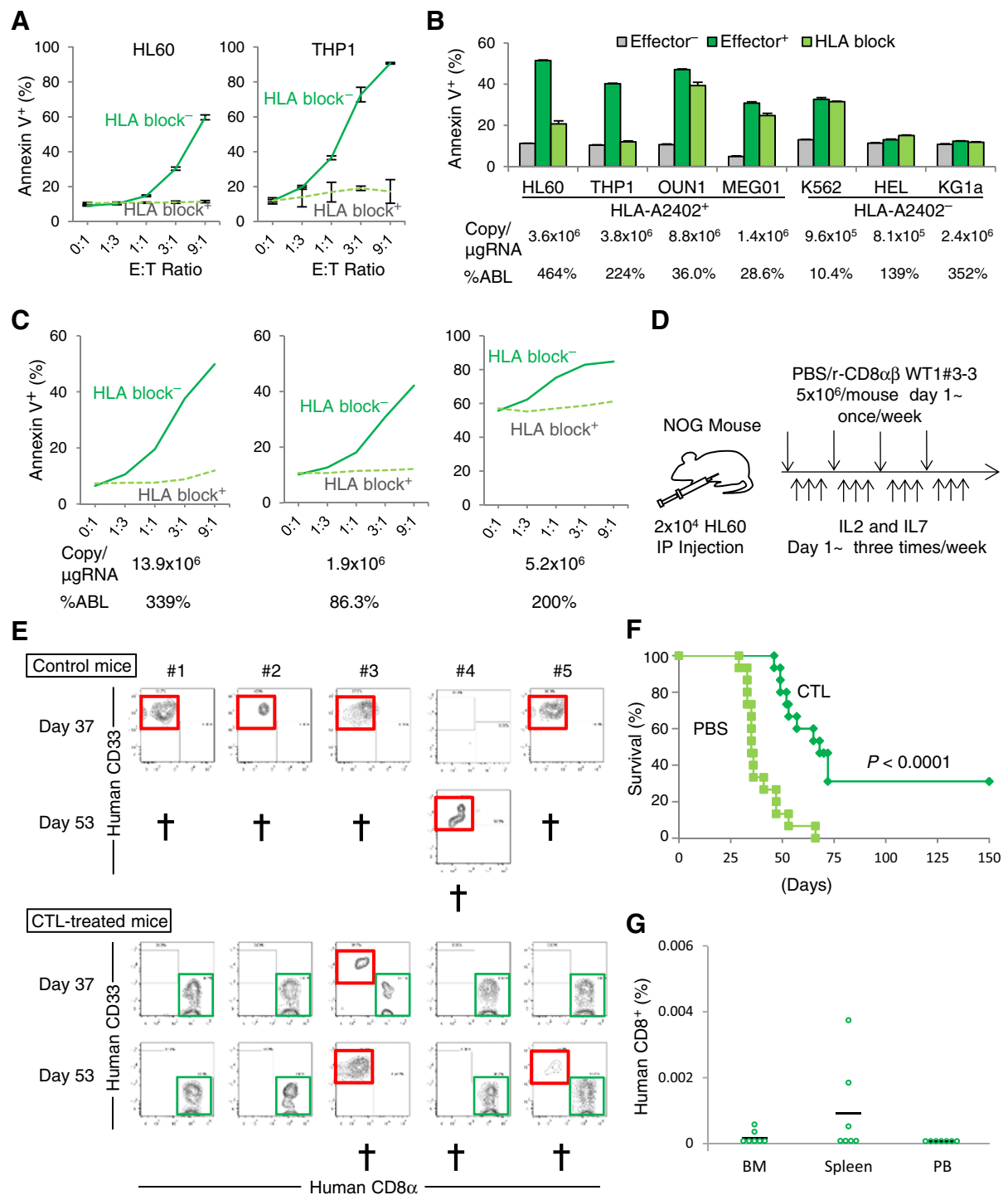


Figure 7. Cytotoxicity of regenerated WT1-specific CD8αβ SP cells *in vitro* and *in vivo*. **A**, HLA-dependent cytotoxicity of r-CD8αβ WT1#3-3 against leukemia cell lines expressing HLA-A*24:02. HL60 and THP1, which express endogenous WT1 protein, were used as target cells. Target cells, pretreated with or without HLA class I blocking Ab, were cocultured with r-CD8αβ WT1#3-3. Representative of three independent experiments. **B**, *In vitro* cytotoxic assay of r-CD8αβ WT1#3-3 against various leukemia cell lines. HLA-A*24:02 positive or negative leukemia cell lines were used as target cells. Target cells, pretreated with or without HLA class I blocking Ab, were cocultured with r-CD8αβ WT1#3-3. The effector-to-target (E:T) ratio was fixed at 3:1. WT1 mRNA expression in each cell line was quantified by real-time PCR and depicted below the graph as absolute value and relative value to ABL. Representative of three independent experiments. **C**, *In vitro* cytotoxic assays of r-CD8αβ WT1#3-3 against primary AML cells expressing WT1 protein and HLA-A*24:02. Primary leukemia cells from each patient, pretreated with or without HLA class I blocking Ab, were co-cultured with r-CD8αβ WT1#3-3. WT1 mRNA expression in each of the primary leukemia cells was measured and shown below each graph as in **B**. **D**, Treatment scheme for the xenograft leukemia model. NOG mice were injected intraperitoneally with 2 × 10⁴ HL60 leukemia cells. (Continued on the following page.)

(Fig. 2D). On day 2, DP cells started to downregulate CD4 without cell division, and then CD8 $\alpha\beta$ cells were generated on day 4 with only one or two cell divisions, indicating that CD8SP cells were induced from DP cells.

DN cells kill DP cells upon stimulation

Thus, it seemed that DN cells might be exerting a suppressive effect on the generation of CD8 $\alpha\beta$ T cells. To test this possibility, isolated DP and DN cells were mixed at various ratios before stimulation, and it was seen that DN cells at a one ninth ratio to DP cells almost completely suppressed the generation of CD8 $\alpha\beta$ T cells (Fig. 3A–C).

The possibility that DN cells suppress DP-SP development by secreting soluble factors is unlikely, because virtually no suppression of CD8 $\alpha\beta$ T-cell generation was seen in a transwell experiment in which DP cells and DN cells were separated by a semipermeable membrane (Fig. 3D and E). We then observed that the DP cells died as early as 5 hours after activation in cultures with a mixture of DN and DP cells, and that the frequency of live DP cells decreased along with an increase in the DN/DP ratio (Fig. 3F–I), indicating that activated DN cells directly kill DP cells. We further found that a significant proportion of DN cells expressed perforin and granzyme A prior to activation (Fig. 3J). These results indicate that failure of DP cells to generate CD8 $\alpha\beta$ T cells after activation is attributable to the direct cytotoxicity of DN cells against DP cells. The entire culture procedure and profiles of generated cells are depicted in Supplementary Fig. S3.

Gene expression profiles of regenerated CD8 $\alpha\beta$ T cells

We analyzed gene expression profiles of regenerated CD8 $\alpha\beta$ T cells (r-CD8 $\alpha\beta$) in comparison with regenerated CD8 $\alpha\alpha$ T cells (r-CD8 $\alpha\alpha$) and primary CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ T cells (p-CD8 $\alpha\beta$, p-CD8 $\alpha\alpha$), using RNA-seq. Not only surface molecules, for example, *CD3*, *CD8 α* , *CD8 β* , and *CD28* but also intracellular functional molecules, for example, *GZMA*, *GZMB*, *PRF1*, *LCK*, and *BCL11B* were commonly expressed in r-CD8 $\alpha\beta$ and p-CD8 $\alpha\beta$ (Fig. 4A). As to immune-checkpoint molecules, *LAG3* was expressed in rCD8 cells but *PDCD1* and *HAVCR2* (*Tim3*) were very low in expression. The signature transcription factors of CD8 T cells including *TBX21*, *RUNX3*, *GATA3*, and *IKZF1* were commonly expressed in all four groups, but *ZBTB16* (*PLZF*), a representative transcription factor for innate type T cells (31–33), was low in both r-CD8 $\alpha\beta$ and p-CD8 $\alpha\beta$ compared with both types of CD8 $\alpha\alpha$ cells (Fig. 4B). Reflecting NK-like cytotoxicity of r-CD8 $\alpha\alpha$, NK-related genes, for example, *NCAM1*, *NCR1*, *NCR2*, *KLRC2*, and *KLRC3* were highly expressed in r-CD8 $\alpha\alpha$. Although r-CD8 $\alpha\beta$ also express some of them (*KLRC2* and *KLRC3*), but others were not expressed (Fig. 4C). However, r-CD8 $\alpha\alpha$ and r-CD8 $\alpha\beta$ shared some common characteristics in terms of chemokine receptor expression, such as high *CXCR3* and low *CCR7* expression, which are reminiscent of effector CD8 T cells (Fig. 4D and E). In global gene expression pattern, r-CD8 $\alpha\beta$ were positioned closer to p-

CD8 $\alpha\beta$ compared to r-CD8 $\alpha\alpha$ (Fig. 4F). Taken together, these observations indicate that gene expression profiles of r-CD8 $\alpha\beta$ resembled with that of p-CD8 $\alpha\beta$, although retaining some innate lymphocyte signature.

Regenerated CD8 $\alpha\beta$ T cells exhibit antigen-specific cytotoxicity comparable to the original CTLs

Regenerated CD8 $\alpha\beta$ T cells killed antigen-loaded target cells at a 1 nmol/L peptide concentration (Fig. 5A), and their antigen-specific cytotoxic activity was found to be slightly weaker than that of primary CTLs, but much stronger than that of regenerated CD8 $\alpha\alpha$ T cells (Fig. 5B, see Fig. 1G for comparison). Regenerated CD8 $\alpha\beta$ T cells showed comparable potential to primary CTLs in producing IFN γ , and produced IFN γ and TNF α at more than 1 nmol/L concentration of peptide (Fig. 5C and D). NK-like cytotoxicity against K562 cells was weaker than regenerated CD8 $\alpha\alpha$ T cells (Fig. 5E). These results collectively indicate that this novel method works well to regenerate functional CD8 $\alpha\beta$ T cells. Another LMP2-specific T-iPSC clone #2-13 also differentiated to CD8 $\alpha\beta$ T cells (Fig. 5E) and exhibited peptide-specific cytotoxicity slightly weaker than the original CTLs (Fig. 5F).

Regeneration of WT1-specific CTLs

We then decided to apply this method against acute myeloid leukemia (AML) cells expressing WT1 antigen, a cancer-testis antigen broadly expressed in various types of solid tumors as well as in leukemia (34, 35). We firstly induced WT1-specific CTLs from a healthy volunteer by using WT1 peptide (Fig. 6A) and measured their cytotoxic activity (Fig. 6B). We then produced iPSCs from these CTLs, and a total of three lines were established (Supplementary Fig. S4 and Supplementary Table S1). Hereafter we will show the data using clone WT1#3-3. Regenerated cells were of the CD8 $\alpha\beta$ type and WT1-tetramer positive (Fig. 6C), and the sequence of the TCR genes in this clone was determined (Supplementary Table S1).

We then expanded CD8 T cells by stimulating with an autologous B LCL carrying WT1 peptide, or with a CD3 Ab, and a six- to eight-fold expansion was seen during 1 week in both cases (Fig. 6D). We decided to use LCL for further studies because they seemed slightly more efficient. During 6 to 8 weeks' culture, CD8 T cells were expanded by ~10,000 fold (Fig. 6E), changing their surface phenotype from a naïve to an effector/memory profile (Fig. 6F). Regenerated CD8 T cells showed the same antigen-specific cytotoxic activity as the original CTLs (Fig. 6G and H).

Regenerated WT1-specific CTLs have cytotoxicity against leukemia cells

Regenerated WT1-CTLs also efficiently killed THP1 and HL60 cells, HLA-matched AML cell lines expressing endogenous WT1 protein (Fig. 7A). The cytotoxic activity was almost completely eliminated by adding a blocking Ab against HLA

(Continued.) Beginning at day 1, PBS or 5×10^6 r-CD8 $\alpha\beta$ WT1#3-3 were injected intraperitoneally into tumor-bearing mice every week for 4 weeks. hIL-2 and hIL-7 were injected intraperitoneally three times a week. **E**, Flow cytometric profiles of peripheral blood from control or CTL-treated mice. CD33 versus CD8 profiles gated on human CD45 positive cells are shown. HL60 and inoculated CTLs were detected as the human CD33⁺ or CD8⁺ fraction. Representative of three independent experiments. **F**, A Kaplan-Meier curve depicting the percent survival of the experimental and control groups is shown ($n = 15$). Accumulation of three independent experiments. $P < 0.05$ by the log-rank test. **G**, Percentage of resident regenerated CTLs in indicated samples from NOG mice inoculated with r-CD8 $\alpha\beta$ WT1#3-3 6 months before ($n = 7$). NOG mice bearing no leukemia cells were inoculated with 5×10^6 r-CD8 $\alpha\beta$ WT1#3-3 and cytokines intraperitoneally in the same manner as shown in **D**. Each dot shows the data from each mouse.

class I, indicating that the killing was MHC restricted and dependent on TCR-MHC binding. Other AML cell lines OUN1 and MEG01 were also killed, but the cytotoxic activity was only partially suppressed by HLA blocking Ab, suggesting that they were also killed by NK-like cytotoxicity (Fig. 7B). Other AML lines, HEL and KG1a, which express WT1 but not express HLA-A*24:02, were not killed, confirming that the TCR of WT1#3-3 cells recognizes WT1 antigen presented on HLA-A*24:02. We further tested whether regenerated CTLs are able to kill primary leukemic cells. Three samples expressing the highest levels of endogenous WT1 antigen selected among several samples from HLA-A*24:02⁺ patients were killed by regenerated CTLs (Fig. 7C).

Finally, we examined CTL activity *in vivo* in a xenograft model. NOD.Cg-Prkdcscid Il2rgtm1Sug/Jic (NOG) mice inoculated with HL60 cells followed by administration of regenerated CTLs (Fig. 7D). Although leukemia cells expressing human CD33 were detected from all the control mice, inoculated regenerated CTLs were present in peripheral blood of all the treated mice (Fig. 7E). The CTL treated mice showed significantly longer survival compared with control mice (Fig. 7F).

Regenerated WT1-specific CTLs are safe

To test the safety of transfused regenerated CTLs, we did long-term observation *in vivo*. NOG mice bearing no leukemic cells were inoculated with regenerated CTLs in the same manner as in xenograft model (Fig. 1D), and observed for 6 months. During observation period, no mice showed diarrhea or body weight loss. After 6 months, all mice were dissected, and mononuclear cells in bone marrow, spleen, and peripheral blood were analyzed. Small amount of regenerated CTLs were detected from spleen and bone marrow of some mice (Fig. 7G). No mice suffered from tumor derived from regenerated CTLs. We also did not see any sign of tissue damage caused by transferred CTLs.

Discussion

Many groups have been struggling to produce CD8 $\alpha\beta$ type T cells from T-iPSCs, but the critical method described here turned out to be rather simple, simply include a DP cell purification step before stimulation. Our results can be summarized as three points: upon TCR stimulation, (i) DP cells give rise to CD8 $\alpha\beta$ T cells, (ii) DN cells immediately kill DP cells, (iii) DN cells give rise to CD8 α T cells. For the first point, the alternative ligand model of thymic selection may provide the explanation. According to this model, DP cells positively selected in the cortex by peptides specifically expressed there can survive in the medulla because they do not encounter the same peptide; thus, the agonist peptide can induce positive selection in the cortex (36, 37). It may be that our culture system reproduces such a cortical, positively selecting microenvironment. As to the second point, we have shown that DN cells already contain mature cells (Fig. 3J), although they do not kill other cells unless activated further via their TCR. It seems likely that DN cells kill DP cells by direct NK-like cytotoxicity; however, it remains to be studied what the target molecules are in this killing. As to the third point, in our system, the TCR is expressed at the DN stage (Supplementary Fig. S3C). When these cells are activated, they may undergo a developmental program toward $\gamma\delta$ T cells, because it is known that the DN cell

fate decision is based on TCR signaling strength. DN cells take the $\gamma\delta$ T cell fate upon receiving a strong TCR signal via the $\gamma\delta$ TCR, whereas they take the $\alpha\beta$ T cell fate when there is a weak signal via the preTCR (38, 39). This model is also in line with previous reports showing that DN cells expressing a transgenic $\alpha\beta$ TCR at the DN stage come to exhibit a $\gamma\delta$ T cell-like phenotype (40).

Regenerated CD8 $\alpha\beta$ T cells showed antigen-specific cytotoxicity slightly less, but nearly comparable to primary CD8 $\alpha\beta$ T cells (Supplementary Table S2). This is a marked improvement compared to regenerated CD8 α T cells and might partly be explained because the CD8 $\alpha\beta$ heterodimer binds to MHC class I and thus functions as a co-receptor to induce Ick signaling, whereas the CD8 α homodimer does not. Gene expression profiles also showed that regenerated CD8 $\alpha\beta$ T cells are closer to primary CD8 $\alpha\beta$ T cells, although still retaining some phenotypic features of innate type T cells. As activated T cells express some NK activation receptors and signal molecules like NKG2D, NKp30, DAP12, and so on (41–43), it is not surprising that these regenerated CTLs cultured *in vitro* also express such NK-associated molecules and possess NK-like cytotoxic potential.

There is growing evidence that naïve T cells, stem cell memory T cells (T_{SCM}), or central memory T cells (T_{CM}) are superior as source of adoptive T-cell transfer for tumor immunotherapy (44–47). In terms of surface markers, regenerated CD8 $\alpha\beta$ T cells expressing low CCR7 and high CXCR3 are considered as effector phenotype T cells, although we are using IL21 to inhibit terminal differentiation (5, 48). Nevertheless, our culture method has advantages that the regenerated CD8 $\alpha\beta$ T cells can be expanded more than 10,000 fold by repeated TCR stimulation while retaining comparable TCR-specific cytotoxic potential. Furthermore, it was reported to be hard to evaluate *in vitro* cultured CTLs just based on their surface markers with regard to the point whether they are terminally differentiated or not (49). In addition, at present, it is also difficult to appropriately address this issue *in vivo* in xenograft model, because microenvironments in immune system of mouse xenograft model are so far from physiological situation in human. In case of murine CTLs regenerated from T-iPSCs, it was recently shown that the regenerated CTLs exhibited memory response *in vivo* in mice bearing syngeneic tumor (50).

One may concern about the safety of regenerated CTLs is tumorigenicity after administration. Although we use SV40 large T antigen to establish T-iPSCs, we confirmed that SV40 was completely deleted from all the iPSCs. Also we examined the long-term safety in transfused mice, and confirmed all the mice were free of tumor derived from regenerated CTLs.

Another concern about safety is off-target specificity. In our method almost all the regenerated CD8 $\alpha\beta$ T cells after expansion by LCL pulsed with cognate peptide retain the same TCR detected as tetramer positive cells (Fig. 2C, 5E, 6C, 6F). To be exact, this is not the case at the DP stage, where RAG1 and RAG2 genes are expressed and secondly rearrangement in TCR alpha chain gene may occur. Indeed, a part of the CD8 $\alpha\beta$ T cells just regenerated by TCR stimulation have lost specificity for original epitope, resulting in the formation of negative population in tetramer staining (Supplementary Fig. S3E, top right). However, these cells can be eliminated during expansion by LCL pulsed with cognate peptide (Supplementary Fig. S3E, bottom right). We actually did not see any sign of Graft-versus-Host

reaction in the mice inoculated with regenerated CTLs (Fig. 7G). In addition, it is also possible to completely ensure clonality by deleting RAG1 or RAG2 gene in T-iPSCs using genome editing technology.

In this method starting from 10^4 T-iPSCs in one 10-cm culture dish, we can eventually obtain 10^9 to 10^{10} regenerated CD8 $\alpha\beta$ T cells in one round culture, which could be sufficient for one transfusion to a patient, thus providing a strong rationale for clinical application of this strategy. We have established antigen-specific T-iPSCs from healthy volunteers, making an allogeneic approach possible. In such an allogeneic setting, it would be preferable to produce T-iPSCs from an HLA-haplotype homozygous donor, because regenerated CTLs could then be used for HLA-haplotype heterozygous patients. In such a strategy, "Off the shelf" T-cell drug would come true by generating CD8 $\alpha\beta$ T cells in advance and preserving as frozen stocks.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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