**A simple method to regenerate CD8αβ type T cells with potent antigen-specific cytotoxic activity from T cell-derived iPSCs**

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**Supplemental methods**

**Preparation of mononuclear cells and LCLs**

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers bearing HLA-A\*24:02 and bone marrow mononuclear cells from leukemia patients were isolated using Ficoll-Paque PLUS (GE Healthcare Bio-sciences AB). Leukemia cells were more than 95% in all patient samples. Autologous B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with Epstein-Barr virus.

**Cell lines and culture**

Cell lines and autologous LCLs were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The HLA-A\*24:02 gene-transduced C1R cell line (C1R-A\*24:02) was cultured with 0.5 mg/mL hygromycin B (Nacalai tesque).

**Expansion of LMP2 and WT1 specific CTLs**

2.5x105 PBMC from healthy volunteers were plated in a well of 96-well U bottom plate (Falcon) and cultured in RPMI 1640 medium supplemented with 10% human AB serum (Sigma), Penicillin (100 U/ml)-Streptomycin (100 μg/ml) mixed solution (Nacalai tesque) and LMP2 (TYGPVFMSL) or WT1 (CYTWNQMNL) (1) synthetic peptide (10 μg/ml; Eurofins). After two days, recombinant IL-2 (12.5 U/ml) (Peprotech), IL-7 (10 ng/ml) (Peprotech) and IL-21 (30 ng/ml) (Peprotech) were added to each well. CD8+ cells containing antigen-specific CTLs detected by tetramer staining were isolated and co-cultured with HLA-A\*24:02+ LCL, which were irradiated (50 Gy) and pre-cultured with 100 nM of LMP2 or WT1 synthetic peptide for further stimulation. After two days of culture, IL-2, IL-7 and IL-21 were added as the same concentration as in the first stimulation. Antigen-specific CTLs were stimulated with LCL every two weeks.

**Comparison of methods of establishing iPSCs from T cells**

Human cord blood T cells or peripheral blood T cells were enriched using CD3 microbeads (Milteny Biotec) and stimulated by CD3/28 T activator (Milteny Biotec). T cells were infected by Sendai Virus vectors of Yamanaka factors (KOSM) or KOSM + SV40 Large T antigen. After four weeks, human ES-like colonies were stained by Vector Black Alkaline Phosphatase Substrate kit II (Vector Laboratories) according to the manufacture’s instruction. Stained colonies were scanned and counted using ImageJ software.

**Establishment of LMP2 and WT1 specific T-iPSCs**

Tumor antigen specific T-iPSCs were established by the method slightly modified from that previously described (2). In brief, tumor antigen specific CTLs were enriched by CD8 microbeads (Milteny Biotec) or FACSAria III cell sorter (BD Biosciences). 1x106 enriched tumor antigen specific CTLs were transduced with Sendai virus vectors containing the four Yamanaka factors (3) and SV40 large T antigen (LTa) (Addgene) at MOI 3. Following spin infection, transduced cells were seeded onto murine embryonic fibroblasts (MEF) feeder cells and cultured in T-cell medium, RPMI-1640 supplemented with 10% human AB Serum and IL-2 (12.5 U/ml), IL-7 (10 ng/ml), IL-21 (30 ng/ml). Beginning at day 2, half the medium was replaced with human iPSC medium, Dulbecco’s modified Eagle’s medium/F12 (Sigma) supplemented with 20% knockout serum replacement (Gibco), nonessential amino acids (0.1 mM) (Gibco), 2-mercaptoethanol (10 mM) (Nacalai Tesque) and basic fibroblast growth factor (5 ng/ml) (Wako). Colonies started to appear from 21-35 days. Each colony was picked up and expanded. All iPSC clones were maintained in iPSC medium.

**Gene expression analysis by RT-PCR**

Total RNA was isolated using RNeasy Plus Mini Kit (QIAGEN) and cDNA was synthesized according to the manufacturer’s protocol using a SuperScript VILO cDNA Synthesis Kit (Thermo Fisher). cDNA was amplified by PCR using various sets of primers as described (2). 409B2, which is a human iPSC established from fibroblasts, was used as a positive control (4).

**Immunostaining**

Human iPSC colonies were fixed for 10 minutes with 4% PFA/PBS. Colonies were incubated overnight at 4°C with SSEA4 (Abcam [MC813]), Nanog (Abcam), Oct4 (Abcam), TRA-1-60 (Abcam [Tra-1-60]) monoclonal antibodies. Next day, colonies were stained with anti-mouse IgG-Alexa488, anti-rat IgG-Alexa488 or anti-rabbit IgG-Alexa488 (In vitrogen).

**Chromosome karyotyping**

Karyotype was determined by Nihon Gene Research Laboratories, Inc. using a standard staining protocol for Giemsa-banding.

**Teratoma formation**

1.0 x 106 human iPSCs were injected into the testis of NOD/ShiJic-scid mice. Twelve weeks after injection, tumors were resected, fixed in 1% formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

**PCR analysis and sequence analysis of Rearranged TCR genes**

For the sequence of TCR alpha and beta chain genes in primary tumor antigen specific CTLs and regenerated CD8SP cells derived from T-iPSCs, isolated total RNA was subjected to 5’RACE cDNA synthesis by SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer’s instructions. The TCR alpha and beta chain genes were amplified by 5’RACE PCR using a Universal Primer mix (Clontech) and TCR-α constant region primer (TCR-A ; 5’-CACAGGCTGTCTTACAATCTTGCAGATC-3’) or TCR-β constant region primers (TCR-B1; 5’-CTCCACTTCCAGGGCTGCCTTCA-3’ and TCR-B2; 5’- TGACCTGGGATGGTTTTGGAGCTA-3’). V/D/J usage was analyzed by IMGT/V-QUEST software.

**Flowcytometry**

The following monoclonal antibodies were used. CD3 (UCHT1), CD4 (RPAT4), CD8α (RPA-T8, HIT8a), CD8β (2ST8.5H7), CD33(P67.6), CD34 (gp105-120), CD43 (1g10), CD45RA (HI100), CD45RO (UCHL1), Perforin (dG9), Granzyme A (CB9), TNFα (MAb11), IFNγ (4S.B3), Nanog (N31-355), Oct3/4 (40/Oct-3), Tra-1-60 (TRA-1-60), Tra-1-81 (TRA-1-81), SSEA3 (MC-631), SSEA4 (MC-813-70), mouse IgG3 isotype control (MG3-35) and mouse IgM isotype control (MM-30) Abs. All Abs were purchased from BioLegend, BD Biosciences or eBioscience. HLA-A\*24:02+ LMP2 (419-427aa TYGPVFMSL) or WT1 (235-243aa CYTWNQMNL) tetramers (MBL) were used for the detection of T cells expressing TCR specific for LMP2 or WT1. Flow cytometry was performed using a FACSCantoII or FACSAriaIII (BD) with FlowJo software (TreeStar). The purity of sorted cells was usually more than 99%.

**Cytotoxicity assays using annexin V**

Target cells were labeled with CFSE (1 μg/ml) and pulsed with LMP2 or WT1 peptide at various concentrations. To assess HLA class I-restricted cytotoxicity, target cells were co-cultured with HLA class I blocking antibody (Clone: W6/32: BioLegend) for 1 hour. Target cells and effector cells were co-cultured at various ratios as indicated in 96 well V-bottomed plates (Nunc) for 6 to 12 hours. Dead and dying cells were detected with the combination of annexin V (BioLegend) and PI as described (5). Annexin V positive cells in the CFSE positive population were defined as dead and dying cells.

**IFNγ ELISA assay**

LMP2 specific p-CTL LMP2#1 or r-CD8αβ LMP2#1-1 (3x104) were co-cultured with THP1 pulsed with LMP2 peptide for 6 hours. The amount of IFNγ in the culture sup were detected using Ready-Set-Go! Human IFNγ ELISA kit (eBioscience).

**Intracellular cytokine assays**

Regenerated CD8 T cells were co-cultured with target cells pulsed with LMP2 or WT1 peptide in the presence of Brefeldin (Sigma) for 6 hours. Cells were stained with CD8a Ab and permeabilized with a BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Becton Dickinson) and then stained for intracellular cytokines with IFNγ Ab (4S.B3) and TNFα Ab (MAb11).

**Quantitative analysis of WT1 mRNA expression**

Total RNA was extracted using an RNeasy Plus mini kit (QIAGEN) and cDNA was synthesized as described above. Quantitative real-time PCR of WT1 mRNA was performed using Taqman probes as described (6). ABL mRNA was detected as an internal control as described (7). These samples were analyzed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems).

**Xenograft model of leukemia**

NOG mice were injected intraperitoneally with 2x104 HL60 leukemia cells. Beginning at day 1, PBS or 5x106 r-CD8αβ 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 for 4 weeks.

**Confirmation of safety of regenerated CTLs in xenograft model**

A total of seven NOG mice bearing no leukemia cells were inoculated with 5x106 r-CD8αβ WT1#3-3 and cytokines intraperitoneally in the same manner as described above. After six months, all the mice were dissected and gross lesions were checked macroscopically. Samples from peripheral blood, spleen and bone marrow were analyzed by flow cytometer. CD45+ CD8α+ cells were considered as residual r-CD8αβ WT1#3-3.

**Statistics**

All data are presented as mean ± SD. Differences were assessed using paired t-test using Prism (GraphPad software). Values of P<0.05 were considered significant.

**Supplemental references**

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