Stem Cell Reports, Volume *5*

Supplemental Information

A Safeguard System for Induced Pluripotent Stem Cell-Derived Rejuvenated T Cell Therapy

Miki Ando, Toshinobu Nishimura, Satoshi Yamazaki, Tomoyuki Yamaguchi, Ai Kawana-Tachikawa, Tomonari Hayama, Yusuke Nakauchi, Jun Ando, Yasunori Ota, Satoshi Takahashi, Ken Nishimura, Manami Ohtaka, Mahito Nakanishi, John J. Miles, Scott R. Burrows, Malcolm K. Brenner, and Hiromitsu Nakauchi

Supplemental Figures

Figure S1, Related to Figure 1

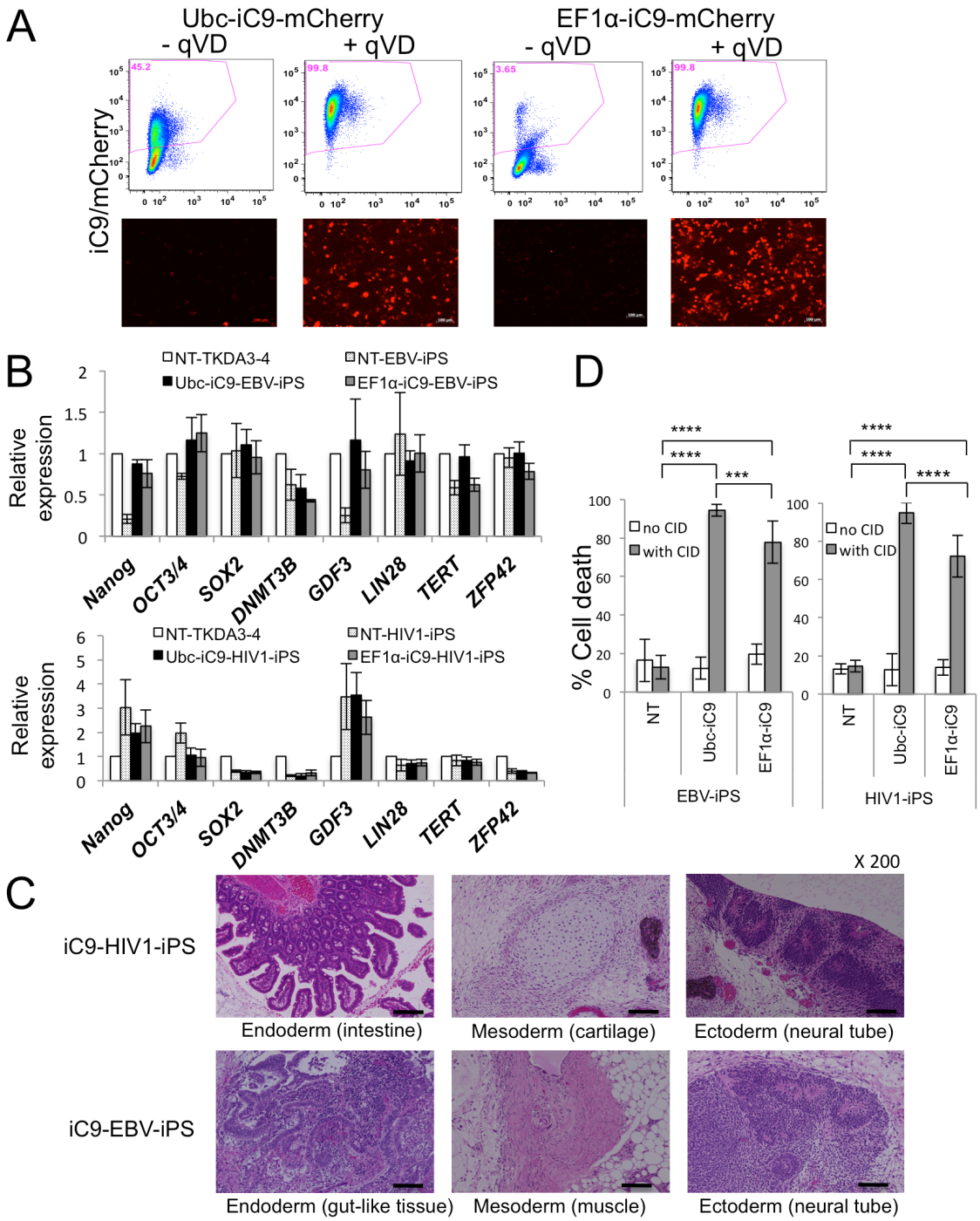

Figure S2, Related to Figure 3

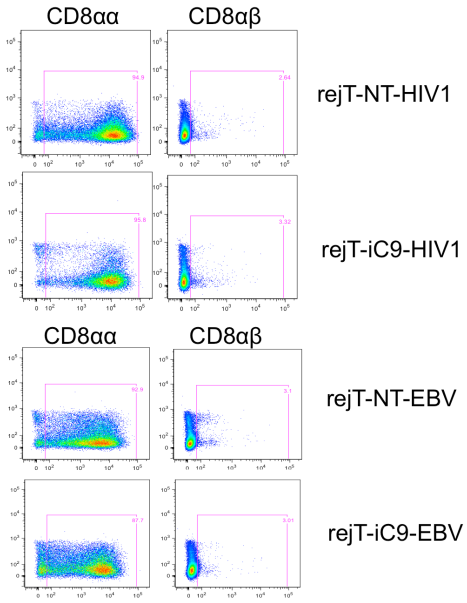

Figure S3, Related to Figure 5

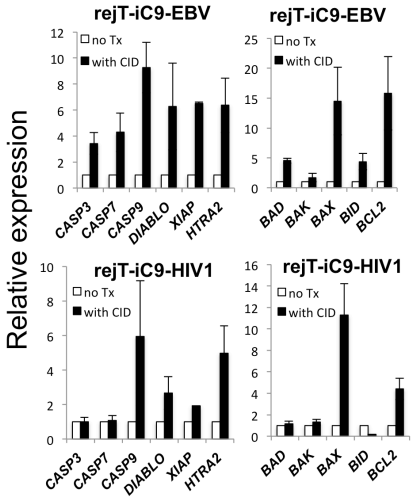

Figure S1, Related to Figure 1

(A) iC9/mCherry expression by lentiviral *iC9*-transduced 293T cells. Forty-eight hours after lentiviral *iC9* transduction of 293T cells, iC9/mCherry expression by 293T cells was analyzed by flow cytometry to compare lentivirus titers with and without administration of qVD, a caspase inhibitor (upper panel).

Fluorescence-microscopy images show iC9/mCherry expression by lentiviral *iC9* transduced 293T cells in each condition. The plots and images are representative of three independent experiments. The scale bar represents 100 μm (lower panel)

(B) Pluripotency of iC9-iPSCs. Quantitative PCR for pluripotency-associated genes in NT-EBV-iPSCs, Ubc-iC9-EBV-iPSCs, and EF1α-iC9-EBV-iPSCs (upper panel) and in NT-HIV1-iPSCs, Ubc-iC9-HIV1-iPSCs, and EF1α-iC9-HIV1-iPSCs (lower panel). Individual PCR results were normalized against endogenous *GAPDH* and are shown as relative expression against levels of NT-TKDA3-4 expression. Data are presented as mean of three independent experiments ± SD.

(C) Representative HE-stained histological sections of a teratoma formed in a NOD-Scid mouse. iC9-HIV1-iPSCs and iC9-EBV-iPSCs differentiated into cell lineages derived from endoderm, mesoderm, and ectoderm. The scale bar represents 100 μm.

(D) Induction of apoptosis in Ubc-iC9 or EF1a-iC9-iPSCs after CID treatment. T-iPSC lines (EBV-iPSC and HIV1-iPSC) were transduced with lentiviral *Ubc-iC9* or *EF1a-iC9*. These transduced iPSC lines and a non-transduced (NT) iPSC line were treated with CID (80 nM) and apoptosis was measured 24 hours later by flow cytometry for annexin V / 7-amino-actinomycin D (7-AAD) marking. Data are presented as mean of three independent triplicate experiments. Error bars represent ± SD. ***P<0.001, ****P<0.0001 by two-way analysis of variance (ANOVA).

Figure S2, Related to Figure 3

CD8αα and CD8αβ expression by iC9-iPSC-derived CTLs. Flow-cytometric analysis of rejT-NT-HIV1, rejT-iC9-HIV1, rejT-NT-EBV, and rejT-iC9-EBV 14 days after stimulation with PBMC, IL7, and IL15. rejT-NT-EBV and rejT-iC9-EBV, EBV-specific CTLs from NT-and iC9-EBV-iPSCs. rejT-NT-HIV1 and rejT-iC9-HIV1, HIV1-specific CTLs from NT-and iC9-HIV1-iPSCs. The plots are representative of three independent experiments.

Figure S3, Related to Figure 5

Apoptosis-associated gene expression in iC9-iPSC-derived CTLs. Quantitative PCR was used to compare apoptosis-associated protein levels in rejT-iC9-EBV (upper panel) and rejT-iC9-HIV1 (lower panel). rejT-iC9-EBV and rejT-iC9-HIV1 were left untreated or were treated with CID. Individual PCR results were normalized against *GAPDH*. Data are presented as mean of three independent experiments ± SEM.

Supplemental Experimental Procedures

Vector construction and virus production

Two iC9-expressing lentiviral vectors (Ubc-iC9 and EF1α-iC9) were generated from CS-CDF-CG-PRE (Yamaguchi et al., 2012). Polymerase chain reaction (PCR)-amplified *Ubc* or -*EF1α* promoter fragments (EcoRI, HpaI, NheI- BsiWI, XbaI, AgeI) were cloned into the EcoRI- AgeI site of CS-CDF-CG-PRE, resulting in CS-Ubc-GFPv2 or Cs-EF1α-GFPv2. To generate a 2A-linked *iC9* and *mCherry* cassette, *iC9* (SphI, NheI- ClaI) and *mCherry* (XbaI- XhoI) were separately amplified by PCR and inserted into T7-mOKS (Yamaguchi et al., 2012) by replacing *Oct4* with *iC9* and with

mCherry. The *iC9-2A-mCherry* cassette was then cloned into the XbaI- XhoI site of the CS-Ubc-GFPv2 or CS-EF1α-GFPv2 vectors. Lentiviral vectors pseudotyped with the vesicular stomatitis virus G glycoprotein were produced as described (Yamaguchi et al., 2012). The pan-caspase inhibitor qVD-Oph (R&D Systems, Minneapolis, MN) (20 μM) was added to the medium to protect 293T cells during lentivirus production. The vector encoding *GFP/FFLuc* is described (Quintarelli et al., 2007).

Generation of iC9-iPSCs

In brief, CTLs were stimulated by 5 mg/ml phytohemagglutinin (PHA-L) (Sigma-Aldrich, St. Louis, MO) and transduced with reprogramming factors via Sendai virus vectors as described (Nishimura et al., 2011; Nishimura et al., 2013; Vizcardo et al., 2013; Wakao et al., 2013). 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, 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 supplemented with 20% knockout serum replacement, 2 mM L-glutamine, 1% nonessential amino acids, 10 mM 2-mercaptoethanol, and 5 ng/ml basic fibroblast growth factor). Two iPSC lines, TKDA3-4 and TKCBSeV-9, were established from human dermal fibroblasts (Takayama et al., 2010) and cord blood (Ochi et al., 2014), respectively. All iPSC lines were maintained in culture with Essential 8^{TM} medium (Gibco, Life Technology, Carlsbad, CA).

T-Cell Differentiation from T-iPSCs

Small clumps of iPSCs (<100 cells) were transferred onto irradiated C3H10T1/2 cells, with co-culture in EB medium (Iscove's modified Dulbecco's medium supplemented with

15% fetal bovine serum [FBS] and a cocktail of 10 mg/ml human insulin, 5.5 mg/ml human transferrin, 5 ng/ml sodium selenite, 2 mM L-glutamine, 0.45 mM a-monothioglycerol, and 50 mg/ml ascorbic acid) in the presence of VEGF. Hematopoietic cells collected from iPSC sac contents were transferred onto irradiated NOTCH ligand-expressing C3H10T1/2 feeder cells. The hematopoietic cells underwent T-lineage differentiation on NOTCH ligand-expressing C3H10T1/2 cells during co-culture in OP9 medium (aMEM supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin) in the presence of SCF, FLT-3L and IL-7. T-lineage cells were then harvested, stimulated with 5 mg/ml PHA-L (Sigma-Aldrich), mixed with irradiated PBMCs, and co-cultured in T-cell medium in the presence of IL-7 and IL-15.

Flow Cytometric Analyses

Flow cytometric analyses and cell sorting were performed using FACS ARIAII(BD Bioscience). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). After cells were incubated with the appropriate concentration of antibody cocktail for 30 min at 4 ℃, they were washed with PBS. Propidium iodide was added to exclude dead cells. Negative controls used for FACS gating were based on unstained cells verified by isotype-matched antibodies as having the same negative staining pattern.

Pluripotency of iC9-iPSCs

To assess expression levels of endogenous pluripotency genes, total RNA from iPSCs was extracted using RNeasy Micro kits (Qiagen, Venlo, The Netherlands) and subjected to reverse transcription using High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Waltham, MA) with random hexamer primers. Quantitative PCR was performed in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using a TaqMan Array Human Stem Cell Pluripotency Card (Applied Biosystems).

Expression was calculated by relative quantification using the ΔΔCt method with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as endogenous control. To prove pluripotency of iC9-iPSCs, undifferentiated T-iPSCs were suspended in Corning Matrigel Matrix containing 10 μM of the Rho-associated kinase (Rock) inhibitor Y-27632 (Wako, Osaka, Japan) and injected (1.3 x 10^6 cells/mouse) into testicular interstitium or subcutaneous tissue of NOD-Scid mice. Six weeks after injection, the animals were sacrificed, the testes (with tumors) were removed and fixed in 5% paraformaldehyde, and samples were embedded in paraffin. Sections stained with hematoxylin / eosin were examined by light microscopy for evidence of tri-lineage germ layer differentiation.

Measurement of apoptosis

CID/AP20187 was purchased from Clontech (Mountain View, CA) as B/B homodimerizer and diluted in 100% ethanol. On the day after 1 x 10^5 iPSCs or 1.5 x 10^5 CTLs were plated in 24-well plates, CID, 80 nmol/l, was added to the wells. Twenty-four hours after CID addition, the cells were stained with annexin-V and 7-AAD for 15 min according to the manufacturer's instructions (BD Biosciences). Negative controls used for FACS gating were based on unstained cells for this assay. Cells that bound annexin V or took up 7-AAD were quantified by flow cytometry (FACS ARIA Ⅱ, BD Bioscience) using FlowJo software (Tree Star, Ashland, OR).

Clonogenic Survival Assays

We slightly modified a published protocol (Franken et al., 2006). On the day after NT- and iC9-iPSCs were plated at 1 x 10^5 cells/well in 6-well plates, CID, 80 nmol/l, was added. iPSC lines were maintained in culture with Essential 8™ medium (Gibco, Life Technology) and allowed to form colonies for 14 days; the medium was aspirated from each well and methylene blue solution, 0.1% w/v in 50% methanol, 1 ml, was added to each well. Twenty minutes later, this was aspirated away and the cells were washed

with PBS at least 3 times. The well contents then were photographed to permit iPSC colony identification. The results reflected survival of clonogenic cells.

ELISPOT and ^{51}Cr Release Assays

Enzyme-linked immunospot (ELISPOT) analysis was performed to determine the frequency of T-cells secreting IFN-γ in response to EBV-antigen peptide or HIV1-antigen peptide (Kawana-Tachikawa et al., 2002; Straathof et al., 2005a). Cytotoxic specificity of each CTL line was analyzed in a standard 4-hour chromium-51 release assay (Kawana-Tachikawa et al., 2002; Rooney et al., 1998).

Real-time Quantitative-PCR

To compare gene expression profiles for rejT-iC9-EBV and rejT-iC9-HIV1 with those for other cell lineages, quantitative PCR was performed using Customized Card or Human Apoptosis (Applied Biosystems). Individual PCR reactions were normalized against 18S rRNA (Customized Card) or *GAPDH* (Human Apoptosis).

Supplemental References

Franken, N.A., Rodermond, H.M., Stap, J., Haveman, J., and van Bree, C. (2006). Clonogenic assay of cells in vitro. Nature protocols *1*, 2315-2319.

Nishimura, K., Sano, M., Ohtaka, M., Furuta, B., Umemura, Y., Nakajima, Y., Ikehara, Y., Kobayashi, T., Segawa, H., Takayasu, S.*, et al.* (2011). Development of defective and persistent Sendai virus vector: a unique gene delivery/expression system ideal for cell reprogramming. The Journal of biological chemistry *286*, 4760-4771.

Ochi, K., Takayama, N., Hirose, S., Nakahata, T., Nakauchi, H., and Eto, K. (2014). Multicolor staining of globin subtypes reveals impaired globin switching during erythropoiesis in human pluripotent stem cells. Stem cells translational medicine *3*, 792-800.

Rooney, C.M., Roskrow, M.A., Suzuki, N., Ng, C.Y., Brenner, M.K., and Heslop, H. (1998). Treatment of relapsed Hodgkin's disease using EBV-specific cytotoxic T cells. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO *9 Suppl 5*, S129-132.

Straathof, K.C., Leen, A.M., Buza, E.L., Taylor, G., Huls, M.H., Heslop, H.E., Rooney, C.M., and Bollard, C.M. (2005a). Characterization of latent membrane protein 2 specificity in CTL lines from patients with EBV-positive nasopharyngeal carcinoma and lymphoma. Journal of immunology *175*, 4137-4147.

Takayama, N., Nishimura, S., Nakamura, S., Shimizu, T., Ohnishi, R., Endo, H., Yamaguchi, T., Otsu, M., Nishimura, K., Nakanishi, M*., et al.* (2010). Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. The Journal of experimental medicine *207*, 2817-2830.

Vizcardo, R., Masuda, K., Yamada, D., Ikawa, T., Shimizu, K., Fujii, S., Koseki, H., and Kawamoto, H. (2013). Regeneration of human tumor antigen-specific T cells from iPSCs derived from mature CD8(+) T cells. Cell stem cell *12*, 31-36.

Wakao, H., Yoshikiyo, K., Koshimizu, U., Furukawa, T., Enomoto, K., Matsunaga, T., Tanaka, T., Yasutomi, Y., Yamada, T., Minakami, H*., et al.* (2013). Expansion of functional human mucosal-associated invariant T cells via reprogramming to pluripotency and redifferentiation. Cell stem cell *12*, 546-558.