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ORIGINAL ARTICLE

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Generation and application of human induced-stem cell memory T cells for adoptive immunotherapy

Taisuke Kondo[1] | Yuki Imura[1,2] | Shunsuke Chikuma[1] | Sana Hibino[1] | Setsuko Omata-Mise[1] | Makoto Ando[1] | Takashi Akanuma[1] | Mana Iizuka[1] | Ryota Sakai[1] | Rimpei Morita[1,3] | Akihiko Yoshimura[1]

[1]Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan

[2]Sohyaku Innovative Research Division, Mitsubishi Tanabe Pharma Corporation, Yokohama, Japan

[3]Department of Immunology, International University of Health and Welfare School of Medicine, Narita, Japan

Correspondence
Akihiko Yoshimura, Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan.
Email: yoshimura@a6.keio.jp

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Adoptive T-cell therapy is an effective strategy for cancer immunotherapy. However, infused T cells frequently become functionally exhausted, and consequently offer a poor prognosis after transplantation into patients. Adoptive transfer of tumor antigen-specific stem cell memory T (T_{SCM}) cells is expected to overcome this shortcoming as T_{SCM} cells are close to naïve T cells, but are also highly proliferative, long-lived, and produce a large number of effector T cells in response to antigen stimulation. We previously reported that activated effector T cells can be converted into T_{SCM}-like cells (iT_{SCM}) by coculturing with OP9 cells expressing Notch ligand, Delta-like 1 (OP9-hDLL1). Here we show the methodological parameters of human $CD8^+$ iT_{SCM} cell generation and their application to adoptive cancer immunotherapy. Regardless of the stimulation by anti-CD3/CD28 antibodies or by antigen-presenting cells, human iT_{SCM} cells were more efficiently induced from central memory type T cells than from effector memory T cells. During the induction phase by coculture with OP9-hDLL1 cells, interleukin (IL)-7 and IL-15 (but not IL-2 or IL-21) could efficiently generate iT_{SCM} cells. Epstein–Barr virus-specific iT_{SCM} cells showed much stronger antitumor potentials than conventionally activated T cells in humanized Epstein–Barr virus transformed-tumor model mice. Thus, adoptive T-cell therapy with iT_{SCM} offers a promising therapeutic strategy for cancer immunotherapy.

KEYWORDS
adoptive immunotherapy, cytokine, Epstein–Barr virus, immunological memory, methodological study

1 | INTRODUCTION

Adoptive T-cell therapy is a promising approach to cancer therapy.[1] Tumor-infiltrating lymphocyte infusion is a classic and effective method for treating melanoma patients.[2,3] Isolated tumor-infiltrating lymphocytes (TILs) are restimulated by tumor-associated antigen

Abbreviations: Bcl-6, B-cell lymphoma 6 protein; CAR, chimeric antigen receptor; CCR7, C-C chemokine receptor type 7; CD45RA, CD45 isoform RA; CFSE, carboxyfluorescein succinimidyl ester; EBV, Epstein–Barr virus; IL, interleukin; iT_{SCM}, induced-T_{SCM}; LCL, lymphoblastoid cell line; MART-1 DC, MART-1 peptide-pulsed monocyte-derived dendritic cell; MART-1, melanoma antigen recognized by T cell-1; TAA, tumor-associated antigen; T_{CM}, central memory T; TCR, T-cell receptor; T_{EM}, effector memory T; T_{EMRA}, CD45RA-positive effector memory T; TIL, tumor-infiltrating lymphocyte; T_{SCM}, stem cell memory T.

(TAA)-loaded antigen-presenting cells, and then expanded TILs are infused into patients. Gene transducing technologies have also recently been applied to adoptive T cell therapy. Artificially designed T-cell receptor (TCR) or chimeric antigen receptor (CAR) against TAA and CAR target antigens are transduced into patients' T cells with TCR stimulation and expansion.[4] As in TIL therapy, these transduced T cells are also re-infused into patients to attack tumor cells. However, the therapeutic effects of tumor-specific T cell transfer have been limited because infused T cells frequently lose functionality after transplantation.[1,5] The TAA-specific T cells become exhausted and dysfunctional in the tumor microenvironment and through the T cell culture procedures.[6,7] Therefore, restoration and enforcement of T cell function and persistency could improve long-term outcomes for cancer patients.

Memory T cells have been classified into two subpopulations: peripheral tissue-homing effector memory T (T_{EM}) cells and lymphatic tissue-homing central memory T (T_{CM}) cells.[8] Novel memory T cell subpopulations, called stem cell memory T (T_{SCM}) cells, were recently identified as the "stem" of memory T cells. Stem cell memory T cells expressed naïve T-cell surface markers, $CD44^{low}CD62L^{high}CCR7^{+}$ in mice and $CD45RA^{+}CD45RO^{-}CCR7^{+}CD62L^{+}$ in humans.[9,10] These cells possess high proliferative and long-term survival abilities in vivo, producing a large number of effector T cells with potent antitumor functions.[11,12] Similar naïve-like memory T cells were discovered in human and called memory T cells with a naive phenotype.[13] A recent paper by Ahmed et al[14] indicated that human T_{SCM} cells are highly proliferative but have long telomeres and high levels of telomerase activity.

Due to these superior features of T_{SCM} cells, methods of generating T_{SCM} cells in vitro for adoptive T cell therapy have been investigated. In 2009, Gattinoni et al[15] first reported that a glycogen synthase kinase 3β inhibitor, TWS119, induces murine and human T_{SCM} cells by activating Wnt signals. TWS119 treatment arrested the cell cycle during TCR stimulation and inhibited the differentiation to T_{CM} and T_{EM} cells. Therefore, this method is only applicable for naïve T cells, and the number of induced T_{SCM} cells is limited. Alternative methods have been proposed by optimizing TCR strength, cytokine supplement (including interleukin [IL]-7, IL-15, and IL-21), and drug treatment. These methods could generate T_{SCM} cells in vitro, but these methods still generate T_{SCM} cells from naïve T cells (Table 1).[16-20] We have established a novel two-step culture system for T_{SCM} induction, which is constituted by a "prime" step and an "induction" step, and have named the induced-T_{SCM} "induced-T_{SCM} (iT_{SCM})" cells.[12] In this paper, we describe a detailed methodology of iT_{SCM} generation and report optimal conditions of priming and cytokine treatment. We also investigate the antitumor efficacy of human iT_{SCM} cells in a humanized mouse model.

2 | MATERIALS AND METHODS

2.1 | OP9-hDLL1 cell coculture

Human T cells were activated using the methods mentioned above. To activate Notch signaling, activated T cells were cocultured with OP9-hDLL1 cells. Human T cells and OP9-hDLL1 cells were cocultured with human IL-2 (20 ng/mL; PeproTech), human IL-7 (10 ng/mL; PeproTech), human IL-15 (20 ng/mL; Biolegend), or human IL-21 (20 ng/mL; PeproTech) in Minimum Essential Medium Eagle-alpha modification for 11 days.

2.2 | Statistics

Statistical analysis was carried out using Student's *t*-test, one-way ANOVA and a long-rank test, using GraphPad Prism version 6.05 software (GraphPad Software, La Jolla, CA, USA). The variance among the groups was estimated using the *F*-test, and *P*-values $<.05$ were considered statistically significant. All data are presented as the mean ± SEM. Mice were randomly assigned to experimental groups. The investigators were not blinded to allocation during experiments and outcome assessment.

Further information regarding materials and methods is included in Tables S1,S2.

TABLE 1 Recently reported methods for stem cell memory T cell generation from naïve T cells

Publication	Year	TCR procedure	TCR strength	Cytokine	Signaling inhibitor
Gattinoni et al *Nat Med*	2011	CD3/CD28 beads	Full	IL-2	GSK3-β (TWS119)
Cieri et al *Blood*	2013	CD3/CD28 beads	Full	IL-7 + IL-15	None
Gomez-Eerland et al *Hum Gene Ther Methods*	2014	CD3/CD28 beads	Full	IL-7 + IL-15	None
Sabatino et al *Blood*	2016	CD3/CD28 beads	Short (4 d)	IL-7 + IL-21	GSK3-β (TWS119)
Scholz et al *EBioMedicine*	2016	CD3/CD28 beads	Full	IL-2	mTOR (rapamycin)
Alvarez-Fernandez et al *J Trans Med*	2016	CD3/CD28 beads	Short (2 d)	IL-21	None
Hurton et al *Proc Natl Acad Sci USA*	2016	APC	Full	mbIL-15	None
Kagoya et al *JCI Insight*	2017	CD3/CD28 beads, APC	Short (1 d)	IL-2 + IL-15	None
Jeza et al *Pan Afr Med J*	2017	APC	Full	IL-21	mTOR (rapamycin)
Zanon et al *Eur J Immunol*	2017	CD3/CD28 beads, APC	Full	IL-7 + IL-15	None
Kaartinen et al *Cytotherapy*	2017	CD3/CD28 beads	Full	Low IL-2	None

APC, antigen presenting cell; d, day; GSK3-β, glycogen synthase kinase 3β; IL, interleukin; mb, membrane bound; TCR, T cell receptor.

3 | RESULTS

3.1 | Overview of iT_{SCM} cell generation

Several methods for generating T_{SCM} have recently been reported. The strength of TCR stimulation, cytokine effects, and signaling inhibitors were associated with T_{SCM} generation from naïve T cells and are summarized in Table 1.[11,16-25] In our two-step culture system, T cells are first primed and expanded by strong TCR stimulation, and are then cocultured with OP9-hDLL1 cells to convert into iT_{SCM} cells (Figure 1A). To acquire a large number of antigen-specific $CD8\alpha^+$ T cells, we first isolated peripheral human $CD8^+$ T cells, labeled with cell trace dye and co-cultured them with autologous Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) for 7 days in the "prime" step. Next, cell trace dye-diluted activated T cells were purified by a cell sorter and were then transferred onto and cocultured with DLL1-expressing OP9 stromal cells (OP9-hDLL1) for 11 days in the "induction" step (Figure 1B). More than 90% of singlet live cells expressed $CD8\alpha$ and more than 80% of $CD8\alpha^+$ T cells highly proliferated (dividing more than six times) and mainly showed $CD45RA^-CCR7^-$ T_{EM} (76.4%) and $CD45RA^-CCR7^+$ T_{CM} (13.4%) phenotypes (Figure 2A). We then isolated phenotypical T_{EM} and T_{CM} cells and transferred them onto OP9-hDLL1 layers with human IL-7. Conversion efficiency from EBV-specific activated human T cells to iT_{SCM} is 10% from T_{EM} cells and >85% from T_{CM} cells (Figure 2B). In addition, the number of T_{EM}-derived $CD8\alpha^+$ T cells was not changed or was only slightly reduced, whereas that of T_{CM}-derived $CD8\alpha^+$ T cells was significantly increased (Figure 2C). To investigate the EBV-specific recall response, we cocultured T_{EM}, T_{CM}, and iT_{SCM} cells with autologous LCL for 60 hours. The EBV-specific iT_{SCM} cells recovered greater number of cells than the T_{EM} and T_{CM} cells did (Figure 2D). The same results could be acquired from several independent experiments using T cells from distinct

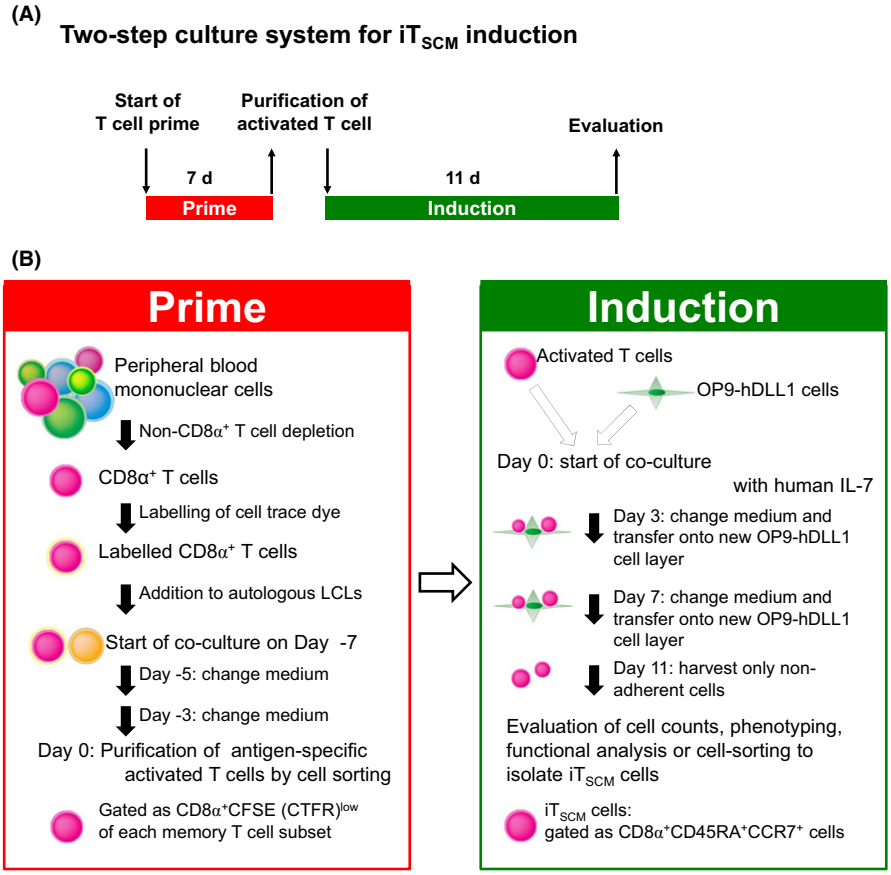

FIGURE 1 Experimental outline for induced stem cell memory T (iT_{SCM}) cell induction. A, Schematic for two-step culture system for iT_{SCM} induction. Peripheral $CD8\alpha^+$ T cells are isolated and activated by T-cell receptor stimulation for 7 d in the first step, called the prime step. Activated T cells are purified by cell sorting and transferred onto OP9-hDLL1 cells for 11 d in the second step, the induction step. Evaluation of iT_{SCM} cells is carried out 11 d after OP9-hDLL1 coculture. B, Detailed protocols for iT_{SCM} generation. Preparation of autologous lymphoblastoid cell lines (LCLs) and OP9-hDLL1 feeder cells are necessary to induce human iT_{SCM} cells. Start with $CD8\alpha^+$ T cell isolation on Day −7 of the prime step (left). Peripheral $CD8\alpha^+$ T cells are negatively isolated from PBMCs and labeled by cell trace dye, followed by the addition of labeled-$CD8\alpha^+$ T cells to irradiated autologous LCL and the start of coculture. Activated T cells, which are defined as cell trace dye-diluted $CD8\alpha^+$ cells, are purified by cell sorting. Next, purified activated T cells (1×10^5 cells/mL) are cocultured with OP9-hDLL1 cells in the presence of human interleukin-7 (IL-7; 10 ng/mL) (right). Harvesting $CD8\alpha^+$ T cells, adjusting cell density (1×10^5 cells/mL), and transferring onto a new OP9-hDLL1 layer are carried out on Days 3 and 7. Coculture with OP9-hDLL1 cells for 11 d induced iT_{SCM} cells, defined as $CD8\alpha^+$ $CD45RA^+$ C-C chemokine receptor type 7 $(CCR7)^+$ cells. Analysis earlier than Day 11 is possible, but the induction of iT_{SCM} cells was completed after more than 10 d of coculture. CFSE, carboxyfluorescein succinimidyl ester; CTFR, cell trace far red dye

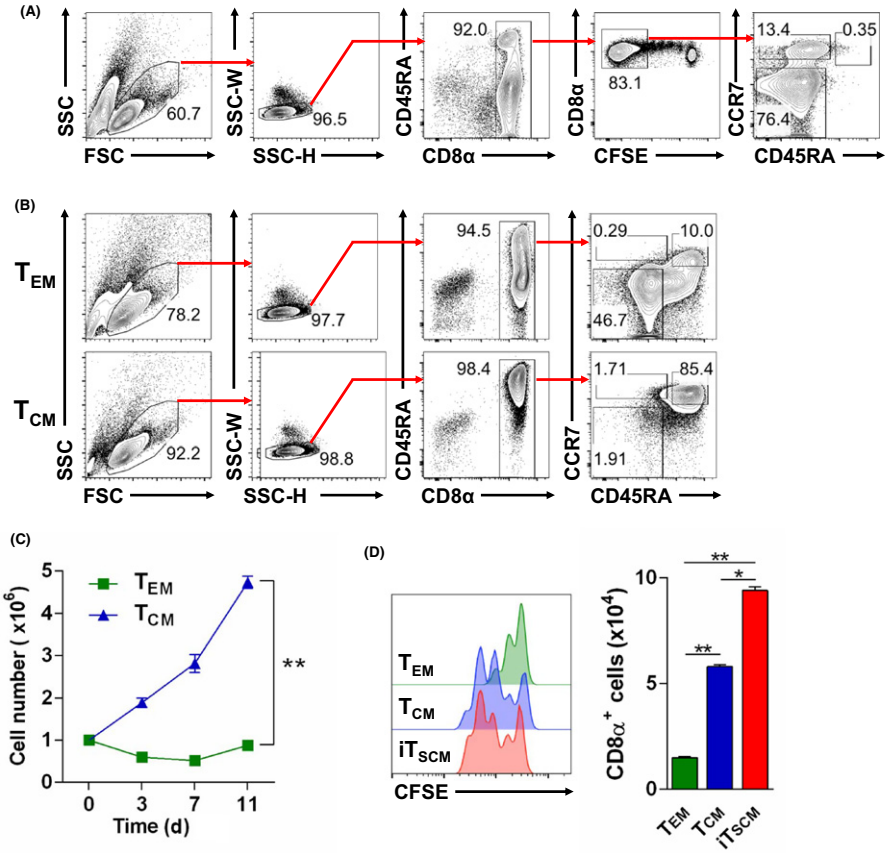

FIGURE 2 Generation of Epstein–Barr virus (EBV)-specific induced stem cell memory T (iT_{SCM}) cells from human peripheral blood T cells. A,B, Generating EBV-specific $CD8^+$ iT_{SCM} cells from human peripheral blood T cells. EBV-specific activated T cells mainly showed effector memory T (T_{EM}) ($CD8\alpha^+$ carboxyfluorescein succinimidyl ester [CFSE]$^{low/-}$ C-C chemokine receptor type 7 [CCR7]$^-$) cell phenotypes and central memory T (T_{CM}) ($CD8\alpha^+CFSE^{low/-}CCR7^+$) cell phenotypes (Day 0) (A). T_{EM} and T_{CM} cells were sorted, and then cocultured with OP9-hDLL1 cells for 11 d. Flow cytometry analysis of $CD8\alpha^+$ cells 11 d after OP9-hDLL1 cell coculture (B). C, Number of $CD8\alpha^+$ cells at 0, 3, 7, and 11 d after coculture with OP9-hDLL1 cells. T_{EM} or T_{CM} cells (1×10^5) were cocultured with OP9-hDLL1 cells on Day 0. D, Recall responses to EBV. Flow cytometry analysis of CFSE dilution in each cell population (left). Column graph shows the fold increase of recovered T cells (n = 3 per group) (right). $^*P < .05$, $^{**}P < .01$ (one-way ANOVA). Data are representative of independent experiments using human samples provided by three healthy donors. Error bars show SEM. CD45RA, CD45 isoform RA; FSC, forward scatter; SSC, side scatter; SSC-H, side scatter-height; SSC-W, side scatter-width

healthy subjects. These data indicate that the coculture with OP9-hDLL1 cells convert into iT_{SCM} from both T_{EM} and T_{CM} cells. However, the efficiency of iT_{SCM} generation from T_{CM} cells is higher than that from T_{EM} cells, and T_{CM} cells do proliferate during iT_{SCM} generation.

3.2 | T-cell receptor stimulating procedures do not alter the efficiency of iT_{SCM} generation

For targeting tumor cells, antigen specificity is critical for adoptive T cell transfer. Expansion of endogenous antigen-specific T cells is used in TIL therapy. Patients' peripheral T cells or tumor-infiltrating T cells were expanded in vitro by culture with TAA-loaded antigen-presenting cells, tumor cells, or tumor tissues.[3] Retroviral or lentiviral TCR or CAR transduction exogenously provides antigen specificity with pan-TCR stimulation using anti-CD3/CD28 beads.[26] To compare the effects of TCR stimuli variations on iT_{SCM} generation, we stimulated peripheral $CD8\alpha^+$ T cells by LCL, CD3/CD28 beads, and melanoma antigen recognized by T cell-1 (MART-1) peptide-pulsed monocyte-derived dendritic cell (MART-1 DC). These activated T cells were cocultured with OP9-hDLL1 cells for 11 days following TCR stimulation (Figure 3A). The $CD8\alpha^+$ T cells activated by LCL and anti-CD3/CD28 beads were expanded, and approximately 13.9% and 30.6% of activated cells showed T_{CM} phenotypes, respectively. Additionally, MART-1 DC induced MART-1-specific T_{CM} cells (Figure 3B). We next isolated these cells and cocultured them with OP9-hDLL1 cells. The induction step efficiently converted the T_{CM} cells into iT_{SCM} cells (Figure 3C), and the efficiency and amplification rates of iT_{SCM} from activated T_{CM} cells are summarized in Table 2. The iT_{SCM} cells from LCL and CD3/CD28 bead-activated T cells were generated in greater number than from MART-1-specific T cells. These data indicate that activated T cells by any TCR stimulating procedure can be converted into iT_{SCM} cells.

3.3 | Cytokine effects in the prime step on iT_{SCM} generation

Interleukin-15 and IL-21 have been reported to enhance T_{SCM} inducing efficiency.[17,21] To investigate T cell stimulating and homeostatic cytokine effects in the prime step, we cocultured T cells with

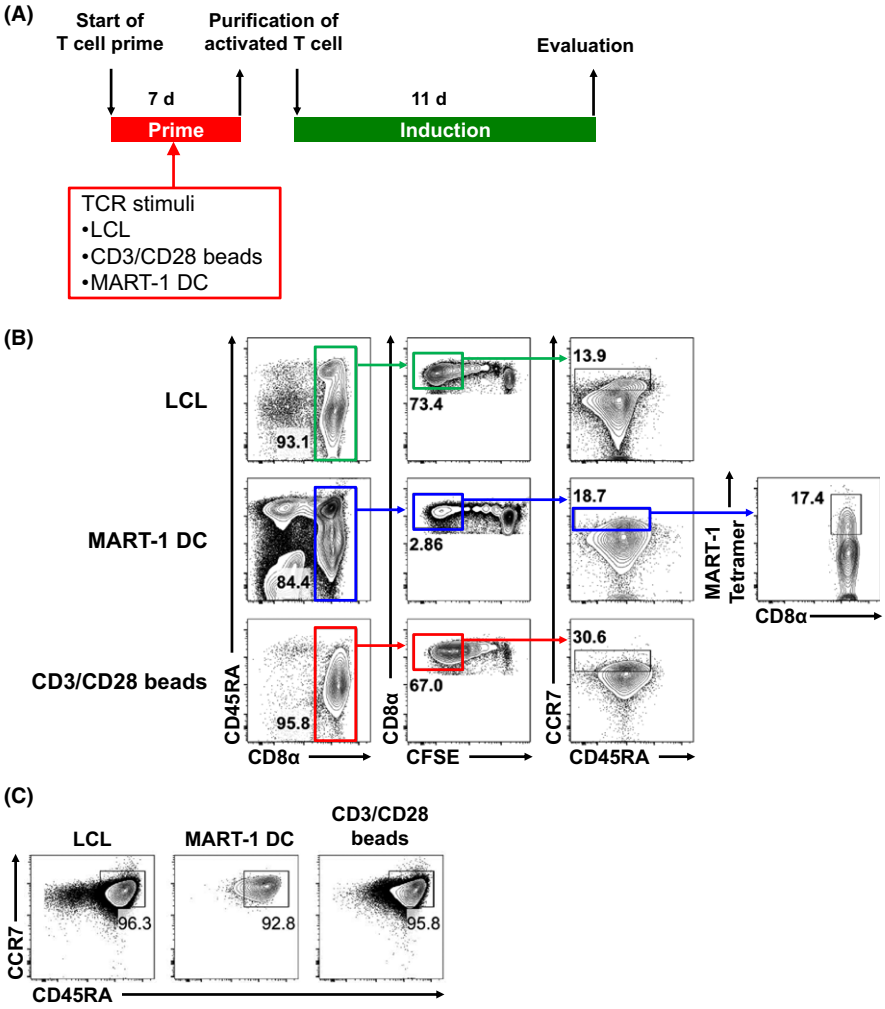

FIGURE 3 Generation of induced stem cell memory T (iT_{SCM}) cells from antigen-specific and CD3/CD28 bead-activated T cells. A, Schematic for generating human iT_{SCM} cells from Epstein–Barr virus and melanoma antigen recognized by T cell-1 (MART-1)-specific and CD3/CD28 bead-activated T cells. B,C, Sorting panels of each $CD8\alpha^+$ central memory T (T_{CM}) cell population stimulated by lymphoblastoid cell lines (LCL), MART-1 peptide-pulsed monocyte-derived dendritic cells (MART-1 DC), and CD3/CD28 beads. MART-1^+ T_{CM} cells were detected by MART-1-loaded MHC class I tetramer (B). CD45RA/C-C chemokine receptor type 7 (CCR7) profiles of the $CD8\alpha^+$ T cells on Day 11 after coculture with OP9-hDLL1 cells (C). Data are representative of independent experiments using human samples provided by two HLA-$A2^+$ healthy donors. CD45RA, CD45 isoform RA; CFSE, carboxyfluorescein succinimidyl ester; TCR, T-cell receptor

autologous LCL in the absence or presence of IL-2, IL-7, IL-15, and IL-21 (Figure S1A). IL-7 and IL-15 made $CD8\alpha^+$ T cells highly proliferate compared with other conditions (Figure S1B). Both T_{EM} and T_{CM} cells stimulated by IL-2 and IL-21 recovered comparable rates and counts of T_{EM} and T_{CM} cells that were activated in the absence of cytokines (Figure S1C,D). CD45RA-positive effector memory T (T_{EMRA}) cells, which are CD45RA-positive effector memory T cells, are thought of as terminally differentiated effector T cells. We also observed low numbers of T_{SCM} and T_{EMRA} cells in IL-2-, IL-21-, and cytokine-absent conditions, whereas large numbers of T_{SCM} and T_{EMRA} cells stimulated by IL-7 and IL-15 were observed (Figure S1C, D). We next cocultured IL-7- and IL-15-stimulated T cells, which were individually isolated as T_{SCM}, T_{CM}, T_{EM}, and T_{EMRA} phenotypes, with OP9-hDLL1 cells (Figure S1E). Both IL-7- and IL-15-stimulated T_{SCM} cells completely retained T_{SCM} cell phenotypes and showed a 1- to 2-fold increase in cell number after coculture with OP9-hDLL1 cells (Figure S1F, Table 3). T_{CM} cells activated in the absence of cytokines were efficiently converted into iT_{SCM} cells and recovered more than 10-fold the number of cells compared with the number before OP9-hDLL1 coculture, whereas a partial conversion into iT_{SCM} cells and 1- to 3-fold increases in cell number compared with before the OP9-hDLL1 coculture were observed in IL-7 and IL-15 stimulated T_{CM} cells from 1×10^5 cells (Figure S1F, Table 3). The absence of cytokines and the presence of IL-7 and IL-15 in the prime step could more efficiently convert into iT_{SCM} cells with a higher degree of proliferation than other T cells.

TABLE 2 Percentages and number of recovered induced stem cell memory T (iT_{SCM}) cells (mean ± SEM) after OP9-hDLL1 coculture from activated T cells by various T-cell receptor stimulation procedures

	iT_{SCM} (%)	iT_{SCM} ($\times 10^5$)
LCL	95.8 ± 0.36	5.44 ± 0.054
MART-1 DC	92.3 ± 0.36	0.52 ± 0.044
CD3/CD28 beads	95.5 ± 0.071	3.86 ± 0.15

Lymphoblastoid cell line (LCL) and melanoma antigen recognized by T cell-1 (MART-1) peptide-pulsed monocyte-derived dendritic cell (DC), n = 3; CD3/CD28 beads, n = 6.

3.4 | Conversion of long-term cultured T cells into iT_{SCM} generation

In conventional T-cell therapy, T cells are repeatedly stimulated for a long period to be expanded before infusion into patients; however,

such long-term TCR stimulation leads to T cell exhaustion or anergy.[27] We assessed whether long-term culture of T cells has any effects on iT_{SCM} generation. We first cocultured carboxyfluorescein succinimidyl ester (CFSE)-labelled T cells with autologous LCL in the presence of IL-7 or IL-15. Seven days after the coculture, we isolated EBV-specific T cells and then restimulated these cells by coculture with LCL for an additional 21 days (Figure S2A,B). Long-term culture strongly induced terminal differentiation of EBV-specific T cells, whereas several T cells maintained T_{SCM} and T_{CM} phenotypes, as shown in Figure S2(C). Cytokine-depleted conditions did not allow T cells to survive for 2 weeks (data no shown). We next isolated each memory T cell subset and then cocultured them with OP9-hDLL1 cells. Both IL-7- and IL-15-stimulated T_{SCM} cells completely retained T_{SCM} cell phenotypes and showed a 0.8-fold increase in cell counts after coculture with OP9-hDLL1 cells (Figure S2D, Table 4). Partial conversion into iT_{SCM} cells and 0.8-fold increases in the number of cells were observed in IL-7-stimulated T_{CM} cells and IL-7- or IL-15-stimulated T_{EMRA} cells (Figure S2D, Table 4). Small percentage of interleukin-15-stimulated T_{CM} cells and T_{EM} cells were converted into iT_{SCM} cells (Figure S2D, Table 4). These data indicate that the presence of IL-7 in long-term priming could efficiently generate iT_{SCM} cells from T_{SCM}, T_{CM}, and T_{EMRA} cells.

TABLE 3 Percentages and number of recovered induced stem cell memory T (iT_{SCM}) cells (mean ± SEM) after OP9-hDLL1 coculture from activated T cells with or without cytokines in the prime step (n = 3 per group)

	None	IL-7	IL-15
iT_{SCM} (%)			
T_{SCM}	NA	99.6 ± 0.033	99.6 ± 0.033
T_{CM}	89.20 ± 0.27	70.9 ± 0.290	39.6 ± 0.620
T_{EM}	4.39 ± 0.32	17.9 ± 0.300	17.0 ± 0.220
T_{EMRA}	NA	60.7 ± 0.660	55.3 ± 3.210
iT_{SCM} ($\times 10^5$)			
T_{SCM}	NA	2.76 ± 0.0110	0.830 ± 0.0260
T_{CM}	10.700 ± 0.4200	3.25 ± 0.1100	1.570 ± 0.0990
T_{EM}	0.019 ± 0.0011	0.070 ± 0.0041	0.042 ± 0.0036
T_{EMRA}	NA	0.43 ± 0.0370	0.260 ± 0.0360

IL, interleukin; NA, not applicable; T_{CM}, central memory T; T_{EM}, effector memory T; T_{EMRA}, CD45RA-positive effector memory T; T_{SCM}, stem cell memory T.

TABLE 4 Percentages and cell count of recovered induced stem cell memory T (iT_{SCM}) cells (mean ± SEM) after OP9-hDLL1 coculture from long-term activated T cells (n = 3 per group)

	IL-7	IL-15
iT_{SCM} (%)		
T_{SCM}	99.40 ± 0.133	98.90 ± 0.34
T_{CM}	58.50 ± 3.920	0.27 ± 0.059
T_{EM}	0.62 ± 0.110	0.63 ± 0.11
T_{EMRA}	29.40 ± 0.150	47.40 ± 2.01
iT_{SCM} ($\times 10^5$)		
T_{SCM}	0.830 ± 0.0440	0.7900 ± 0.00760
T_{CM}	0.840 ± 0.0640	0.0097 ± 0.00220
T_{EM}	0.006 ± 0.0010	0.0020 ± 0.00044
T_{EMRA}	0.140 ± 0.0063	0.0900 ± 0.00370

IL, interleukin; T_{CM}, central memory T; T_{EM}, effector memory T; T_{EMRA}, CD45RA-positive effector memory T; T_{SCM}, stem cell memory T.

3.5 | Cytokine effects in the induction step on iT_{SCM} generation

To assess cytokine effects in the induction step, we next cocultured T cells with autologous LCL for 7 days, and EBV-specific T

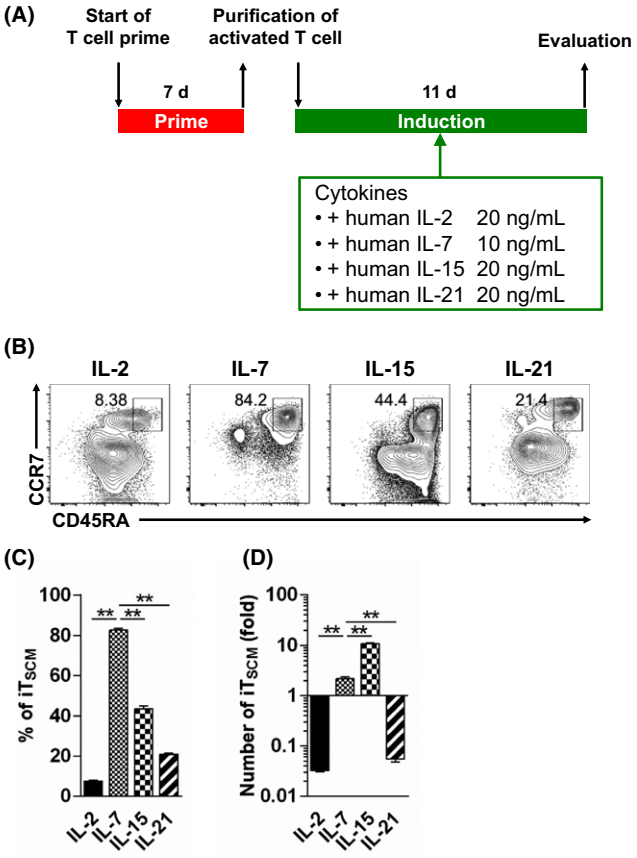

FIGURE 4 Effects of cytokines in the induction step for induced stem cell memory T (iT_{SCM}) cell generation. A, Schematic for the induction of human iT_{SCM} cells by human interleukin (IL)-2, IL-7, IL-15, and IL-21. Coculture with OP9-hDLL1 layers was undertaken in the presence of human IL-2 (20 ng/mL), human IL-7 (10 ng/mL), human IL-15 (20 ng/mL), or human IL-21 (20 ng/mL). B, Flow cytometry analysis of $CD8\alpha^+$ T cells after OP9-hDLL1 cell coculture with multiple cytokines. C,D, Percentages (C) and cell counts (D) of recovered iT_{SCM} cells after OP9-hDLL1 coculture (n = 3 per group). $^{**}P < .01$ (one-way ANOVA). Data are representative of at least two independent experiments. Error bars show SEM

cells were then transferred onto OP9-hDLL1 layers for 11 days in the presence of IL-2, IL-7, IL-15, and IL-21 (Figure 4A). The absence of cytokines induced T cell death in the induction step (data not shown). The conversion efficiency rates of iT_{SCM} cells were 8% (IL-2), 84% (IL-7), 44% (IL-15), and 21% (IL-21) (Figure 4B, C). Although IL-7 and IL-15 generated a greater number of iT_{SCM} cells than the initial cell number on Day 0, fewer IL-2- and IL-21-induced iT_{SCM} cells were recovered than the initial cell count (Figure 4D). These results indicate that both IL-7 and IL-15 generate a high number of iT_{SCM} cells in the induction step, but IL-7 generates iT_{SCM} cells with high purity and certain cell expansion, whereas IL-15 generates a larger number of iT_{SCM} cells with lower purity than IL-7 does. Next, to validate synergistic effects of cytokines, we induced iT_{SCM} cells in the presence of IL-7 with or without IL-2, IL-15, and IL-21. The combination of IL-7 and IL-15 effectively converted into iT_{SCM} cells and a larger number of the cells were recovered by the combination, compared with other conditions (Figure S3).

3.6 | Characterization of iT_{SCM} populations generated by different priming methods

It has been shown that the transcriptional program strictly controls effector and memory T cell fate and functions. Blimp-1 (encoded by *PRDM1*) and T-bet (encoded by *TBX21*) positively regulated terminal differentiation, whereas Eomes and B-cell lymphoma 6 protein (Bcl-6) promote memory formation and retain memory homeostasis.[28] These transcriptional programs govern not only effector/memory formation but also T cell survival and proliferative and effector ability.

To characterize iT_{SCM} cells derived from CD3/CD28 bead-activated T cells (beads-iT_{SCM}) and those cells derived from LCL-activated T cells (LCL-iT_{SCM}), we assessed these gene profiles and proliferative ability. Blimp-1 and T-bet were poorly expressed in all iT_{SCM} populations compared with T_{EM} and T_{CM} cells (Figure 5A,B). High expression of *EOMES* and low expression of *BCL6* were observed in beads-iT_{SCM} cells, whereas the opposite results were observed in LCL-iT_{SCM} cells either induced in the presence of IL-7

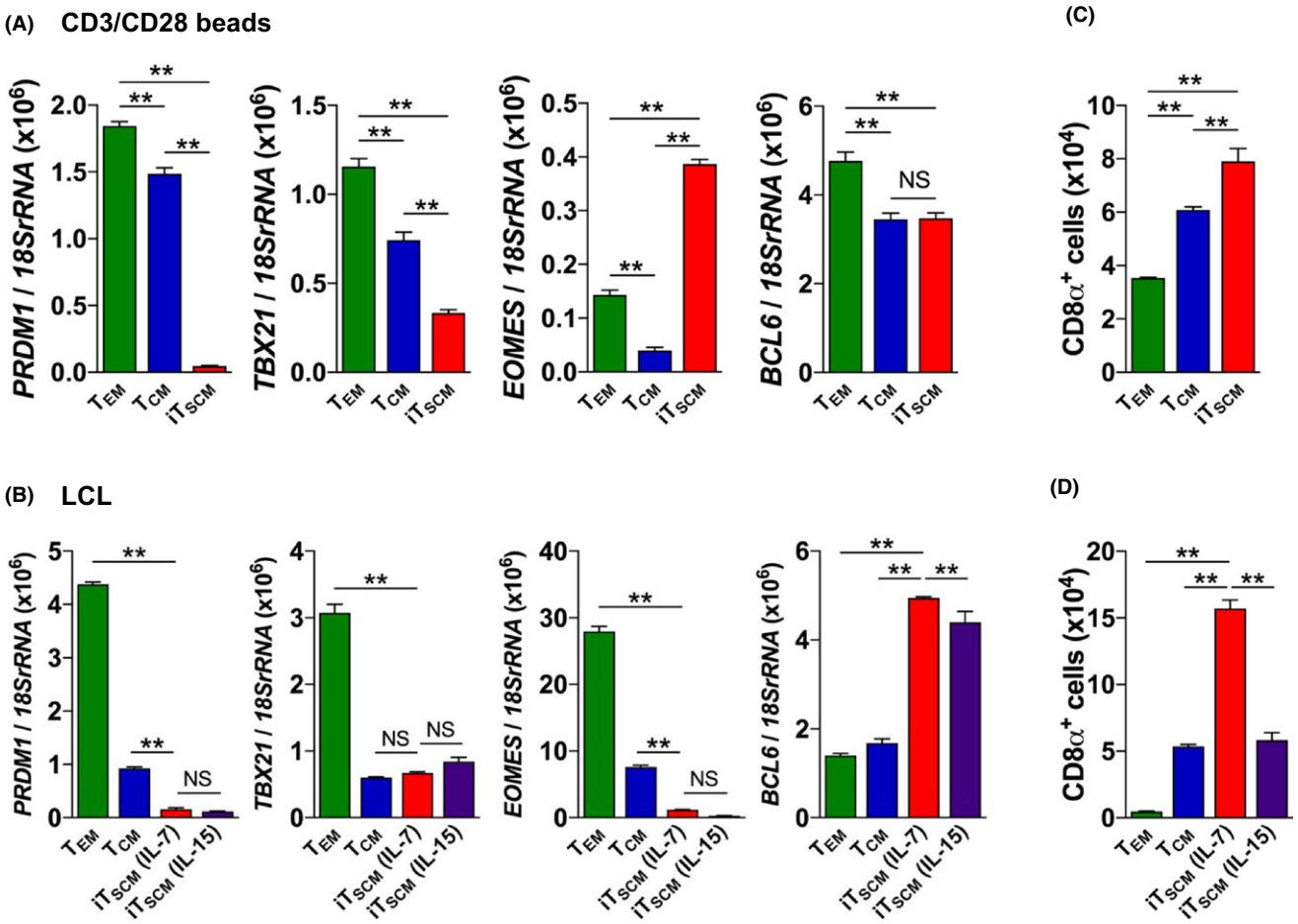

FIGURE 5 Gene profile and proliferative ability of induced stem cell memory T (iT_{SCM}) cells. A,B, Gene expression in bead-generated effector memory T (T_{EM}), central memory T (T_{CM}), and iT_{SCM} cells, and lymphoblastoid cell line-generated T_{EM}, T_{CM}, and iT_{SCM} cells induced by interleukin (IL)-7 (iT_{SCM} (IL-7)) or IL-15 (iT_{SCM} (IL-15)) (n = 3 per group). Each gene expression was normalized by 18S rRNA expression level. C, D, Recall responses by T-cell receptor stimulation. Each T cell population (5×10^4) was activated by CD3/CD28 beads for 60 h. Column graphs show the fold increase of recovered T cells (n = 3 per group). $^{**}P < .01$ (one-way ANOVA). Data are representative of at least two independent experiments. Error bars show SEM

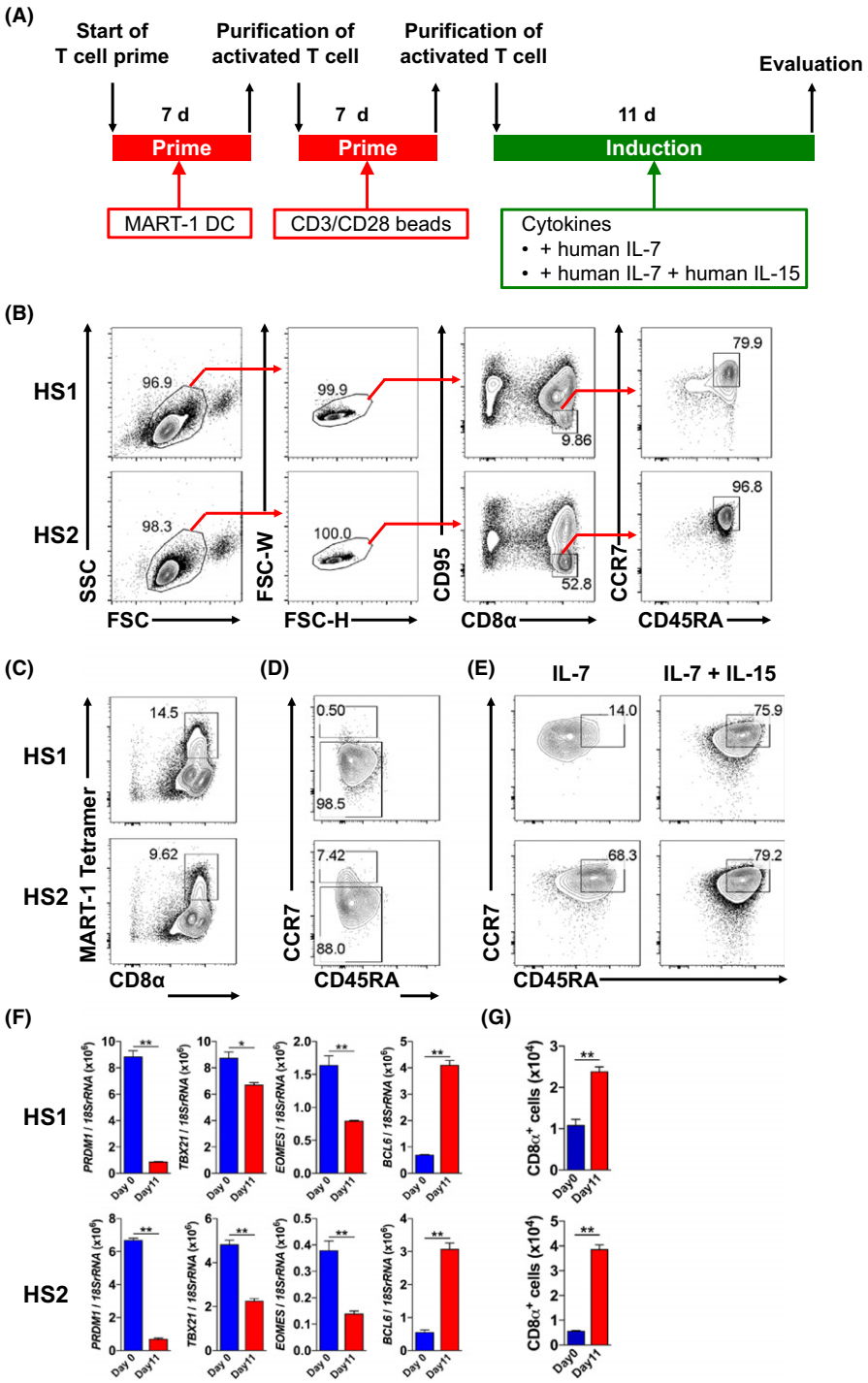

FIGURE 6 A, Schematic for generating human induced stem cell memory T (iT_{SCM}) cells from melanoma antigen recognized by T cell-1 (MART-1)-specific activated T cells. Purified naïve $CD8\alpha^+$ T cells were activated by MART-1 peptide-pulsed monocyte-derived dendritic cells (MART-1 DC) for 7 d. MART-1-specific T cells were then purified and restimulated by CD3/CD28 beads for 7 d. After 14 d of stimulation, whole MART-1-specific T cells were cocultured with OP9-hDLL1 cells for 11 d in the presence of interleukin (IL)-7 or both IL-7 and IL-15. B-E, Sorting panels of each step of $CD8\alpha^+$ T cell populations. Naïve T cells were purified from $HLA\text{-}A2^+$ PBMCs by cell sorting, gated as $CD8\alpha^+CD95^-CD45RA^+$ C-C chemokine receptor type 7 $(CCR7)^+$ cells (B). $MART\text{-}1^+$ central memory T (T_{CM}) cells were detected by MART-1-loaded MHC class I tetramer (C). CD45RA/CCR7 profiles of the $CD8\alpha^+$ T cells on Day 0 before coculture with OP9-hDLL1 cells (D) and on Day 11 after coculture with OP9-hDLL1 cells (E). F, Gene expression in MART-1-specific T cells before (Day 0) or after (Day 11) OP9-hDLL1 coculture. G, Recall responses by T-cell receptor stimulation. MART-1-specific activated (Day 0) and iT_{SCM} cells (Day11) (5×10^4) were activated by CD3/CD28 beads for 60 h. Column graphs show the fold increase of recovered T cells (n = 3 per group). $^*P < .05$, $^{**}P < .01$ (Student's *t*-test). Data are representative of independent experiments using human samples provided by two $HLA\text{-}A2^+$ healthy donors. Error bars show SEM. CD45RA, CD45 isoform RA; FSC, forward scatter; HS, healthy subject; SSC, side scatter; SSC-H, side scatter-height; SSC-W, side scatter-width

(designated as "iT_{SCM} (IL-7)") or IL-15 (designated as "iT_{SCM} (IL-15)") (Figure 5A,B). Beads-iT_{SCM} and iT_{SCM} (IL-7) cells showed strong proliferative ability after recall response, but weak proliferation was observed in iT_{SCM} (IL-15) cells (Figure 5C,D). Proliferation of iT_{SCM} (IL-7) cells was higher than beads-iT_{SCM} cells (Figure 5C,D). These results indicate that effector-associated programs are suppressed in all iT_{SCM} populations and iT_{SCM} (IL-7) cells have superior proliferative ability compared to other iT_{SCM} cells.

We then tried to generate TAA-specific iT_{SCM} cells. In Figure 3, MART-1-specific iT_{SCM} cells could be converted from MART-1-specific T_{CM} cells, but the recovered cell number was much lower than LCL- and beads-iT_{SCM} cells. To overcome this low yield, we optimized culture conditions for generating MART-1-specific iT_{SCM} cells. We first isolated naïve T cells from PBMC (endogenous T_{SCM} cells were depleted by anti-CD95 antibody) and cocultured with autologous MART-1 DCs for 7 days (Figure 6A,B). Next, we purified MART-1 $tetramer^+$ T cells and restimulated them using CD3/CD28 beads for additional 7 days (Figure 6C). Fourteen days after stimulation, most of the activated MART-1-specific T cells, which showed T_{EM} phenotypes, were transferred onto an OP9-hDLL1 layer in the

presence of IL-7 alone or IL-7 and IL-15 (Figure 6D). The combination of IL-7 and IL-15 could effectively induce LCL-iT$_{SCM}$ cells with more than 80-fold expansion (Figure S3). The combination could effectively induce MART-1-specific iT$_{SCM}$ cells regardless of donors; IL-7 alone could not effectively induce iT$_{SCM}$ cells from one donor (Figure 6E, Table 5). Gene profiles of the iT$_{SCM}$ cells resembled those of LCL-iT$_{SCM}$ cells and showed strong proliferative ability compared with the cells before iT$_{SCM}$ induction (Figure 6F,G). These results confirm that this two-step iT$_{SCM}$ induction system can be applied regardless of activation methods.

3.7 | Application of human iT$_{SCM}$ cells for cancer immunotherapy

The iT$_{SCM}$ cells showed strong proliferation following recall response and long-term persistence after adoptive transfer.[29] Thus, we investigated the antitumor effects of human iT$_{SCM}$ cells using human LCL-bearing mice. We s.c. inoculated LCL into NOD.Cg-*PrkDC* ^{cid}*Il2rg*^{tm1Wjl} (NSG) mice. Eight days after tumor inoculation, we transferred EBV-specific T$_{EM}$, T$_{CM}$, and iT$_{SCM}$ cells into autologous LCL-bearing mice (Figure 7A). As shown in Figure 7(B), EBV-specific iT$_{SCM}$ cells showed significantly stronger suppressive effects on LCL growth than EBV-specific T$_{EM}$ and T$_{CM}$ cells. Consequently, EBV-specific iT$_{SCM}$ cells improved the survival rates of the mice (Figure 7C). Tumor antigen-specific human iT$_{SCM}$ cells are more likely to have potent antitumor effects and are appropriate for adoptive cancer immunotherapy.

4 | DISCUSSION

Stem cell memory T cells have functional advantages for adoptive T-cell therapy compared with other memory T cell populations.[11] Thus, T$_{SCM}$ cells should play a significant role in cancer immunotherapy. We previously reported a novel T$_{SCM}$ generating method, converting memory and effector T cell subsets into T$_{SCM}$ cells. In this report, we optimized the conditions for generating iT$_{SCM}$ cells for potential adoptive immunotherapy. Our method constitutes two steps, the prime step and the induction step. This method can induce iT$_{SCM}$ cells regardless of the priming method: from activated T cells by non-specific TCR stimulation (CD3/CD28 beads), expanded T cells from existing memory T cells with a specific antigen (EBV), or expanded antigen-specific T$_{EM}$ cells from naive T cells (MART-1). As T cells expand during both priming and induction steps, we achieved a high iT$_{SCM}$ cell yield. In our experiments, more than 1 × 10^6 MART-1-specific iT$_{SCM}$ cells were recovered from 100 mL whole blood. In addition, we showed the conceptual advantage of human tumor antigen-specific iT$_{SCM}$ cells for antitumor adoptive T-cell therapy.

Efficiency and expansion of antigen-specific iT$_{SCM}$ cells are highly dependent on cytokines. For the induction of EBV-specific iT$_{SCM}$ cells, IL-7 was sufficient to generate a large number of EBV-specific iT$_{SCM}$ cells (Figures 4,S1). Combined stimulation with IL-7 and IL-15 efficiently induced MART-1-specific iT$_{SCM}$ cells from MART-1-specific T$_{EM}$ cells during the induction step (Figure 6). The cultural settings provided high yield (15-fold) of the iT$_{SCM}$ cells compared with only IL-7 stimulation (Table 5). The reason why different cytokines are required for LCL-induced iT$_{SCM}$ cells and MART-1 DC-induced iT$_{SCM}$ cells is not clear at present. This may be because of the

TABLE 5 Percentages and cell count of recovered induced stem cell memory T (iT$_{SCM}$) cells (mean ± SEM) after OP9-hDLL1 coculture from melanoma antigen recognized by T cell-1-specific T cells (n = 3 per group)

	HS1	HS2
iT$_{SCM}$ (%)		
IL-7	13.4 ± 0.42	68.1 ± 0.44
IL-7 + IL-15	76.4 ± 0.71	79.7 ± 0.26
iT$_{SCM}$ (×10^5)		
IL-7	0.004 ± 0.00058	1.16 ± 0.087
IL-7 + IL-15	2.250 ± 0.03900	11.9 ± 0.860

HS, healthy subject; IL, interleukin.

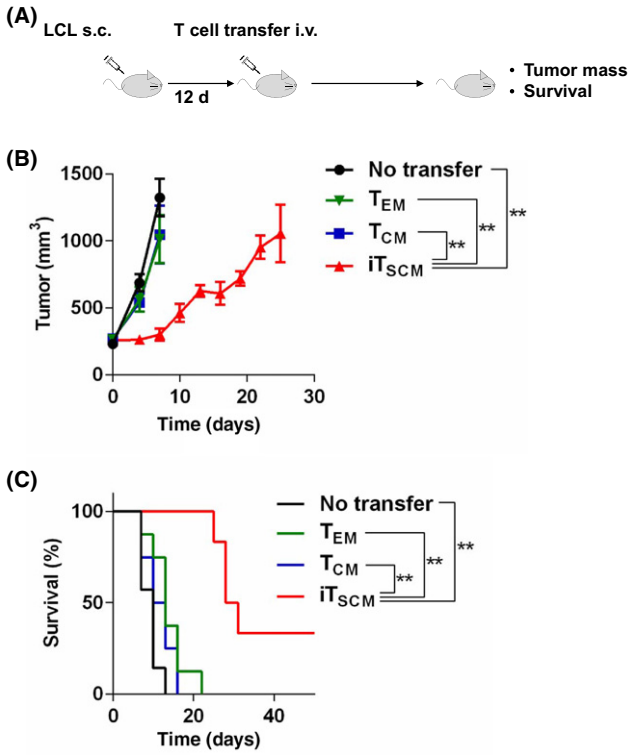

FIGURE 7 Antitumor potential of human induced stem cell memory T (iT$_{SCM}$) cells. A, Schematic for generating a humanized tumor model mice for adoptive T-cell therapy. Severe immunodeficient (NOD.Cg-*PrkDC* ^{cid}*Il2rg*^{tm1Wjl}/*Szj*, NSG) mice were s.c. inoculated with 5 × 10^6 Epstein–Barr virus-transformed lymphoblastoid cell line (LCL). Effector memory T (T$_{EM}$), central memory T (T$_{CM}$), and iT$_{SCM}$ cells (5 × 10^5) were adoptively transferred into LCL-bearing mice 12 d after LCL inoculation. B, Tumor volumes of LCL-bearing mice. C, Survival rates of LCL-bearing mice (no transfer and T$_{EM}$, n = 7; T$_{CM}$, n = 4; iT$_{SCM}$, n = 6) **P < .01 (one-way ANOVA [B]; Long-rank test [C]). Data are representative of at least two independent experiments. Error bars show SEM

difference in antigen-presenting cells (B cells vs DCs) or the difference in the origin of primed T cell phenotypes (in vivo memory T cells in LCL-iT_{SCM} and naïve T cells in MART-1 iT_{SCM}).

Expression of key transcription factors for memory subset differentiation and functions was compared in iT_{SCM} cells induced from different sources (Figures 5,6). Blimp-1 and T-bet, which positively regulate terminal effector formation were low in three different types of iT_{SCM} cells. Although Eomes and Bcl-6 mRNA expression appear to be variable in iT_{SCM} cells induced from CD3/CD28 bead-activated T cells, reduced expression of *EOMES* and increased expression of *BCL6* were observed in both MART-1 DC-induced iT_{SCM} cells and LCL-induced iT_{SCM} cells, suggesting that iT_{SCM} phenotypes are mostly conserved, regardless of the priming method.

One could argue that iT_{SCM} cells might be a result of selective expansion of pre-existing T_{SCM}-like cells. However, we generated MART-1-specific iT_{SCM} cells from naïve T cells that excluded T_{EMRA}, T_{EM}, T_{CM}, and T_{SCM} cells, from healthy donors. Thus, the possibility of expanding pre-existing T_{SCM} cells is unlikely, although it is very difficult to completely exclude this possibility of contamination. In addition, it is hard to show a direct generation of iT_{SCM} cells from pre-existing T_{EM} cells and T_{CM} cells in vivo. We showed that iT_{SCM} cells can be generated from activated T cells from immunized mice, which include T_{EM} cells. However, it is difficult to show the direct conversion of human existing T_{EM} cells to iT_{SCM} cells from healthy donors without immunization. Nevertheless, it is a great advantage of our method for immunotherapy that iT_{SCM} cells can be generated from T_{EM} and T_{CM} cells primed from any type of T cell, regardless of naive or memory.

The functional role of Notch signaling in iT_{SCM} cells remains to be clarified. Previously, we showed that iT_{SCM} cells can be induced by coculture with OP9-DL1 but not with OP9 cells. In addition, Notch signaling inhibitors strongly suppressed generation of iT_{SCM} cells.[12] These data indicate that Notch signals are indispensable for the induction of iT_{SCM} cells. Previous work by Maekawa et al[30] also reported that Notch signaling plays a central role in maintaining $CD4^+$ memory T cells. Therefore, we think that Notch signaling is important not only for induction but also for maintenance of iT_{SCM} cells.

As a next step for cancer immunotherapy, establishing the method to generate iT_{SCM} cells from exhausted T cells within the tumor. As we could not obtain TILs from patients at present, we have not addressed the question whether iT_{SCM} cells can be generated directly from TILs. However, as TILs can be expanded in vitro by IL-2 or TCR stimulation, we speculate that iT_{SCM} cells will be induced from TILs after expansion by our methods, like LCL-activated T cells or MART-1 DC-activated T cells. We also need to improve the method as the good manufacturing practice-graded methods without the use of OP9-hDLL1 feeder cells and stimulator LCLs.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

ORCID

Taisuke Kondo http://orcid.org/0000-0002-8545-1251
Akihiko Yoshimura http://orcid.org/0000-0001-5676-8949

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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