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Antigen-specific CD8⁺ memory stem T cells generated from human peripheral blood effectively eradicate allogeneic targets in mice

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Abstract

Background: As the implantation and long-term existence of tumor-specific T cells in host are the prerequisite for adoptive immunotherapy, memory stem T cells (T_{SCM}) with self-renewal and differentiation capacity show the greatest potential to implant and long-term exhibit function in vivo, compared with other T cells of differentiation stages. Hence, tumor-specific T_{SCM} have become potential candidate for adoptive T cell therapy of cancer. Here, we reported a protocol to generate allogeneic antigen-specific CD8⁺ T_{SCM} cells from human PBLs.

Methods: To prepare allogeneic antigen-specific CD8⁺ T_{SCM}, we used an LCL named E007 of defined HLA allotyping as simulator, a co-culture of E007 and allogeneic PBLs was carried out in the presence of differentiation inhibitor TWS119 for 7 days. Sorting of proliferation cells ensured the E007-specificity of the prepared T_{SCM} cells. The sorted lymphocytes underwent further expansion by cytokines IL-7 and IL-15 for further 7 days, making the E007-specific CD8⁺ T_{SCM} expanded in number. The stem cell and T memory cell properties of the prepared CD8⁺ T_{SCM} were observed in NOD-SCID mice.

Results: Our protocol began with 1×10^7 PBLs and resulted in 2×10^7 E007-specific CD8⁺ T_{SCM} cells in 2 weeks of preparation. The prepared T_{SCM} cells exhibited a proliferative history and rapid differentiation into effector cells upon the E007 re-stimulation. Importantly, the prepared T_{SCM} cells were able to exist long and reconstitute other T cell subsets in vivo, eradicating the E007 cells effectively after transferred into the LCL burden mice.

Conclusions: This protocol was able to prepare allogeneic antigen-specific CD8⁺ T_{SCM} cells from human PBLs. The prepared T_{SCM} showed the properties of stem cells and T memory cells. This study provided a reference method for generation of antigen-specific T_{SCM} for T cell adoptive immunotherapy.

Keywords: T memory stem cells, Allogeneic antigen specificity, Preparation in vitro, Adoptive immunotherapy

Background

Adaptive immunity is an effective strategy for cancer treatment. T cells are the main force to combat microbes and cancer cells in adaptive cells [1, 2]. However, T cell is heterogeneous, which exists in a continuum of differentiation states. According to differentiation stages, T cells can be divided into naive T cells (T_N), memory stem cells (T_{SCM}), central memory cells (T_{CM}), effector

memory cells (T_{EM}) and differentiated effectors (T_{EF}) [3, 4]. Adoptive T cell therapy (ACT) for clinical application is normally based on the use of terminally differentiated T_{EF} cells, which have short lifespan and inferior engraftment capacities [5–7]. T_{SCM} is defined as the earliest developmental stage of memory T cells, which possesses capacities of self-renewal and differentiation [5, 8]. Phenotypically, T_{SCM} can be identified by the expression of CD3⁺ CD45RA⁺ CD62L⁺ CD95⁺ CCR7⁺ CD28⁺ [5, 9–11]. T_{SCM} has longer lifespan, as it is reported T_{SCM} is able to be tracked in vivo for 12 years after infusion of genetically modified lymphocytes [7]. In particular, T_{SCM}

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mediates longer and more robust efficiency of tumor rejection *in vivo* compared to other memory and effector subsets [5, 6, 8, 12–14]. It is these characteristics that make T_{SCM} a good candidate for immunological cytotherapy of cancer.

T_{SCM} constitutes a small proportion of the T cell subset, 2–4% of the total T cells in peripheral blood [5, 8]. The premise of antigen-specific T_{SCM} used for clinical therapy is to accomplish *in vitro* T_{SCM} preparation in a large number. It is demonstrated that small molecule chemical inhibitor TWS119, a potent inhibitor of the serine–threonine glycogen synthase kinase 3 β (GSK-3 β) able to induce the Wnt- β -catenin signaling, helps the enrichment of T_{SCM} through differentiation inhibition both in mice and in human [15–17]. On the other hand, T_{SCM} can be amplified with cytokines IL-7 and IL-15; IL-21 is also reported to promote the generation of T_{SCM} under *ex vivo* culture conditions [6, 18–26].

Allogeneic hematopoietic stem cell transplantation (allo-SCT) is an effective immunotherapeutic approach with curative potential in patients with malignancies. The therapeutic basis is mainly dependent on the donor T cell alloresponses against the recipient's malignant cells named as graft versus leukemia (GVL) or graft versus tumor (GVT) effect [27]. T cell responses to alloantigen are of peptide-MHC complex (pMHC) specificity in the same way as those to nominal antigen [28, 29]. Transfer of alloreactive T cells with defined specificity, such as for leukemia- or tumor-associated antigens, is proposed to separate the GVL or GVT effect from the deleterious graft versus host disease (GVHD) [30, 31]. As the implantation and long-term existence of tumor-specific T cells in host are the prerequisite for adoptive immunotherapy, it is of importance to prepare alloantigen-specific T_{SCM} cells *in vitro*.

In this study, we explored a methodology of alloantigen-specific T_{SCM} (allo-specific T_{SCM}) preparing *in vitro*. Although both CD4⁺ and CD8⁺ T_{SCM} cells are reported, we focused on CD8⁺ T_{SCM} cells. The T_{SCM} cells were generated in an allogeneic co-culture, enriched by TWS119, then sorted with proliferation, and finally expanded by IL-7 and IL-15. Our protocol prepared 2×10^7 allo-specific T_{SCM} cells from 1×10^7 PBLs. An LCL burden mouse model was introduced in for the T_{SCM} cell behavior *in vivo*. LCL cells were human B lymphoblastoid cells immortalized by EB virus infection. Although this model would reflect lymphoproliferative disorders, the LCL cells in mice could act as allogeneic targets to measure eradication efficacy of T cells *in vivo* in our study. Importantly, the prepared T_{SCM} cells exhibited both stem cell and memory T cell properties, and were able to implant and effectively eradicate the targets after adoptive transfer into mice. This study provided a practical method for generation of allo-specific

T_{SCM} cells *in vitro*, which would be expected to be referred in preparation of allogeneic T_{SCM} grafts with defined antigen specificity for the purpose of adoptive immunotherapy.

Materials and methods

Peripheral blood lymphocyte isolation

Peripheral blood was obtained from healthy donors after informed consent under a protocol approved by the Ethics Committee of Tongji Medical College, Wuhan, China. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation through a ficoll-hypaque gradient (Ficoll-Hypaque density 1.077 g/ml) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), then placed in dish for 2 h to remove the adherent cells. The non-adherent cells were collected as peripheral blood lymphocytes (PBLs) for the co-culture.

Cell lines and antibodies

The EB virus (EBV) transformed B lymphoblastoid cell lines (LCLs) E007 and E001 were established in our lab according to reported protocol [32]. HLA typing for LCLs was performed with PCR-SSP [33]. The HLA class I alleles of E007 and E001 are mismatched, E007 carried A*110x, 310x; B*510x, 550x; C*030x, 150x; and E001 A*020x, 240x; B*460x, 540x; C*010x, 080x.

Fluorescent antibodies for cells staining included human CD3-APC-Cy7 (clone HIT3a), CD8-BV510 (clone SK1), CD3-Percp-Cy5.5 (clone HIT3a), CD62L-PE-Cy7 (clone DREG-56), CD45RA-APC (clone HIT100), CD45RA-BV421 (clone HI100), CD95-PE (clone DX2), CXCR7-PE (clone 10D1-J16), CD28-APC (clone CD28.2), IL-2-APC (clone MQ1-17H12), IFN- γ -PE (clone 4S.B3), TNF- α -PE (clone MAb11), the above antibodies were purchased from BioLegend, USA. Corresponding isotype for each antibody were used as isotype control. Cells were analyzed on a BD LSR flow cytometer. T cell subsets were determined using fluorescence minus one (FMO) controls for interesting antibody.

Allogeneic co-culture and allo-specific T_{SCM} preparation

Allogeneic PBLs were co-cultured with the E007 for raising alloreactive T_{SCM} cells. PBLs were stained with 5 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE, sigma) for 8 min at 37 °C and washed with RPMI1640 supplemented with 10% FBS three times. E007 were inactivated by irradiating (2.0 Gy). PBLs were mixed with E007 on day 0 at a ratio of 5:1 with 5 μ M TWS119 in the co-culture for the first week. PBLs cultured alone used as control. TWS119 and medium were replaced on day 4, and cells were counted by Trypan blue dye exclusion. As the allo-specific T cells showed

proliferation in the co-culture, CFSEdim cells were sorted by FACS with BD FACS AriaII on day 7. Sorted cells were expanded by cytokines IL-7 and IL-15 (Pepro-tech, USA) of 25 ng/ml each for a further week.

T cell subsets in the co-culture bulks were identified by their surface markers, CD3⁺ CD8⁺ CD45RA⁺ CD62L⁺ CCR7⁺ CD95⁺ CD28⁺ for T_{SCM}, CD3⁺ CD8⁺ CD45 RA⁻ CD62L⁺ for T_{CM}, CD3⁺ CD8⁺ CD45RA⁻ CD62L⁻ for T_{EM}, and CD3⁺ CD8⁺ CD45 RA⁺ CD62L⁻ for T_{EF}.

Preparation of allo-specific T_{EM} and T_{EF} for mouse transfer

Allo-specific T_{EM} and T_{EF} cells were prepared by a co-culture of allogeneic PBLs with E007 set up as above, but no TWS119 was added. CFSEdim cells were sorted on day 7. Sorted cells were further expanded by 300 U/ml IL-2 (Pepro-tech, USA) instead of IL-7 and IL-15 for a further week.

In vitro T_{SCM} cells differentiation assay and intracellular cytokine staining

The prepared T_{SCM} cells were incubated with E007, E001 cells and Dynabeads Human T-Activator CD3/CD28 (α-CD3/CD28) (Gibco, USA), respectively, in a 5:1 ratio. Cells were collected at 6, 12, and 24 h, and labeled with corresponding fluorescent antibodies for differentiation assay. The T_{SCM} cells incubated with the above stimulations in the presence of 1 × BFA (eBioscience, USA) were collected for intracellular IFN-γ, IL-2, and TNF-α staining after 4 h incubation. Samples were analyzed on BD Verse flow cytometer. The data analysis was performed with Flowjo software version 10.0 (Tree Star).

Quantitative real-time PCR

T cell subsets in the co-culture were sorted by BD Aria II flow cytometer based on the corresponding phenotypes on day 7. Total DNA of the T cells was isolated using a genomic Extraction kit (Takara, Japan), following manufacturer's instructions. TRECs of T cell subsets were detected using fluorescently quantitative PCR kit (Takara, Japan) with TREC-specific primers [34] on Bio-Rad CFX Sequence detection system.

Animal experiments

6-week-old female NOD-SCID mice were purchased from Vital River Lab Animal Co, Ltd. (Beijing; a distributor of the Jackson Laboratory). Animal experiments in this study were approved by the Ethical Committee of Tongji Medical College. The mice were irradiated with 2.0 Gy and randomized in five groups ($n = 5$), then intravenous injection with 8×10^6 E007 or E001 on day -3. The mice were injected with the 1×10^7 allo-specific T_{SCM} or allo-specific T_{EM} and T_{EF} on day 0. Blood samples were taken every week from mice after the T cell

transfer. The number and phenotype of human T cells in mouse peripheral blood were determined by flow cytometry. The signs of GVHD were monitored daily. Mice were euthanized on day 35 and bone marrow and spleen were removed to detect human T cells and residual LCL cells. The LCL cells were detected by the latent membrane protein 1 (LMP1) of EB virus using antibodies LMP1-FITC (primary antibody: mouse anti-EBV LMP1, clone CS1-4, Abcom; secondary antibody: goat anti-mouse IgG-FITC, clone poly 4053, BioLegend) and flow cytometry.

Immunofluorescence analysis for mouse spleen

Formaldehyde-fixed spleen specimens were embedded in paraffin and cut into sections for immunofluorescent detection of human CD3⁺ T cells with antibodies CD3-488 (primary antibody: rabbit anti-human CD3, clone SP7, ThermoFisher; secondary antibody: goat anti-rabbit IgG H&L-Alexa Fluor 488, Abcom) and LMP1-CY3 (secondary antibody: goat anti-mouse IgG H&L-Cy3, Abcom) respectively. Nuclei were stained with DAPI (BioLegend).

Statistical analysis

The statistical significance of differences between two groups was assessed with a 2-tailed paired or unpaired *t* test. Comparisons of more than two groups were performed by one-way ANOVA with multiple comparison tests. Data are shown as the mean ± standard deviation (SD). Difference were marked as NS, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$, and *** $P < 0.001$. All the data obtained from the study was analyzed using SPSS 22.0 (IBM, USA).

Results

Our in vitro protocol is able to prepare allo-specific CD8⁺ T_{SCM} cells effectively

To prepare allo-specific T_{SCM}, this study began with a co-culture of a simulator cells and allogeneic PBLs on day 0. The stimulator was an LCL named as E007 with defined HLA allotyping. Due to the difference in HLA alleles among random donors, allo-specific T_{SCM} cells were generated from T_N through proliferation during the co-culturing (Fig. 1a). The T_{SCM} were enriched in the presence of differentiation inhibitor TWS119, of which the optimal concentration was 5 μM in the allogeneic co-culture (Additional file 1: Figure S1A B). On day 7, the T_{SCM} cells in the co-culture bulks were defined by the phenotype CD3⁺ CD8⁺ CD45RA⁺ CD62L⁺ CD95⁺ CCR7⁺ CD28⁺ (Fig. 1b). The inhibition of differentiation in the allogeneic co-culture enriched the T_{SCM} numbers by 100 folds on day 7 (Fig. 1c). As the allo-specific T cells showed proliferation in the co-culture, sorting of proliferation cells ensured the antigen-specificity of the prepared T_{SCM} cells. The

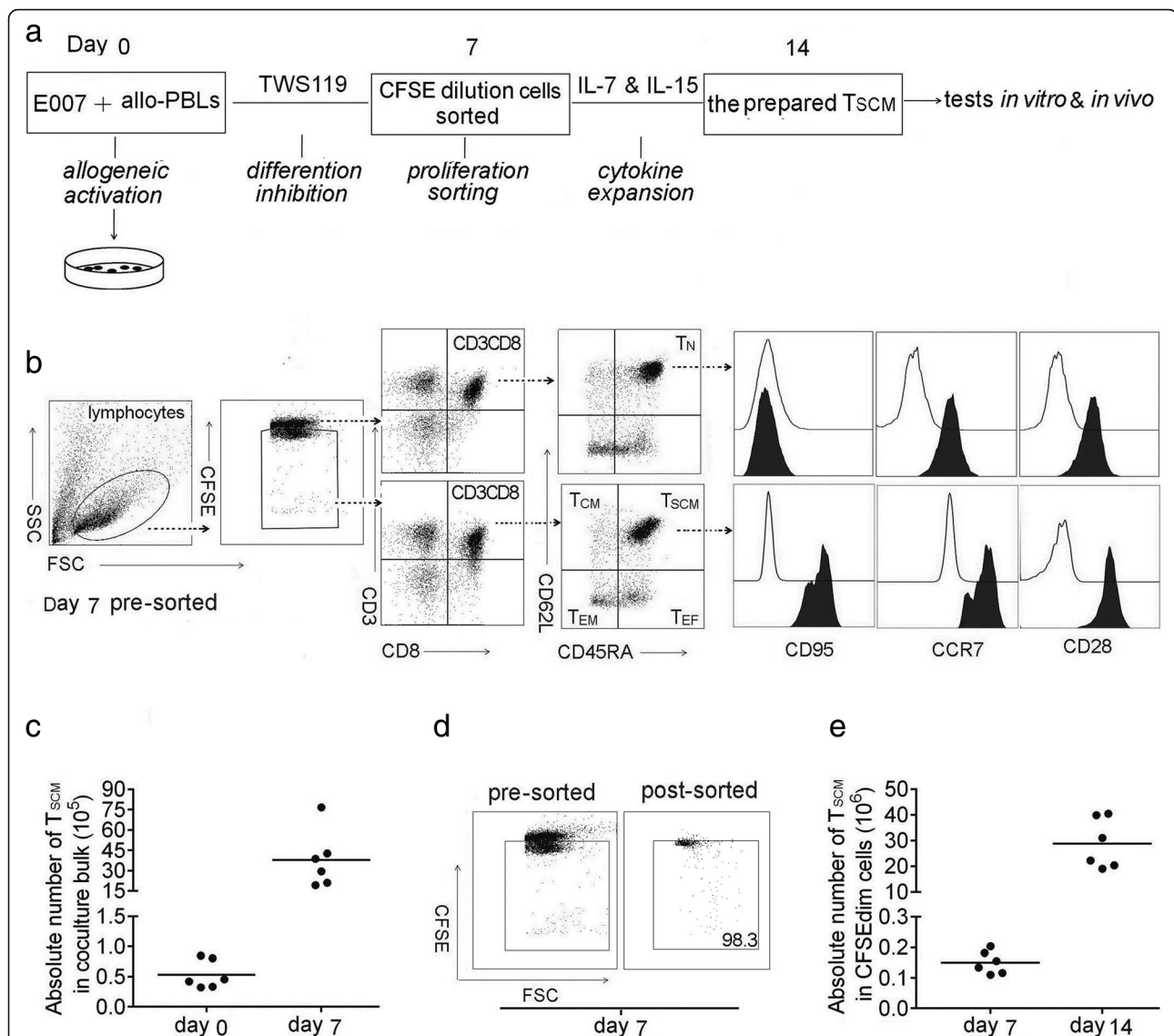


Fig. 1 Allo-specific T_{SCM} cells are effectively prepared *in vitro*. **a** Protocol for preparation of allo-specific T_{SCM}. The method began with setting up an allo-reactive co-culture by mixing of 2×10^6 E007 cells and 1×10^7 allogeneic PBLs (allo-PBLs) on day 0 (allogeneic activation), to generate allo-specific T cells. The presence of 5 μ M TWS119 enriched T_{SCM} in the co-culture (differentiation inhibition). The CFSE diluted cells in the co-culture bulks were sorted by FACS on day 7 (proliferation sorting). The sorted cells were cultured with IL-7 and IL-15 (25 ng/ml each) to expand the T_{SCM} cells for the next 7 days (cytokine expansion). Then, the cultural bulks were used as the prepared T_{SCM} cells for the tests *in vitro* and *in vivo* included in this study. **b** Gating strategy for identification of CD8+ T_{SCM} subset in the co-culture bulks. Lymphocytes were identified based on SSC versus FSC, and proliferating cells were on a reduced fluorescence intensity of CFSE (CFSEdim). In the gated CFSEdim population, cells expressing CD3 and CD8 were selected for further CD45RA and CD62L analysis. CD8+ T_{SCM} showed the phenotype of CFSEdim CD3+ CD8+ CD45RA+ CD62L+, T_{CM} of CD45RA- CD62L+, T_{EM} of CD45RA+ CD62L-, T_{EF} of CD45RA+ CD62L-, and CD95 was further detected to distinguish T_{SCM} from T_N, the latter showed the phenotype of CFSE high CD95- in the co-culture bulks, but T_{SCM} of CFSEdim CD95+. The illustration was a representative of co-culture bulks analyzed by FCM on day 7 before sorting, the white peaks in CD95, CCR7, and CD28 plots were isotype controls. The broken arrows indicated the sequential gating strategy. **c** T_{SCM} numbers increased by 100 folds in the co-culture bulks with TWS119 enrichment for 7 days. Data are represented as mean \pm SD of six individual experiments. **d** E007-specific T cells underwent proliferation in the co-culture, the CFSEdim cells were sorted by FACS with purity above 98%. **e** The sorted cells were further expanded by IL-7 and IL-15 for the next 7 days, the allo-specific T_{SCM} increased by another 150 folds. Data are represented as mean \pm SD of six individual experiments

sorting reached above 98% purity of the proliferative cells (Fig. 1d). It would be rational that the prepared T_{SCM} cells were E007 specific. After sorting, the cells were cultured in the presence of IL-7 and IL-15 (25 ng/

ml each) for the next 7 days. The allo-specific T_{SCM} increased by another 150 folds on day 14 (Fig. 1e). The lymphocyte distribution at this time not only showed the cultural bulks were mainly CD8+ T_{SCM} ($60.1 \pm 11.2\%$),

but also contained a few CD4⁺ T_{SCM} (10.4 ± 8.16%), CD3⁻ cells (6.15 ± 5.23%), CD8⁺ non-T_{SCM} (12.6 ± 3.48%), and CD4⁺ non-T_{SCM} (10.2 ± 8.66%) cells (Additional file 1: Figure S1D E F). By allogeneic activating, inhibiting differentiation with TWS119, sorting CFSEdim cells, and expansion with IL-7 and IL-15, our in vitro protocol was able to prepare about 2 × 10⁷ allo-specific CD8⁺ T_{SCM} cells from 1 × 10⁷ PBLs. The number of the T_{SCM} cells was sufficient to meet the needs of the following studies.

The T_{SCM} preparation strategy used in this study could be translated into a single epitope-specific T_{SCM} preparation. When the E007 co-cultured with allogeneic PBLs, the precursor frequencies were much higher than those of T cell responses to a single allogeneic epitope. To prepare a single epitope-specific T_{SCM} cells in a large number, a modification with a prolonged cytokine expansion was required (Additional file 2: Figure S2).

The prepared T_{SCM} cells show stem cell and memory T cell characteristics in vitro

To examine the self-renewal capacity, T_N, T_{SCM}, T_{CM}, and T_{EM} cells were sorted by their corresponding phenotypes from the co-culture on day 7 (Fig. 1b). The content of T cell receptor rearrangement excision circles (TRECs) in each subset was examined by real-time qPCR. As TRECs cannot be replicated while cell division, the content of TRECs reflects the frequency of T cell proliferation. Results showed that T_N had the highest content of TRECs. T_{SCM}, T_{CM}, and T_{EM} cells possessed less TRECs, confirming they were differentiated from T_N through T cell clonal proliferation (Fig. 2a). The content of TRECs in T_{SCM} cells was between those of T_N and T_{CM} cells, suggesting T_{SCM} was at the earliest stage after T_N activation.

The prepared T_{SCM} cells were relabeled with CFSE and exposed to IL-2 (200 U/ml) for 10 days. As IL-2 is a potential promoter for T cell proliferation and differentiation, seven generations of CFSE-diluted daughter cells developed. A portion of T_{SCM} cells (CD45RA⁺ CD62L⁺) were found in each generation, indicating that the prepared T_{SCM} cells were able to maintain a stem cell phenotype during proliferation and differentiation (Fig. 2b, c). Collectively, the prepared T_{SCM} cells showed characteristics of self-renewal.

To check memory T cell characteristics, the prepared T_{SCM} cells were incubated with the cognate stimulator E007, HLA class I mismatched E001, and α-CD3/CD28 beads. Phenotypic analysis revealed that the E007-restimulated T_{SCM} cells showed vigorous differentiation in 6 h, which became more intensive with prolonged stimulation time. A similar response was observed in the α-CD3/CD28-stimulated T_{SCM} cells. While the E001-stimulated T_{SCM} cells showed no

significant differentiation after 24 h, the response was similar to that of non-stimulated T_{SCM} cells (Fig. 3a, b). In addition, after 24 h of incubation with E007 and α-CD3/CD28, we found that T_{SCM} cells in the incubation showed no significant change in absolute number (Additional file 3: Figure S3E), suggesting that T_{SCM} cells were able to self-renew during proliferation and differentiation.

CD8⁺ T cell mediated immune effectors include production of cytokines and killing of target cells. To investigate production of cytokines, the T_{SCM} cells were stimulated with E007, E001, and α-CD3/CD28 beads, then intracellular IL-2, TNF-α, and IFN-γ production was measured. The E007-restimulated T_{SCM} cells showed more positive cells of these cytokines. Gating with CD62L, the incubated bulks were divided into CD62L⁺ (T_{SCM} and T_{CM}) and CD62L⁻ (T_{EM} and T_{EF}) cells, most of the cytokine positive cells were T_{EM} and T_{EF} cells (Fig. 3c). Meanwhile, gating with CD45RA, we found that both T_{EM} and T_{EF} cells showed similar frequency of the cytokine positive cells (Additional file 3: Figure S3A-D). Whereas, the T_{SCM} cells in the incubation produced almost background IL-2, and only a low level of TNF-α and IFN-γ. The T_{SCM} cells responded against α-CD3/CD28 in a similar profile of the cytokine production to that against E007. In contrast, the T_{SCM} cells stimulated with E001 showed frequency of cytokine-positive cells in the same way as that with non-stimulation (Fig. 3c-f). Next, we moved to cytotoxicity assay, the T_{SCM} co-cultured with E007 or E001 for 24 h at a ratio 5:1. Killing rate of the T_{SCM} and daughter cells against E007 was higher than that against E001 after 8 h (Additional file 3: Figure S3F, G). These results showed that the prepared T_{SCM} cells were E007-specific and able to differentiate rapidly into effector T cells after stimulated by the same antigen.

The prepared T_{SCM} cells are able to implant and effectively remove target cells in vivo

To evaluate capacity of persistence and rejecting target cells in vivo of the prepared T_{SCM} cells, human LCL cells E001 or E007 were inoculated intravenously into NOD/SCID mice to establish LCL-burden mouse model. After 3 days, the LCL-burden mice were treated with either E007-specific T_{SCM} cells or E007-specific T_{EM} and T_{EF} cells (Fig. 4a, b). On days 7, 14, 21, 28, and 35 after T cell infusion, caudal vein peripheral blood samples were taken to detect the frequency and phenotype of human T cells. We found human CD3⁺ CD8⁺ T cells in the peripheral blood of the T_{SCM}-treated mice at all sampling times. However, in the T_{EM}+EF-treated mice (E007-T_{EM}+EF), human T cells were detected in peripheral blood on day 7, but not on day 14 or later (Fig. 4c). In the T_{SCM}-treated mice, the frequency of T_{SCM} cells in

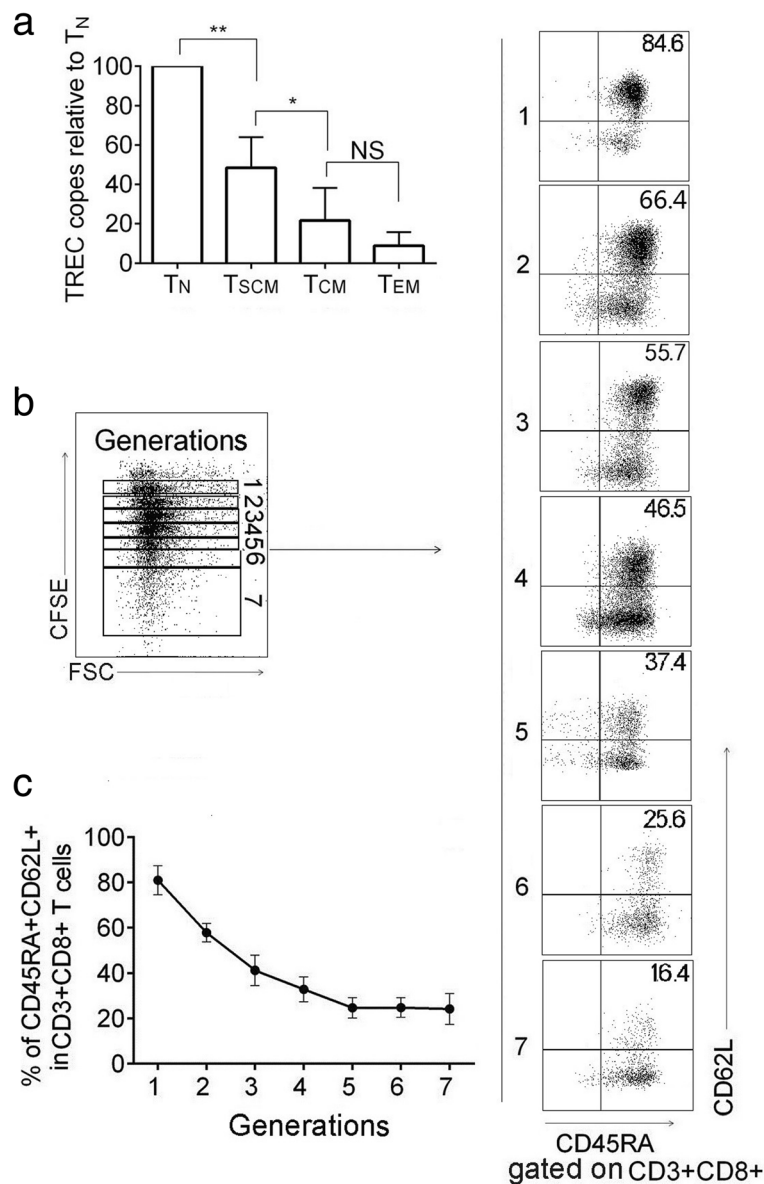
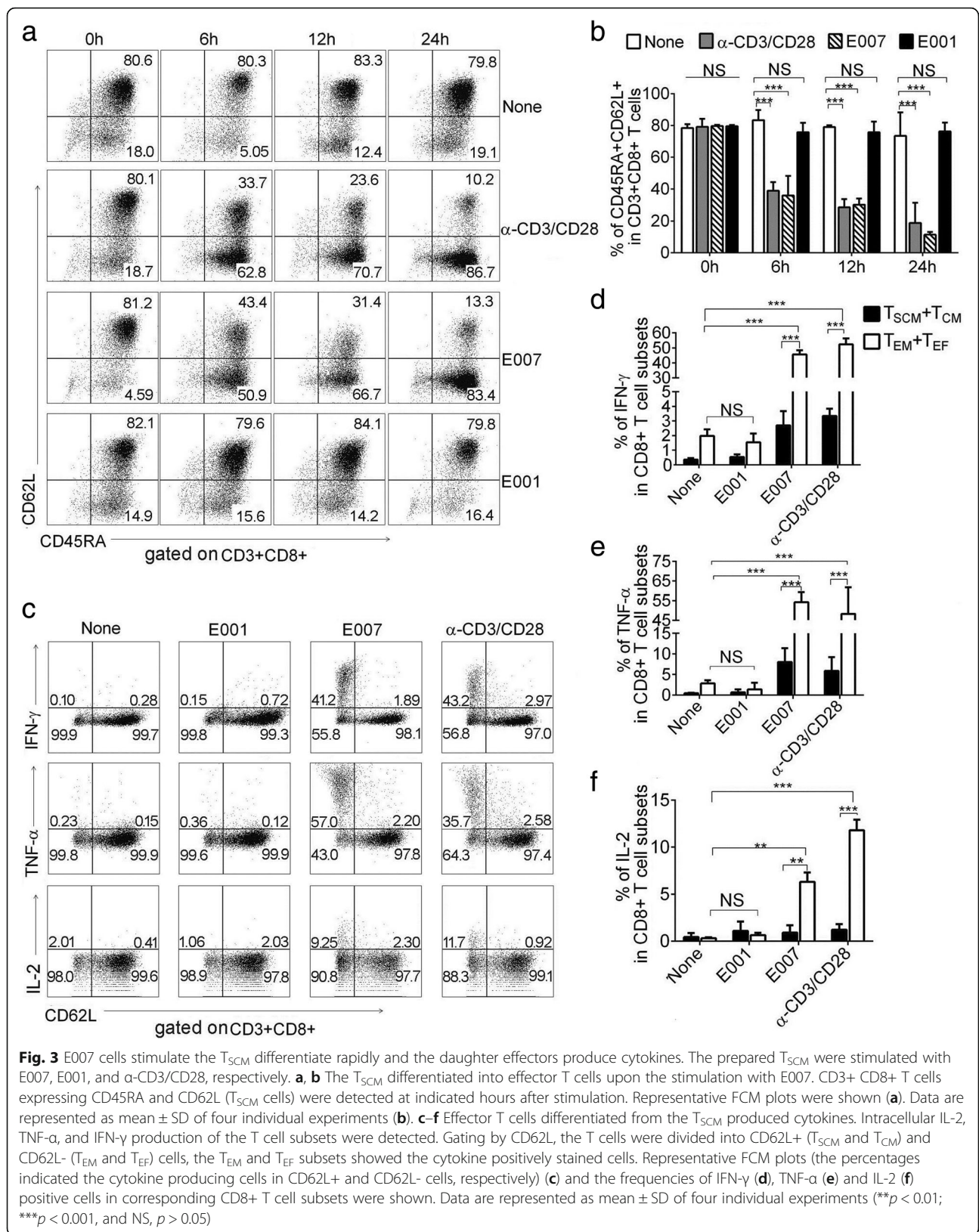


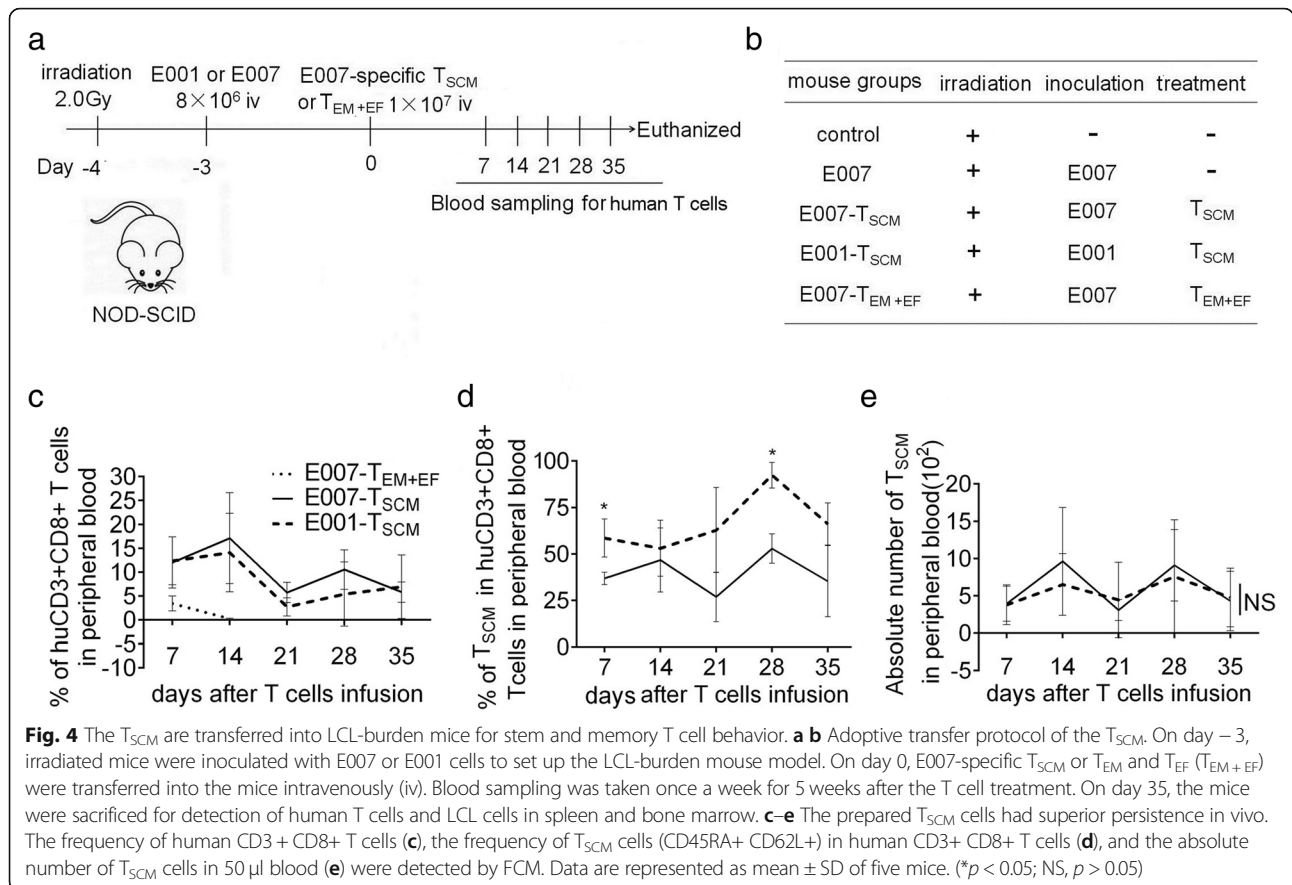
Fig. 2 The prepared T_{SCM} cells bear the ability of self-renewing. **a** T_N , T_{SCM} , T_{CM} , and T_{EM} cell subsets in CD8+ T cells were sorted by their corresponding phenotypes from the co-culture on day 7. TREC copy number in the sorted subsets relative to T_N cells was shown. Data are represented as mean \pm SD of four individual experiments. **b** The prepared T_{SCM} cells were relabeled with CFSE and exposed to 200 U/ml of IL-2 for 10 days, the proliferating daughter cells divided into 7 generations (left panel). In each generation, there were a portion of CD8+ T cells expressing CD62L and CD45RA (right panel). Representative FCM plots were shown. **c** The frequency of T_{SCM} (CD45RA+ CD62L+) cells in CD8+ T cells was analyzed at each CFSE dilution peak. Data are represented as mean \pm SD of four individual experiments. (* $p < 0.05$; ** $p < 0.01$, and NS, $p > 0.05$)

the E007-burden mice (E007- T_{SCM}) were lower than that in the E001-burden mice (E001- T_{SCM}), but no difference was found in the absolute number of T_{SCM} cells between E007-burden and E001-burden mice (Fig. 4d, e). On day 35, more frequent human T cells were found in the spleen and bone marrow of the T_{SCM} -treated mice revealed by flow cytometry (Fig. 5a), and the immunofluorescent staining of the spleen sections showed the similar results (Fig. 6d, e). These findings indicated that

the T_{SCM} cells were able to survive long through self-renewal in the mice, and differentiate into other T cell subsets as stimulated with the specific antigens of E007.

To investigate the immune reconstitution capacity of the T_{SCM} cells, peripheral blood, spleen, and bone marrow of T_{SCM} -treated and $T_{EM} + EF$ -treated mice were tested for human T cells on day 35. The human T cells in the above specimens consisted of T_{SCM} (CD45RA +





CD62L+), T_{CM} (CD45RA-CD62L+), T_{EM} (CD45RA-CD62L-), and T_{EF} (CD45RA+ CD62L-). The distribution of T cell subsets revealed more T_{EM} and T_{EF} cells in the E007-burden mice, whereas more T_{SCM} and T_{CM} cells in the E001-burden mice (Fig. 5b). As T_{EM} and T_{EF} cells are at terminal differentiation stage while T_{SCM} and T_{CM} cells at early differentiation stage, our findings suggested the E007-specific T_{SCM} cells differentiated into the other T cell subsets when they came cross the same antigen in the E007 burden mice.

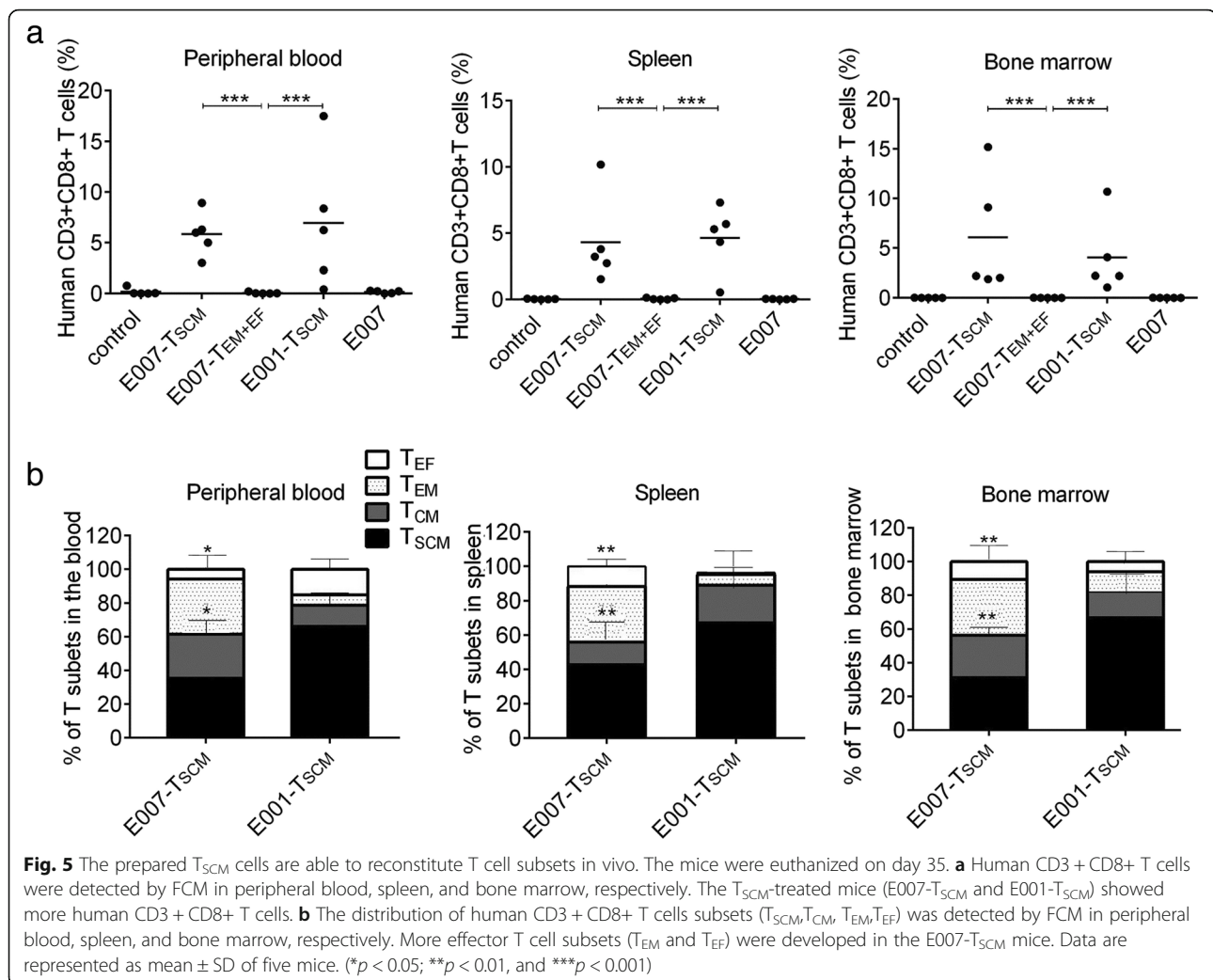
To analyze killing efficacy of the T_{SCM} cells in vivo, on day 35 of the T cell treatment, the residual LCL cells in mice were detected by intracellular LMP1 staining. The LCL cells were mainly found in the spleen and bone marrow of the LCL-burden mice. In the E007-burden mice, the residual LCL cells in spleen and bone marrow of the T_{SCM} -treated mice were similar to that of the control mice without LCL infusion (control). Nevertheless, the T_{EM+EF} -treated mice carried a significant amount of the residual LCL cells compared to the control mice, but the amount was lower than the E007-burden mice without T cell treatment (E007) (Fig. 6a-c). More residual LCL cells were also found in the immunofluorescent spleen sections of the T_{EM+EF} -treated mice than those of the T_{SCM} -treated mice (Fig. 6d, e) These data demonstrated the T_{SCM} cells

equipped with superior capacity of killing targets of specific antigens in vivo compared to the T_{EM} and T_{EF} cells.

In contrast to the E007-burden mice, the E001-burden mice bore not only less T_{EM} and T_{EF} cells after the T_{SCM} treatment, but also the residual LCL cells as high as that of the E007-burden mice without T cell treatment (Fig. 6a-c). Results of immunofluorescence staining of spleen sections for human T cells and LCLs showed residual LCL cells in E007-burden mice treated with T_{SCM} were similar to those of the control mice. Although the spleen of the E001-burden mice showed a significant number of human T cells, the residual LCL cells were similar to that of the E007-burden mice without T cell treatment (Fig. 6d, e). By the way, the human T cell-treated mice showed no sign of xenogeneic graft-versus-host disease (xeno-GVHD), such as loss of body weight, back arched, and shed. Collectively, these results indicated that the prepared T_{SCM} cells were able to survive over a long-term in vivo and differentiate into effector T cells to eradicate the target bearing the cognate antigens.

Discussion

Mature T cells are comprised of cells that are at various stages of differentiation, which are discernible by the expression of surface molecules. T_N cells are



conventionally defined by the co-expression of the CD45RA, CCR7, and the lymph node homing molecules L-selectin (CD62L) [3]. Similar to T_N cells, CD62L and CCR7 are maintained on T_{CM} cells, whereas these molecules are lost on more differentiated T_{EM} cells [3, 35]. T_{SCM} cells, the least differentiated of all distinct memory populations, are identifiable through the expression of markers, including CD62L, CCR7, CD95, and the chemokine (C-X-C motif) receptor 3 [5, 10, 36]. Naïve T cells downregulate CD62L expression after stimulation, as clonal expansion goes on, both CD62L^{LOW} and CD62L^{HIGH} T cell subsets are developed. With real-time tracking of CD8 T-cell divisions in vitro, Kinjyo and co-workers define memory T cells among CD62L^{HIGH} cells. The memory T cells stay CD62L^{HIGH} and proliferate in vitro driven by IL-2, although the division duration is slow, the phenotype and cell cycle duration are inherited by the progeny of the T cells [37]. We identified CD8+ T_{SCM} subset in the co-culture bulks by the phenotypes CD3+ CD8+ CD45RA+ CD62L+ CCR7+

CD95+ CD28+, as our results showed the cells with the phenotypes in the co-culture bulks were of stem cell and memory T cell properties.

A frequently used method to enrich T_{SCM} cells is differentiation inhibition. With antigen priming, antigen-specific CD8+ T_N cells proliferate and develop into T_{SCM} cells first and then the other subsets. It is reported that T_{SCM} differentiation can be inhibited by TWS119, which inhibits the GSK-3 β and activate Wnt/ β -catenin. The inhibition improves the maintenance of 'stemness' in mature memory CD8+ T cells which mediated a better anti-tumor response after transferred into mice [15, 38]. Rapamycin is also reported to help the formation of T cell subset at an early stage of differentiation by modulation of mTOR signaling [39–41]. However, rapamycin tended to promote the generation of T_{CM} cells in our study (Additional file 1: Figure S1C), in line with other report [41]. In our co-culture of E007 with allogeneic PBLs, the E007-specific T_{SCM} cells were generated from T_N and enriched in the presence of

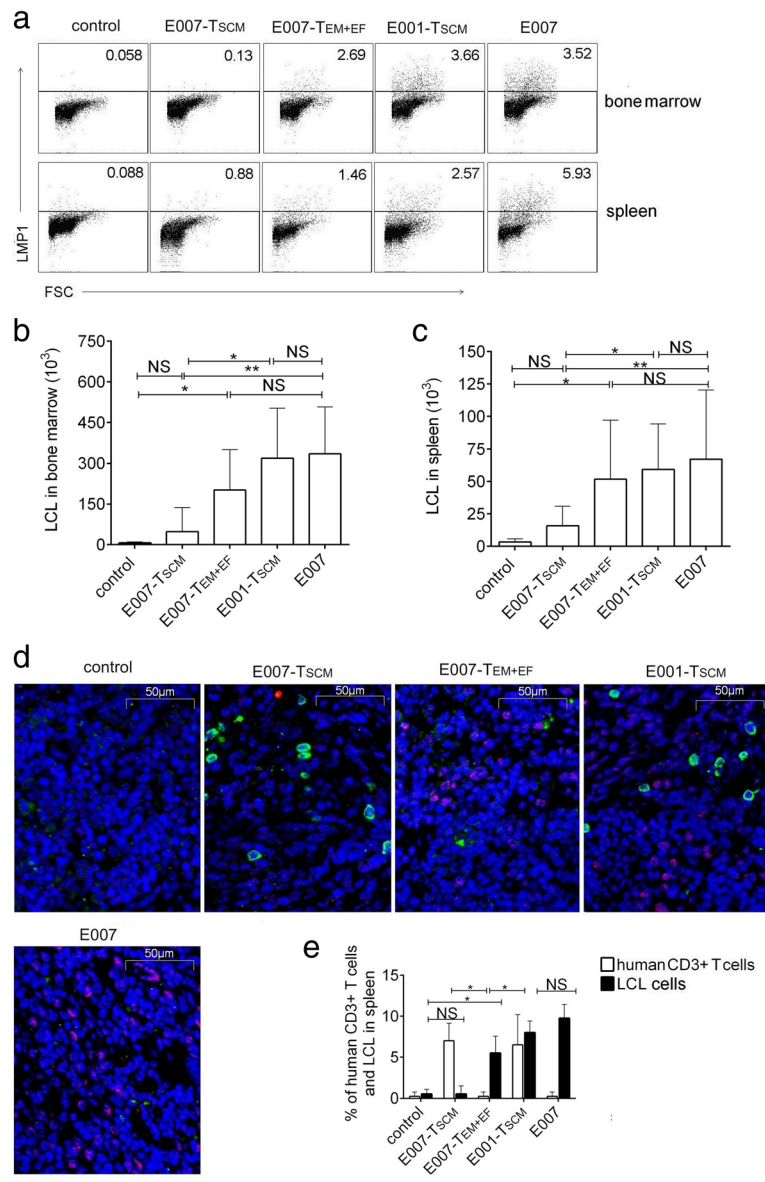


Fig. 6 The T_{SCM} cells are able to eradicate allo-antigen-specific targets in vivo. The spleen and bone marrow were detected for the residual LCL cells by intracellular LMP1 staining using FCM. **a–c** Representative FCM plots (**a**) and the absolute number of LCLs in bone marrow of two femurs (**b**) and in 100 mg spleen tissue (**c**) were analyzed. **d** Spleen sections were observed for LCLs and human T cells by immunofluorescence. Representative illustrations of immunofluorescent labeling of human CD3 (green), LMP1 (red), and nucleus staining by DAPI (blue) for spleen sections were shown (×400). **e** Data showed the mean frequencies of human CD3 and LMP-1-positive cells in 4 fields chosen randomly (100 cells each) for each mouse spleen section. The residual LCLs in the E007-T_{SCM} mice were similar to that in the control mice without LCL inoculation, the rest mouse groups showed relatively more LCL cells. Data are represented as mean ± SD of five mice (**p* < 0.05; ***p* < 0.01; ****p* < 0.001, and NS, *p* > 0.05)

TWS119 which inhibited the T_{SCM} further differentiation. As inhibition by TWS119 results in a T_{SCM} phenotype in human CD8+ T cells [5, 15, 16], our protocol preferred expansion of CD8+ T_{SCM} to that of CD4+ T_{SCM}.

The administration of cytokines is another method to expand T_{SCM}. Cytokines have important functions related to T cell expansion, differentiation, survival, and

homeostasis. Common γ-chain (γc) family cytokines are commonly used in clinical trials, including IL-2, IL-7, IL-15, and IL-21. IL-7 is instrumental for the generation of T_{SCM} cells by binding to IL-7 receptor expressing naive and memory T lymphocytes [42, 43]. Expansion of T_{SCM} required either IL-15 or IL-2; IL-15 proves superior to IL-2 in supporting expansion coupled to preservation of the T_{SCM} phenotype [18, 19]. Activated naive T

cells show a higher expression of IL-21 receptor, and IL-21 has been reported to be able to enrich less differentiated T_{SCM} within the total T_{SCM} subset [20, 22, 23, 44]. However, IL-21 exerted few effects on the T_{SCM} expansion in our study. Therefore, IL-7 and IL-15 were used to expand the T_{SCM} cells after the proliferation sorting, resulting in a large number of the T_{SCM} sufficient for the experiments in vitro and in vivo included in our study.

An important feature of stem cells is the self-renewal capacity. Although self-renewal abilities of the prepared T_{SCM} cells were revealed by TREC copy numbers and proliferative history driven by IL-2 in vitro, long-term in vivo implantation is the gold standard for identification of stem cells. Long-lasting survival of human T_{SCM} cells in mice is reported. In serial transplantations model, T_{SCM} cells prove able to persist in secondary recipients suggesting self-renewal abilities [5, 6, 19, 45]. Here, the prepared T_{SCM} migrated to secondary lymphoid organs, such as bone marrow and spleen, showed long-lasting survival potential at least for 35 days after transferred in our model. Importantly, the number of T_{SCM} cells in the blood samples was observed consistent during this study, suggesting the self-renewal capacity of the transferred T_{SCM} cells in vivo. The implantation and long-term existence of the T_{SCM} cells in the host would be expected to mount durable T cell responses after transferring.

Governing antigen specificity is important for T_{SCM} preparation; various strategies are used for this purpose. For example, HCMV-specific T_{SCM} cells are isolated from peripheral blood T_{SCM} cells of HCMV seropositive donors and enriched in vitro by incubation with HCMV antigen [26]. CD19-specific T_{SCM} cells are prepared from peripheral blood T_N and T_{SCM} by transduction with a γ -retroviral vector encoding the CD19-chimeric antigen receptor (CAR) [24]. TCR-transgenic mice are also a source of antigen-specific T_{SCM} cells [46]. In our approach, the E007-specific T cells underwent proliferation in the co-culture bulks, making it feasible to be sorted by CFSE dilution. In our pre-experiment, IL-7 or IL-15 was able to make PBLs proliferation without the E007 stimulation. Hence, no cytokine was added to the co-culture before the sorting, to ensure the E007-specificity of the prepared T_{SCM} cells.

Two HLA class I mismatched LCLs, E007 and E001, were used to examine the antigen specificity of the prepared CD8+ T_{SCM} cells. Rapid differentiation into effector T cells and robust effector functions were observed when the prepared T_{SCM} cells were stimulated with the E007, but not with the E001. Although the E007-specificity was suggested by these in vitro findings, it would be more important to observe the behavior of the prepared T_{SCM} cells in vivo. In our LCL burden

mouse model, LCL cells could be found in spleen and bone marrow up to 45 days after transfer into the immunodeficiency mice in our preliminary test. The LCL burden mice showed no measurable side effect during our study, such as loss of body weight, back arched, and shed. Interestingly, the prepared T_{SCM} cells exhibited intensive differentiation into other T cell subsets and effective eradication of LCL targets in the E007-burden mice instead of the E001-burden mice. These observations reflected the prepared T_{SCM} cells were E007-specific.

Conclusions

Functional allo-specific CD8+ T_{SCM} cells were prepared from human PBLs in a procedure of allogeneic co-culture, differentiation inhibition, proliferation sorting, and cytokine expansion. Although our study provided a practical protocol for allo-specific T_{SCM} cell preparation, this method would be adapted to prepare T_{SCM} cells specific for antigen of interesting. As T_{SCM} cells show the implantation and long-term existence in the host after transferring, the preparation of antigen-specific T_{SCM} cells is crucial for T cell adoptive immunotherapy.

Additional files

Additional file 1: Figure S1. Enrichment of T_{SCM} cells by differentiation inhibitors and the lymphocyte distribution changes in the cultural bulks over the T_{SCM} preparation course. **A, B** TWS119 exhibited concentration-dependent enrichment of T_{SCM} in the alloreactive co-culture, 5 μ M of TWS119 was the best concentration. T_{SCM} cells in frequency (**A**) and in absolute number (**B**) were shown. **C** Differentiation inhibition by rapamycin was more likely to enrich T_{SCM} instead of T_{SCM} . **D-F** After allogeneic activation, differentiation inhibition, proliferation sorting, and cytokine expansion, lymphocyte distribution in the cultural bulks was revealed by FCM over the T_{SCM} preparation course. CD3+CD4+ T cells, CD3+CD8+ T cells and CD3- cells in the cultural bulks were detected, and the proportion of CD3+CD8+ T cells was increased over time (**D**). The majority of cultural bulks were CD3+CD8+ T_{SCM} (60.1 \pm 11.2%) on day 14 (**E**). The proportion of CD3+CD8+ T_{SCM} in CD8+ T cells was increased over time, about 80% of CD8+ T cells were T_{SCM} on day 14 (**F**). Data are represented as mean \pm SD of four individual experiments. (JPG 170 kb)

Additional file 2: Figure S2. Our T_{SCM} preparation strategy can be used to generate a single epitope-specific T_{SCM} cells. **A** Procedure for preparation of AFP-specific, allogeneic T_{SCM} . T2 cells express only empty HLA-A2 allele and no other HLA allele. Alpha fetoprotein (AFP) is a tumor associated antigen of hepatocarcinoma, the hAFP₁₅₈₋₁₆₆ (FMNKFYIEI) is an HLA-A2 restricted peptide. When pulsed with the AFP peptide, T2 cells were able to present the AFP/HLA-A2 complex. The AFP-specific T_{SCM} were raised by co-culturing HLA-A2 negative (HLA-A2-ve) PBLs and the T2 cells pulsed with the AFP peptide (T2/AFP). In a procedure of an allogeneic co-culture, differentiation inhibition, proliferation sorting and cytokine expansion, the AFP/HLA-A2 complex-specific T_{SCM} cells were produced. **B** Co-culture by mixing of 1×10^7 PBLs and 2×10^6 T2/AFP on day 0, a prolonged cytokine expansion was required to generate 1×10^6 AFP-specific T cells. Data are represented as mean \pm SD of four individual experiments. **C-E** The prepared T_{SCM} cells were AFP-specific. The prepared T_{SCM} cells were incubated with the T2/AFP and T2 cells pulsed with an irrelevant peptide HBCAg₁₈₋₂₇ (T2/HBC), respectively. After 4 h incubation, the T cell subsets and their intracellular IFN- γ production were detected.

Representative FCM plots (C). T_{SCM} cells differentiated more when incubated with the T2/AFP (D). The daughter cells showed more frequent IFN- γ positive cells when incubated with the T2/AFP (E). Data are represented as mean \pm SD of four individual experiments (** $p < 0.01$). (JPG 404 kb)

Additional file 3: Figure S3. The prepared T_{SCM} differentiated into effector T cells stimulated by E007. The prepared T_{SCM} were stimulated with E007, E001 and α -CD3/CD28, respectively. The T cell subsets and their intracellular IL-2, TNF- α and IFN- γ production were detected. The T_{SCM} differentiated into effector T cells upon the stimulation with E007 and α -CD3/CD28. **A–D** After 4-h stimulation, both T_{EM} and T_{EF} cells exhibited the similar frequency of the cytokine positive cells. Representative FCM plots (A). Gating by CD3+ CD8+ CD62L-, the T cells were divided into CD45RA- (T_{EM}) and CD45RA+ (T_{EF}) cells. The T_{EM} and T_{EF} subsets showed the cytokine positively stained cells of IFN- γ (B), TNF- α (C) and IL-2 (D). **E** After 24-h stimulation, the absolute number of T_{SCM} remained stable during differentiation. **F, G** The cytotoxicity of the T_{SCM} and the daughter cells were E007-specific. E007 and E001 labeled with celltrace, co-cultured with the T_{SCM} at ratio 1:5. Dead cells stained by PI dye were detected by FCM. Representative FCM plots (F) and the frequencies of dead cells (G) were shown. Data are represented as mean \pm SD of four individual experiments (NS, $p > 0.05$; ** $p < 0.01$, and *** $p < 0.001$). (JPG 722 kb)

Abbreviations

ACT: Adoptive cell therapy; GVHD: Graft versus host disease; HLA: Human leukocyte antigen; LCL: B lymphoblastoid cell lines; LMP1: Latent membrane protein 1; PBL: Peripheral blood lymphocytes; PBMC: Peripheral blood mononuclear cells; pMHC: Peptide major histocompatibility complex; TCR: T cell receptor; TREC: T cell receptor rearrangement excision circle

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

XWW designed the study, analyzed the data, and wrote the manuscript. LG was a major contributor in the experimental performance, analyzed the data, and wrote the manuscript. XL performed and analyzed the cell culture experiments. JW contributed to the animal experimental performance. XFW contributed to the design of study. ZL contributed to the data analysis. JY contributed to the manuscript preparation. All authors read and approved the final manuscript.

Ethics approval

All donors' samples were obtained after informed consent according to a protocol approved by the Ethics Committee of Tongji Medical College, Wuhan, China. Animal experiments were approved by the Ethics Committee of Tongji Medical College. All animal procedures were performed in strict accordance with the guidelines of the Chinese Council on Animal Care.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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