

ORIGINAL RESEARCH

Comparison of naïve and central memory derived CD8⁺ effector cell engraftment fitness and function following adoptive transfer

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ABSTRACT

Human CD8⁺ effector T cells derived from CD45RO⁺CD62L⁺ precursors enriched for central memory (T_{CM}) precursors retain the capacity to engraft and reconstitute functional memory upon adoptive transfer, whereas effectors derived from CD45RO⁺CD62L⁻ precursors enriched for effector memory precursors do not. Here we sought to compare the engraftment fitness and function of CD8⁺ effector T cells derived from CD45RA⁺CD62L⁺ precursors enriched for naïve and stem cell memory precursors (T_{N/SCM}) with that of T_{CM}. We found that cytotoxic T cells (CTLs) derived from T_{CM} transcribed higher levels of CD28, FOS, INF γ , Eomesodermin (Eomes), and lower levels of BCL2L11, maintained higher levels of phosphorylated AKT, and displayed enhanced sensitivity to the proliferative and anti-apoptotic effects of γ -chain cytokines compared to CTLs derived from T_{N/SCM}. Higher frequencies of CTLs derived from T_{CM} retained CD28 expression and upon activation secreted higher levels of IL-2. In NOD/Scid IL-2R γ C^{null} mice, CD8⁺ T_{CM} derived CTLs engrafted to higher frequencies in response to human IL-15 and mounted robust proliferative responses to an immunostimulatory vaccine. Similarly, CD8⁺ T_{CM} derived CD19CAR⁺ CTLs exhibited superior antitumor potency following adoptive transfer compared to their CD8⁺ T_{N/SCM} derived counterparts. These studies support the use of T_{CM} enriched cell products for adoptive therapy of cancer.

Abbreviations: BM, bone marrow; CD19CAR, CD19-specific chimeric antigen receptor; CTLs, cytotoxic T cells; EGFRt, truncated human EGFR; fLuc⁺, firefly luciferase; IL-15R, IL-15 Receptor; LCL, lymphoblastoid cell lines; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; REM, rapid expansion method; T_{CM}, central memory T cells; T_{N/SCM}, naïve and stem cell memory T cells

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Introduction

Adoptive transfer of *in vitro* expanded T cells is a therapeutic approach, that when coupled to genetic modification to express tumor targeting antigen receptors, can result in dramatic regressions of leukemia and lymphoma.¹⁻⁴ While early data in the CD19-specific chimeric antigen receptor (CD19CAR) field is demonstrative of the efficacy of this approach in selected patients, the full potential of this emerging modality is hampered by the therapeutic failures arising from attenuated engraftment of CAR redirected T cells. Most active trials use patient derived peripheral blood mononuclear cells (PBMC) as a source of T cells for product manufacturing. Consequently, each product is composed of a heterogeneous population of T cells that is unique to the repertoire of the patient at the time of peripheral blood acquisition. It is reasonable to expect that the patient's immune status based on underlying tumor type and tumor burden, prior cytotoxic therapies, and patient age will significantly affect the composition of T cells from which products are generated. Insufficient number of CAR redirected T cells capable of engrafting, amplifying, and persisting in the cell products is therefore a significant impediment to achieving reproducible and uniform therapeutic potency. We hypothesize

that this untoward variable might be ameliorated by manufacturing T cell products of defined composition and specifically enriched for T cell subsets that harbor intrinsic capacity for sustained engraftment and antitumor functional outputs.

The attributes of T cells that confer engraftment fitness as manifested by the capacity to sustain a functional immune response following adoptive transfer of *in vitro* propagated effector T cells has been the subject of intensive investigation. We have demonstrated in a non-human primate model and human T cell NOD/Scid IL-2R γ C^{null} (NSG) mouse model that CD8⁺ effector T cells derived from macaque CD62L⁺CD95⁺ or CD62L⁺CD45RO⁺ central memory T cells (T_{CM}), respectively, have the capacity to persist following adoptive transfer and re-populate functional memory niches.^{5,6} Consistently, Busch et al. demonstrated the self-renewal capacity and multipotency of single T_{CM} in serial transfer design, indicating the stemness of T_{CM}.^{7,8}

Here, we compared the relative engraftment performance of human CD8⁺ effector cells derived from CD45RA⁺CD62L⁺ naïve/T_{SCM} enriched precursors (T_{N/SCM}) and CD45RO⁺CD62L⁺ T_{CM} enriched precursors *in vitro* and *in vivo* using a NSG mouse engraftment model. Our data using a clinical applicable IL-2 based

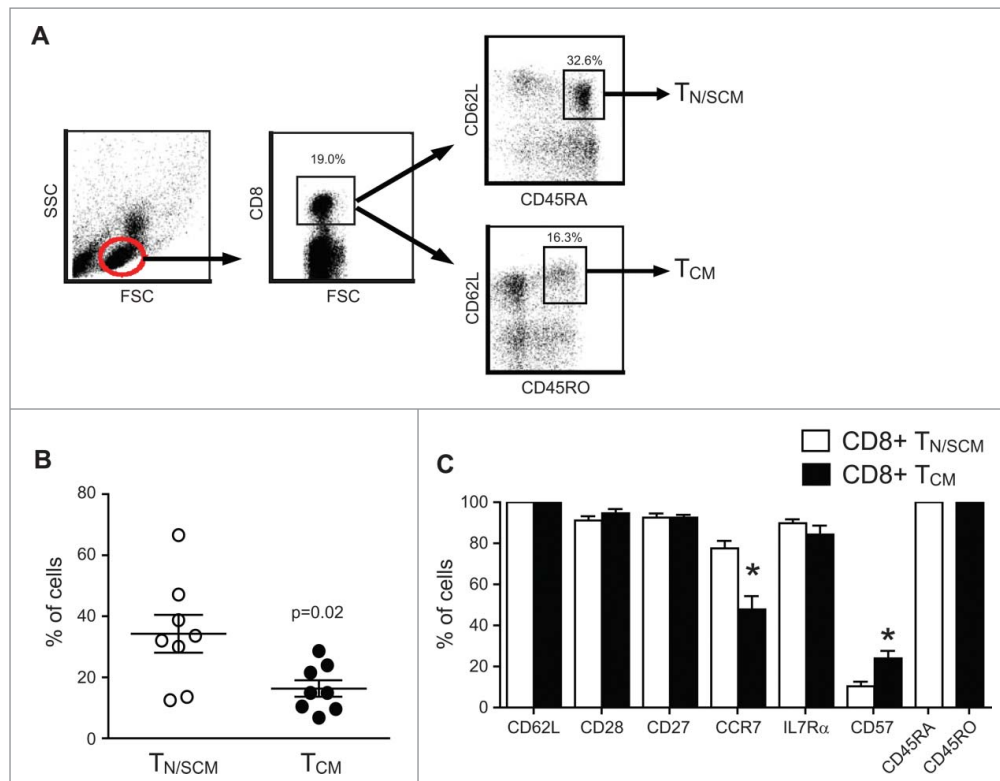


Figure 1. Frequency and phenotypic attributes of CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} from healthy donor peripheral blood. (A) Representative gating/sorting strategy for CD8⁺CD45RA⁺CD62L⁺ T_{N/SCM} vs. CD8⁺CD45RO⁺CD62L⁺ T_{CM}. (B) Analysis of PBMC from eight different healthy donors gated on the CD8⁺ population and analyzed for CD45RA⁺CD62L⁺ T_{N/SCM} and CD45RO⁺CD62L⁺ T_{CM} by multicolor flow cytometry. (C) Percentages of immunoreactive cells in each population are indicated (mean + SEM, n = 8). * p < 0.05 when comparing T_{N/SCM} and T_{CM} using a paired Student's t-test.

regimen demonstrate that CD8⁺ effector cells arising from polyclonal preparations of CD45RO⁺CD62L⁺ T_{CM} enriched precursors exhibit superior performance in homeostatic cytokine driven engraftment, vaccine driven proliferation, and CD19CAR redirected antitumor potency in the NSG mouse model system, as compared to their CD45RA⁺CD62L⁺ T_{N/SCM} enriched counterparts. Superior engraftment performance of CD45RO⁺CD62L⁺ T_{CM} derived CD8⁺ effector cells in response to IL-15 *in vivo* was correlated with higher levels of IL-15 Receptor (IL-15R) expression and responsiveness, while augmented proliferation *in vivo* in response to vaccine challenge correlated with sustained CD28 expression on activated effector cells and enhanced autocrine IL-2 secretion. Lastly, T_{CM} derived CD8⁺ effector cells lentivirally transduced to express a second generation CD19CAR exhibited enhanced antitumor efficacy as compared to their T_{N/SCM} derived counterparts in a xenogeneic model of human lymphoma and leukemia. These data provide the rationale for embarking on clinical trials of CD19CAR T cell adoptive therapy using cell products derived from CD45RO⁺CD62L⁺ T_{CM} enriched PBMC precursors.

Results

Phenotypic attributes and purification of CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} from healthy donor peripheral blood

Human T cells can be segregated into T_{N/SCM} and T_{CM} based on differential expression of CD45 isoforms CD45RA and CD45RO.^{9,10} Using multiparameter flow cytometry, we analyzed peripheral blood samples from 8 healthy donors to determine the

frequencies of CD8⁺ T cells expressing CD45RA⁺CD62L⁺ versus CD45RO⁺CD62L⁺. CD45RA and CD45RO double positive cells were excluded in these two populations. Based on forward and side scatter profiles to gate on CD8⁺ lymphocytes (Fig. 1A), we found that CD45RA⁺CD62L⁺ cells in the blood are more frequent (34.3±6.2%) than CD45RO⁺CD62L⁺ cells (16.3±2.7%) (p = 0.02) (Fig. 1B), which is consistent with other reports.¹¹⁻¹³ Despite the differential expression of CD45RA and CD45RO, these resting/unstimulated CD62L⁺CD8⁺ T cells exhibited comparable frequencies of cells expressing CD28, CD27, and IL7R α (Fig. 1C). As expected, significantly higher frequencies of CCR7⁺ cells were observed within the CD45RA⁺CD62L⁺ enriched T_{N/SCM} cells (77.5±3.7% for T_{N/SCM} and 47.8±6.5% for T_{CM}, p < 0.01).¹⁴ Likewise, CD45RO⁺CD62L⁺ CD8⁺ T cells have a significantly higher frequency of CD57 expression, a molecule associated with memory T cells with high cytolytic potential,¹⁵ and the expression of which is up-regulated in cells with a replicative history (10.4±2.2% for T_{N/SCM} and 23.9±6.5% for T_{CM}; Fig. 1C).¹⁶ We next sort-purified CD8⁺ cells (> 97% purity) from leukapheresis products obtained from the cohort of 4 healthy donors into CD45RA⁺CD45RO⁻CD62L⁺ and CD45RA⁻CD45RO⁺CD62L⁺ subsets for subsequent testing (Table 1).

In vitro differentiation of T_{N/SCM} and T_{CM} precursors generates effector cytotoxic T cells (CTLs) with divergent gene expression profiles, and phenotypic/functional attributes

Purified CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} were subject to a 14 day *in vitro* rapid expansion method (REM) stimulation¹⁷ including

Table 1. Purity of isolated CD8⁺CD45RA⁺CD62L⁺ CD8⁺ T_{N/SCM} and CD8⁺CD45RO⁺CD62L⁺ CD8⁺T_{CM} cells from healthy donors (HD).

| HD | %CD8 ⁺ T _{N/SCM} | %CD8 ⁺ T _{CM} |
|----|--------------------------------------|-----------------------------------|
| 1 | 98 | 99 |
| 2 | 98 | 99 |
| 3 | 97 | 100 |
| 4 | 100 | 100 |

anti-CD3 ϵ (OKT3), irradiated PBMC, and lymphoblastoid cell lines (LCLs) as feeders in the presence of recombinant human IL-2 (rhIL-2) 50 U/mL that has been successfully used in many applications^{18,19} (Fig. S1). We observed equivalent proliferative responses and cytolytic activity of effector CTLs derived from CD8⁺ T_{N/SCM} and T_{CM} precursors. These findings were reproduced even after additional REM stimulation cycles (Fig. S2A, B). In addition, each subset maintained equal telomere length through repetitive stimulations (Fig. S2C) and therefore subsequent differences in replicative potential, persistence and functional outputs in the *in vivo* NSG model could not be attributed to differences in the proclivity of either subset to enter replicative senescence.

To investigate the impact of different stimuli on the proliferative capacity of the T cell subsets, we subjected purified CD8⁺ T_{N/SCM}, CD8⁺ T_{CM}, and CD8⁺ memory stem cells (T_{SCM}) characterized as CD45RA⁺CD62L⁺CD95⁺^{12,20} (Fig. S3A) to either REM stimulation (Fig. S3B, C) or OKT3 stimulation in the presence of irradiated allogeneic PBMC and rhIL-2 300U/mL (Fig. S3D, E) as described by Hinrichs et al.¹¹ We observed comparable levels of cell growth of all three subsets and CD28 expression was better retained in CD8⁺ T_{CM} subset independent of the culture conditions.

Consistent with effector cell differentiation during the first 14-day REM stimulation, we observed down regulation of CD45RA expression on T_{N/SCM} derived CTL and incremental decreases in frequencies of T cells from both T_{N/SCM} and T_{CM} subsets of CD62L, CCR7 and IL-7R α expression (Fig. 2A). In contrast, despite the initial comparable frequencies of CD28 expression on unstimulated CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} precursors (91 \pm 2% and 94 \pm 2%, respectively), following REM stimulation, CD28 expression was retained preferentially on cultured cells derived from T_{CM} precursors (78.3 \pm 4.9%) than that of T_{N/SCM}, (40 \pm 2.8%) ($P < 0.01$) (Fig. 2A, B and Table 2). While both precursor subsets expressed CD28 and have equal

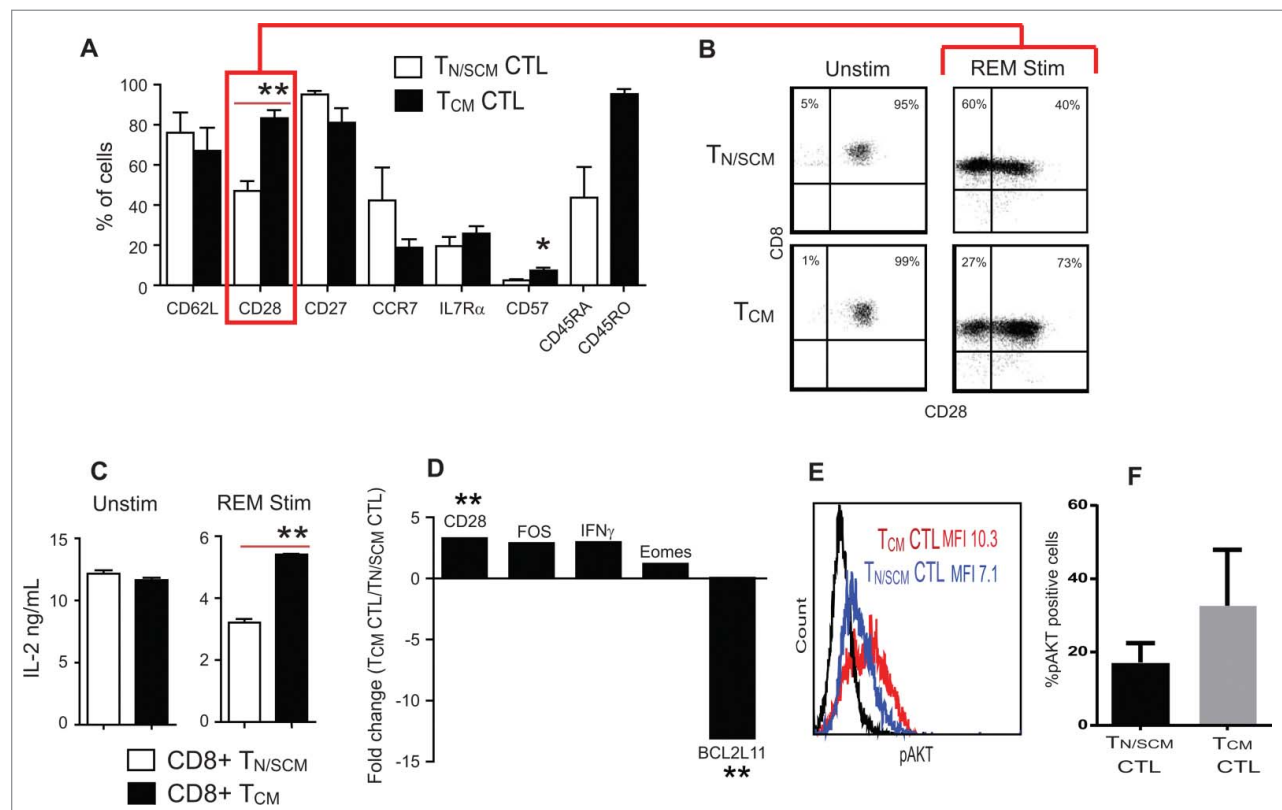


Figure 2. Effector cells derived from CD8⁺ T_{CM} precursors expressed higher levels of CD28 and IL-2 production upon stimulation. Purified CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} were subject to 14 d *in vitro* REM stimulation in the presence of rhIL-2 (50U/mL). (A) Phenotypic analysis of differentiated effector cells derived from CD8⁺ T_{N/SCM} (T_{N/SCM} CTL) and CD8⁺ T_{CM} (T_{CM} CTL) following expansion. Mean percentages of immunoreactive cells + SEM from 4 different donors are presented (** $p < 0.01$, * $p < 0.05$). (B) Representative flow cytometric analysis of CD28 on the CTLs derived from CD8⁺ T_{N/SCM} and CD8⁺ T_{CM}. (C) Supernatants were collected after overnight co-incubation of either unstimulated CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} or stimulated CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} with OKT3 expressing LCL. Cytokine levels (means + SEM of triplicate wells) were determined using cytometric bead array. ** $p < 0.01$. Representative data of 4 experiments are depicted. (D) After 14 d of stimulation, RNA was extracted and analyzed for genes that confer differentiation, survival, and apoptotic qualities of effector T cells and 4 control genes including housekeeping genes, RT control and positive PCR control. Medians of fold change of CD28, FOS, IFN γ , Eomes and BCL2L11 mRNA from cells derived from CD8⁺ T_{CM} (T_{CM} CTL) vs. CD8⁺ T_{N/SCM} (T_{N/SCM} CTL) from 4 individual donors are presented. ** $p < 0.01$. (E) Effector T cells derived from CD8⁺ T_{N/SCM} (T_{N/SCM} CTL) (blue) and CD8⁺ T_{CM} (T_{CM} CTL) (red) were stained for intracellular phosphorylated AKT (pAKT). Fluorochrome conjugated isotype matched antibody stained cells are shown in black. (F) Percentages of AKT⁺ cells from 4 donors are presented.

Table 2. CD28 expression on CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} CTL.

| HD | %CD28 CD8 ⁺ T _{N/SCM} | %CD28 CD8 ⁺ T _{CM} |
|----------------|---|--|
| 1 | 36.1 | 78.7 |
| 2 | 35.3 | 70.2 |
| 3 | 47.4 | 92.0 |
| 4 | 39.6 | 72.8 |
| Mean±SEM | 40.3±2.8 | 78.3±4.9 |
| <i>p</i> value | 0.008 | |

| HD | MFI CD28 CD8 ⁺ T _{N/SCM} | MFI CD28 CD8 ⁺ T _{CM} |
|----------------|--|---|
| 1 | 20 | 29 |
| 2 | 35 | 65 |
| 3 | 31 | 64 |
| 4 | 42 | 80 |
| Mean±SEM | 32±4.6 | 59.5±10.8 |
| <i>p</i> value | 0.0015 | |

capacity to secrete IL-2 upon TCR/CD28 triggering, decreased CD28 expression by T_{N/SCM} derived CTLs upon differentiation to effector CTLs correlates with their reduced capacity to secrete IL-2 upon subsequent polyclonal activation using OKT3-LCL stimulator cells (Fig. 2C).

To further define the differences between the two effector CTL populations, we evaluated expression levels of transcriptionally regulated genes involved in CD28 signaling and T cell survival by RT-qPCR. Consistent with their higher CD28 expression by flow cytometric analysis, CD8⁺ T_{CM} derived CTL cells contained a two-fold higher content of mRNA

encoding CD28 and FOS, a downstream signaling molecule of CD28 which leads to IL-2 production (Fig. 2D).²¹⁻²³ In line with these findings, intracellular phosphorylated AKT (pAKT), an effector molecule of co-stimulatory receptors such as CD28,²⁴ was present in higher abundance in CD8⁺ T_{CM} derived CTLs than in CD8⁺ T_{N/SCM} derived CTLs (Fig. 2E, F) and the mRNA encoding the apoptosis facilitator BCL2L11 was 13-fold lower in T_{CM} derived CTLs than that of T_{N/SCM} derived CTLs. These data reveal cell intrinsic divergence of CD28 gene expression and CD28 costimulatory function in effector cells derived from T_{CM} vs. T_{N/SCM} precursors. As intrinsically programmed, CD8⁺ T_{CM} derived CTLs transcribed higher levels of IFN γ and Eomesodermin (Eomes) than that from CD8⁺ T_{N/SCM} after *in vitro* activation and expansion.^{25,26}

CTLs derived from T_{CM} precursors exhibited increased common γ -chain cytokine receptors and responsiveness to rhIL-2 and rhIL-15 than that from T_{N/SCM}

The capacity of T_{CM} derived CD8⁺ CTLs to engraft and persist following adoptive transfer has been correlated with their sensitivity to the anti-apoptotic effects of homeostatic cytokines, in particular, IL-15.²⁷⁻²⁹ This augmented sensitivity has been attributed to cell intrinsic differences in expression levels of the γ c-chain cytokine receptor subunits, including CD25 and CD132. To further compare T_{CM} and T_{N/SCM} derived CTLs, we evaluated the expression levels of the γ -chain cytokine

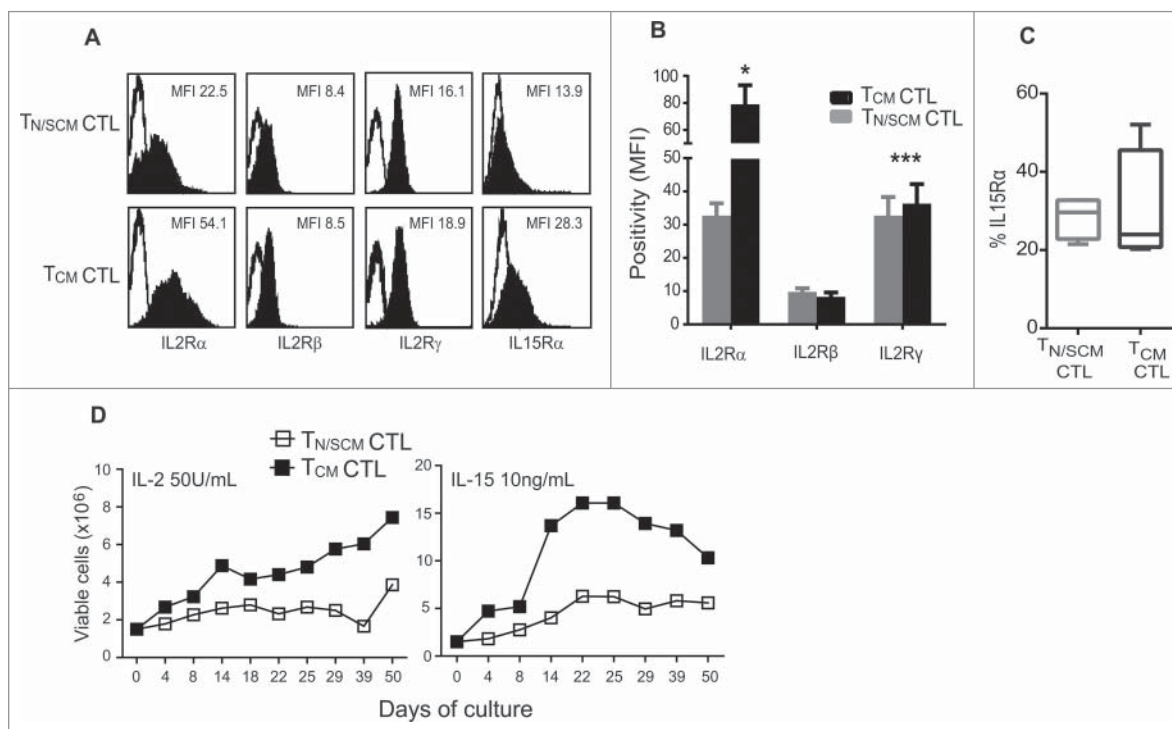


Figure 3. Effector cells derived from CD8⁺ T_{CM} precursors displayed greater ability to persist and expand *in vitro*. Purified CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} were expanded 14 d *in vitro* using a REM in the presence of rhIL-2 (50U/mL). (A) After 14 d of *in vitro* stimulation, the effector cells from each population (T_{N/SCM} CTL and T_{CM} CTL) were stained with antibodies to the indicated cytokine receptor and analyzed by flow cytometry. Histograms show the mean fluorescence intensities (MFIs) of γ -chain cytokine receptor positive cells (black) after subtraction from the isotype controls (open). Representative data of 4 experiments are depicted. (B) Positivity of IL-2 receptors from four different donors is presented. **p* < 0.05, ****p* < 0.001. (C) Percentages of IL-15R α from a cohort of four donors are presented. (D) After the initial expansion, the T_{N/SCM} CTL and T_{CM} CTL cells were maintained in rhIL-2 (50U/mL) (left) or rhIL-15 (10ng/mL) (right). Cytokines were supplemented every other day. Viable cell numbers were determined by Guava ViaCount at the indicated time points.

receptor complex. We found that T_{CM} derived CTLs expressed significantly higher levels of the α -chain ($p < 0.05$) and γ -chain ($p < 0.001$) of the IL-2 receptor, while the β -chain expression levels were equivalent (Fig. 3A and B). IL-15 receptor α -chain was higher on the T_{CM} derived CTLs (Fig. 3A and C). We next subjected the REM expanded CTLs to prolonged culture (50 days) in either rhIL-2 (50U/mL) or recombinant human IL-15 (rhIL-15) (10ng/mL) without restimulation.

Higher numbers of viable T_{CM} derived CTLs persisted in both rhIL-2 and rhIL-15 supplemented cultures as compared to $T_{N/SCM}$ derived CTL (Fig. 3D). Similar findings were observed using CTLs from different donors (Fig. S4). These data demonstrate that T_{CM} derived effectors retain higher expression levels of γ -chain cytokine receptor and have enhanced capacity to survive and expand in the presence of rhIL-2 or rhIL-15 than $T_{N/SCM}$ derived CTLs.

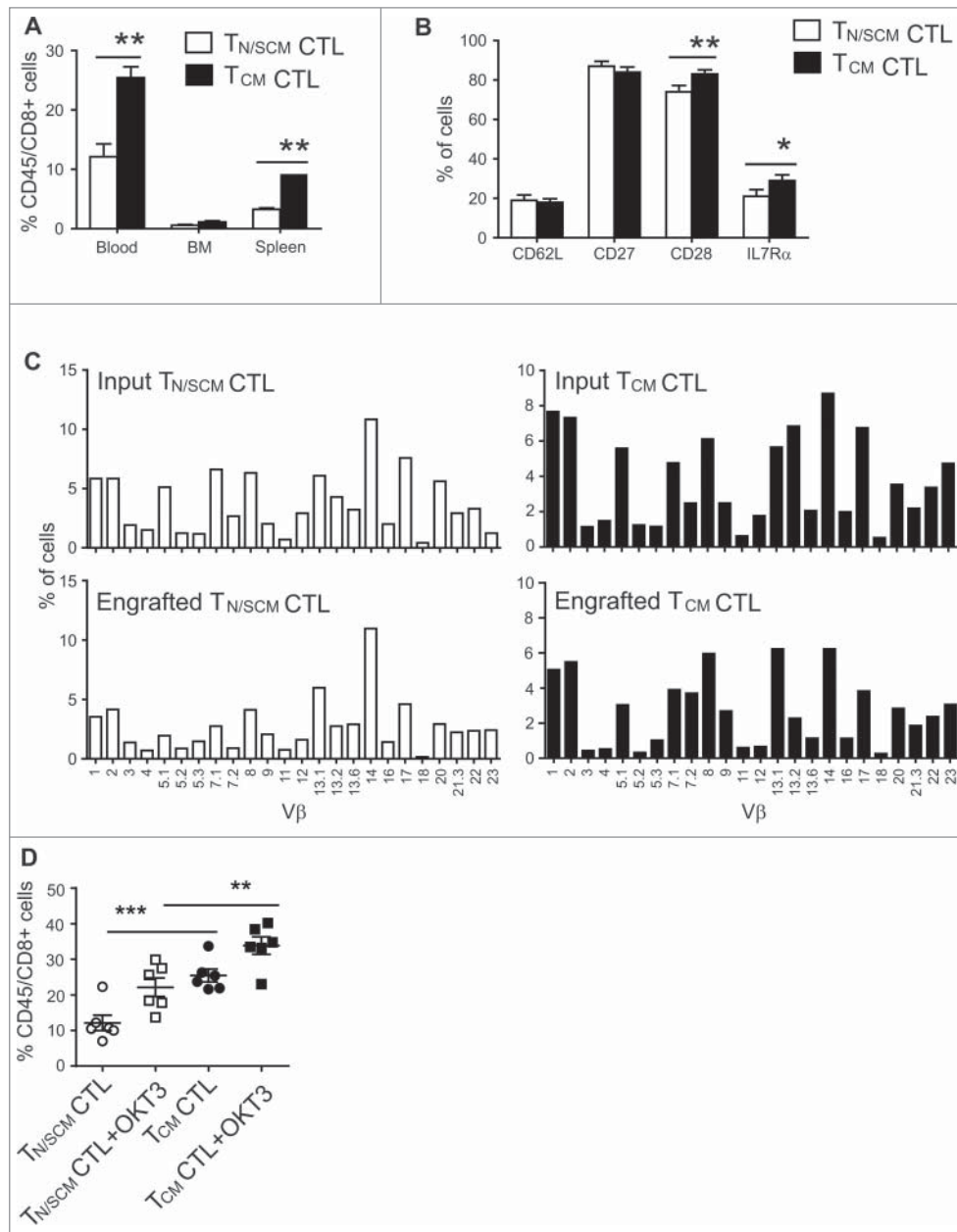


Figure 4. Effector cells derived from $CD8^+ T_{CM}$ precursors exhibited superior engraftment fitness in NSG mice. After 14 d of *in vitro* stimulation, effector cells derived from $CD8^+ T_{N/SCM}$ ($T_{N/SCM}$ CTL) and $CD8^+ T_{CM}$ (T_{CM} CTL) (10^7) were infused i.v. into huIL-15-replete NSG mice. (A) Three weeks after adoptive transfer, human T cells in the peripheral blood, BM and spleen of recipient mice were determined by flow cytometric analysis using antibodies specific for human CD45 and CD8. Means+SEM of a total of 6 mice per T cell subset in a representative experiment are depicted. ** $p < 0.01$, using a Mann–Whitney test. (B) Percentages of CD62L, CD27, CD28, and IL7R α positive cells on gated human CD45 $^+$ positive cells of pooled peripheral blood, BM and spleens are indicated. * $p < 0.05$ ** $p < 0.01$ using a Mann–Whitney test. (C) TCR $v\beta$ repertoire of the effectors derived from $CD8^+ T_{N/SCM}$ and $CD8^+ T_{CM}$ before and after infusion. Percentage (%) of human $CD3^+$ cells that were positive for the indicated TCR $v\beta$ genes was determined by flow cytometry. (D) For *in vivo* stimulation, 10^7 irradiated OKT3-expressing LCL (+OKT3) were injected i.v. into mice that had been engrafted (3 days) with $CD8^+ T_{N/SCM}$ or $CD8^+ T_{CM}$ derived CTLs. Human T cells in peripheral blood were determined 7 d post *in vivo* challenge. *** $p < 0.001$. ** $p < 0.01$.

In vitro expanded T_{CM} derived $CD8^+$ CTLs exhibit superior in vivo engraftment in response to homeostatic huIL-15 as compared to $T_{N/SCM}$ derived $CD8^+$ CTLs

We examined the extent to which human $CD8^+$ T_{CM} and $CD8^+$ $T_{N/SCM}$ derived CTLs engraft and persist *in vivo*. Three weeks after i.v. infusion, $CD8^+$ T_{CM} derived CTLs were detected in blood, bone marrow (BM) and spleen at levels significantly higher than in mice receiving $T_{N/SCM}$ derived CTLs ($p < 0.01$) (Fig. 4A). We did not observe a remarkably divergent phenotypic profile for the engrafting cells from each cohort of mice, besides a consistent and statistically higher frequency of $CD28^+$ and $IL-7R\alpha^+$ T cells in T_{CM} derived CTL infused mice (Fig. 4B). To rule out the selective engraftment of an oligoclonal population that might be expected from a rare T_{SCM} , and to establish that the superior engraftment fitness of T_{CM} derived $CD8^+$ CTLs is a general feature of $CD45RO^+CD62L^+CD8^+$ T cells, we performed flow-based TCR V β spectratyping of T cells prior to adoptive transfer and on 21 day post engraftment. We demonstrate a highly polyclonal input cell population from each subset and an equally polyclonal, minimally skewed population of engrafting T cells (Fig. 4C). Unlike our previous observation that $CD45RO^+CD62L^-$ effector memory derived $CD8^+$ CTLs in the same model did not engraft, $T_{N/SCM}$ derived $CD8^+$ CTLs exhibited engraftment fitness as reflected by these data, however, not at the level exhibited by T_{CM} derived CTLs, presumably due in part to the greater sensitivity of these cells to limiting amounts of huIL-15 *in vivo*.

We next compared the extent to which CTLs proliferate *in vivo* in response to an immunostimulatory vaccine. We quantitated the frequency of engrafted human T cells immediately before and seven days after OKT3-LCL challenge. Both subsets have equivalent fold expansion to vaccine (1.8 folds for $CD8^+$ $T_{N/SCM}$ derived CTL and 1.3 folds for $CD8^+$ T_{CM} derived CTL in peripheral blood (Fig. 4D). These data demonstrate that once engrafted, both T_{CM} and $T_{N/SCM}$ derived CTLs are able to mount a proliferative response to TCR stimulation.

CD19CAR CTLs derived from $CD8^+$ T_{CM} displayed superior persistence and antitumor potency as compared to that from $CD8^+$ $T_{N/SCM}$

To further compare the therapeutic potential, purified $CD8^+$ $T_{N/SCM}$ and $CD8^+$ T_{CM} were genetically modified by lentiviral transduction to express a second generation CD19CAR using CD28 as the costimulatory domain and a selectable marker, truncated EGFR (EGFRt) (CD19R:CD28: ζ /EGFRt).³⁰ EGFRt⁺CD19CAR T cells were immunomagnetically enriched to 90% purity and underwent 3 cycles of REM stimulation and expansion. Studies were then performed using the CD19CAR CTLs derived from $CD8^+$ $T_{N/SCM}$ ($T_{N/SCM}$ -CD19R) and $CD8^+$ T_{CM} (T_{CM} -CD19R) (Fig. 5A) populations. The T_{CM} -CD19R displayed higher fold expansion *in vitro* with comparable telomere length to that of $T_{N/SCM}$ -CD19R (Fig. 5B, C). They possessed similar phenotypic characteristics other than CCR7 expression (Fig. S5A) and exhibited equivalent CD19-specific effector function *in vitro* (Fig. S5B, C). Interestingly, after adoptive transfer into huIL-15 supplemented mice, human T cells were only detected in the BM of NSG mice that had been infused with

T_{CM} -CD19R (Fig. 5D). Therapy experiments were also performed using CD19⁺ ffluc⁺ LCLs that were inoculated i.v. into NSG mice. *In vitro* expanded $T_{N/SCM}$ -CD19R or T_{CM} -CD19R were adoptively transferred into the tumor bearing, huIL-15-supplemented mice. After 20 days, tumor signals were significantly inhibited ($p < 0.05$) in the mice that received T_{CM} -CD19R as compared to mice receiving $T_{N/SCM}$ -CD19R (Fig. 5E).

Consistently, when the purified $CD8^+$ T_{CM} and $CD8^+$ $T_{N/SCM}$ cells were transduced with lentivirus encoding CD19R (EQ) to ensure enhanced potency after adoptive transfer,^{31,32} T_{CM} -CD19R displayed higher fold expansion *in vitro* (30 vs. 8 folds for total cells and 47 vs. 12 folds for CAR⁺ T cells) (Fig. S6A, B) and superior antitumor activity to $T_{N/SCM}$ -CD19R (Fig. S6C). Antitumor efficacy of 1×10^6 T_{CM} -CD19R is comparable to that of 2×10^6 $T_{N/SCM}$ -CD19R as demonstrated in a titration *in vivo* experiment (Fig. S7).

Discussion

The nature of the infused T cells is known to be one major determinant for the persistence of transferred cells and correlates with therapeutic efficacy.⁵ Our previous studies in macaques and xenogeneic mouse models demonstrated that human $CD8^+$ effector T cells derived from macaque $CD62L^+CD95^+$ T_{CM} or human $CD45RO^+CD62L^+$ T_{CM} precursors, respectively, retain the capacity to engraft and reconstitute functional memory upon adoptive transfer, whereas effectors derived from $CD62L^-$ T_{EM} precursors do not.^{5,6,33} In support of this, clinical experience with adoptive transfer using bulk PBMC derived effector T cells for the treatment of CLL and ALL have suggested that some of the gene-engineered cells detected long-term in patients exhibit characteristics of T_{CM} .¹⁻⁴ Although it is impossible to track the precursors of the persisting cells in those trials, these results indicate that the efficacy of adoptively transferred T cells might be augmented by specifically engineering the T cell subset that exhibits the intrinsic capacity to persist *in vivo*. As an extension of our previous studies, in the present investigation, we sought to compare the *in vivo* performance of the T_{CM} and $T_{N/SCM}$ derived effector cells using an IL-2 based REM system that is widely used in clinical trials of T cell therapy.^{18,19} Our data demonstrate that effector cells arising from polyclonal preparations of $CD45RO^+CD62L^+$ T_{CM} enriched precursors exhibited superior engraftment fitness and remained the capacity to respond to *in vivo* stimulation via TCR, and display better CD19CAR redirected antitumor efficacy in the xenogeneic model of human lymphoma and leukemia, as compared to CTLs derived from the $CD45RO^+CD62L^+$ $T_{N/SCM}$ enriched precursors.

It is increasingly evident that *in vitro* attributes of *ex vivo* expanded T cells may correlate with their ability to persist after adoptive transfer.^{11,34} In our study, stimulation with REM resulted in the equivalent proliferative response and cytolytic activity of CTLs derived from T_{CM} and $T_{N/SCM}$ precursors and each subset maintained equal telomere length through multiple rounds of stimulation. However, the qualitative difference of the two populations was revealed in more detailed analysis. Despite the comparable levels of CD28 expression on freshly isolated $CD8^+$ T_{CM} and $CD8^+$ $T_{N/SCM}$ and the ability to secrete IL-2 upon OKT3 stimulation, after 14 days of *in vitro*

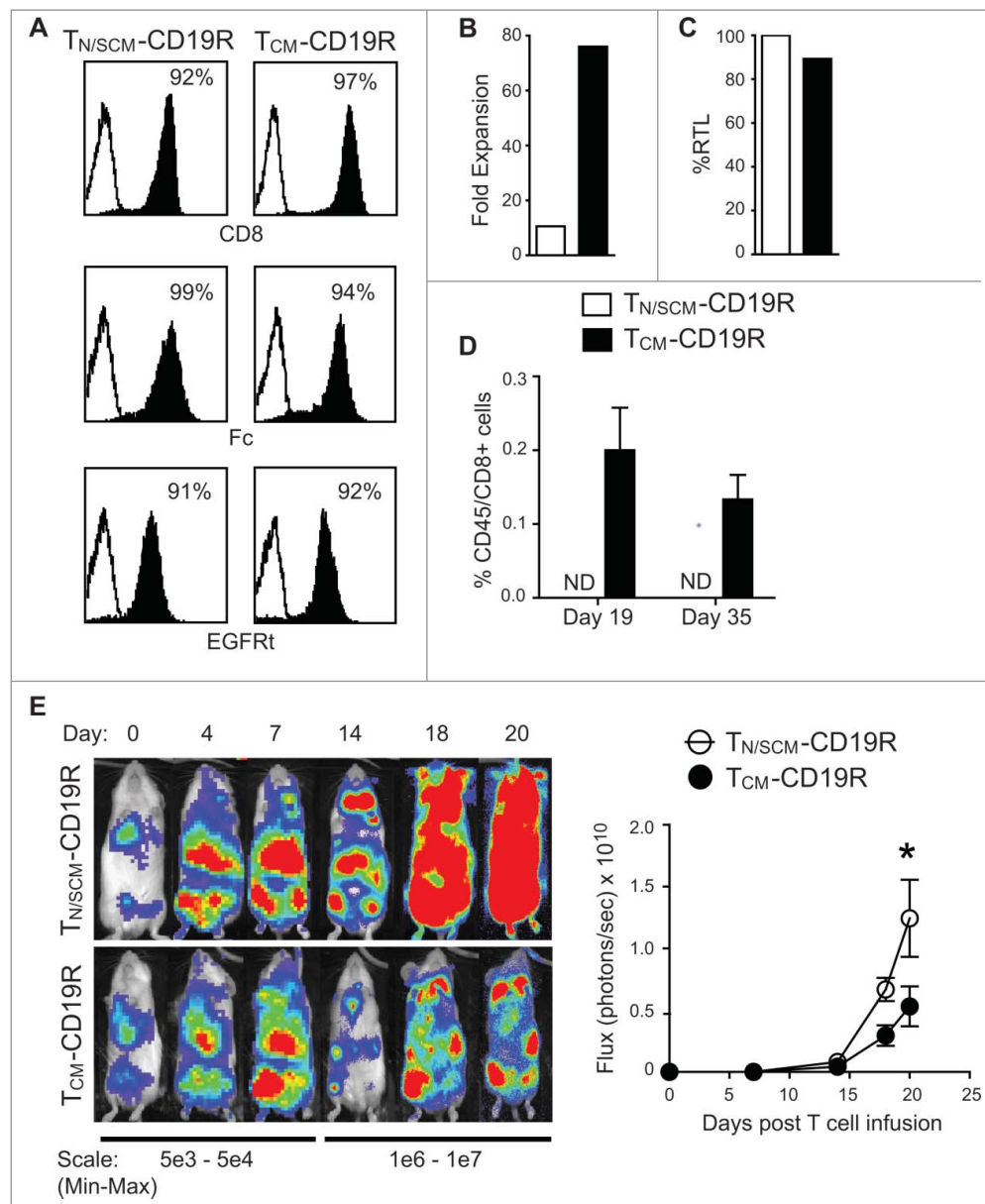


Figure 5. Engineered CD19 specific T cells derived from CD8⁺ T_{CM} (T_{CM}-CD19R) displayed superior engraftment fitness and antitumor activity as compared to that from CD8⁺ T_{N/SCM} (T_{N/SCM}-CD19R). CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} that had been transduced to express a CD19-specific CAR (CD19:CD28:ζ) and huEGFRt selection marker, were enriched for EGFRt⁺ cells and further expanded by REM stimulation in the presence of rIL-2 and rIL-15. (A) Purified EGFRt⁺ populations after 3 cycles of REM were flow cytometrically analyzed for expression of CD8⁺ as well as the CAR and EGFRt transgenes (using anti-Fc and Erbitux reagents, respectively) (B) Fold expansion of the purified EGFRt⁺ T_{N/SCM}-CD19R and T_{CM}-CD19R cells within the third cycle of REM stimulation. (C) Relative telomere length of the expanded cells. (D) The expanded T_{N/SCM}-CD19R and T_{CM}-CD19R cells (10⁷) were adoptively infused into hull-15 reconstituted NSG mice. Human T cells in the BM were analyzed by flow cytometry at indicated time points (3 mice per time point). ND, not detectable. (E) CD19⁺fluc⁺LCLs (10⁶) were inoculated into NSG mice i.v. on day -4. Expanded T_{N/SCM}-CD19R and T_{CM}-CD19R cells (10⁷) were adoptively transferred (i.v.) into the hull-15-reconstituted, tumor-bearing mice on day 0. Tumors were monitored with biophotonic imaging. Representative mouse images and mean photon flux ± SEM of each group (N = 6) are depicted. *, p < 0.05 using Mann–Whitney t-test. Representative data from two separate experiments are presented.

expansion, the CD8⁺ T_{CM} derived CTLs maintained higher levels of CD28, phosphorylated AKT, and autocrine IL-2 secretion than that of CD8⁺ T_{N/SCM} derived CTLs. Additionally, CD8⁺ T_{N/SCM} expressed higher levels of apoptotic facilitator BCL2L11 and low expression of CD28-associated FOS signaling at molecular levels. Apparently, these intrinsic attributes of the CTLs derived from T_{CM} was not compromised by the acquisition of effector cell transcriptional program such as higher IFN γ and Eomes.³⁵ These results led to the postulation that T_{CM} derived CTLs would perform better in both persistence and function upon adoptive transfer compared to T_{N/SCM} derived CTLs.

In contrast to our findings, others' studies have suggested that after *in vitro* stimulation, CD8⁺ naïve derived effector cells possess better features for immunotherapy as compared to CD8⁺ T_{CM} derived effector cells such as longer telomere and higher expression of costimulatory molecules.¹¹ The discrepancy could be ascribed to differences among the factors, such as selection of the T cell subsets from frozen/ overnight rested PBMC, which has been showed to induce CD62L shedding with potential biological changes,³⁶ and subsequent gene modification in those studies. We speculate that different culture conditions such as addition of IL-7 and IL-15²⁰ and various

means of stimulation would also result in differential impacts on the activation and differentiation of T_{SCM} , naive and memory T cells.^{12,37,38}

To further study whether the phenotypic and functional attributes of CTLs derived from $CD8^+T_{CM}$ correlate with their ability to persist and mount an effective antitumor response *in vivo*, *ex vivo* expanded T cells were adoptively transferred into NSG mice. CTLs arising from $CD8^+T_{CM}$ cells exhibited an engraftment advantage with broad $V\beta$ TCR repertoire in hUL15-supplemented NSG mice, consistent with higher expression of IL-15 α and γ -chain receptors on the infused cells. Moreover, the persisting CTLs derived from $CD8^+T_{CM}$ were also capable of expanding *in vivo* robustly in response to OKT3 vaccine challenge, indicating CTLs derived from $CD8^+T_{CM}$ possess essential characteristics for improved engraftment, long term survival and retention of memory function after adoptive transfer. We also consistently found that lentiviral transduced and expanded $CD19CAR^+$ CTL derived from $CD8^+T_{CM}$ exhibited superior engraftment fitness and enhanced antitumor activity compared to $T_{N/SCM}$ derived $CD19$ CAR CTL upon transfer into $CD19^+$ tumor bearing mice.

Memory stem cells (T_{SCM}) defined by Restifo's group displayed the potential for the induction of long-term memory T cells *in vivo*, therefore, have direct relevance to the design of T cell immune therapy. However, like other somatic stem cells, the naturally occurring "memory stem cells" are rare in PBL, the frequency is around 2% of $CD8^+$ cells in peripheral blood. Isolation of such a rare subset and obtaining a clinically relevant therapeutic dose without prolonged *ex vivo* culture are the obvious challenges.³⁹ It is possible that T_{SCM} , which are characterized as $CD45RA^+CD62L^+CD95^+$ were captured in the $T_{N/SCM}$ that were examined in our study. However, we found that T_{CM} derived cells still exhibited the best engraftment and antitumor activity in our mouse model. In addition, the culture conditions used in our study lack the preferential signals such as Wnt signaling required for the infrequent T_{SCM} to expand. This might further contribute to the superior therapeutic activity of $CD8^+T_{CM}$ derived cells.

Overall, this work supports the idea that the $CD8^+T_{CM}$ described here is a promising candidate T cell population to consider for immediate immunotherapeutic use. We have developed a semi-closed manufacturing process for reproducibly generating genetically modified $CD19$ -specific T cells from a defined population of T_{CM} in a short period of time for clinical applications.⁴⁰

Materials and methods

Cell lines

Unless stated otherwise, all cell lines were maintained in RPMI 1640 (Irvine Scientific) supplemented with 2mM L-glutamine, 25mM HEPES, and 10% heat-inactivated FCS (Hyclone), hereafter referred to as complete media (CM). PBMCs were transformed with Epstein–Barr virus to generate lymphoblastoid cell lines (LCL) as previously described.⁴¹ OKT3-LCL cells express membrane bound OKT3 and are grown in CM supplemented with 0.4mg/mL hygromycin.³⁰ K562 and Sup B15 cells were obtained from ATCC and cultured as recommended.

Antibodies and flow cytometry

Human T cells were analyzed by flow cytometry after staining with fluorochrome-conjugated monoclonal antibodies (mAbs) to $CD8^+$, $CD62L$, $CD45RO$, $CD45RA$, $CD127$, $CD28$, $CD27$, $CD57$, $CD45$, $CD3$, $IFN\gamma$, $CD25$ (IL-2 α), $CD122$ (IL-2 β), and $CD132$ (IL-2 γ) (BD Biosciences); $CCR7$ and $IL-15\alpha$ (R&D Systems); and phosphorylated AKT (pAKT) (Cell Signaling Technology). The IOTestBeta Mark T cell receptor (TCR) $V\beta$ Repertoire Kit was purchased from Beckman Coulter. Isotype-matched mAbs served as controls. Data acquisition was performed on a FACSCalibur (BD Biosciences) and analyzed using FCS Express, Version 3 software (De Novo Software). Telomere length analysis was performed using flow-fish technique with Telomere PNA Kit/FITC obtained from Dako (Dako Denmark A/S, Denmark). Biotinylated Erbitux (Cetuximab) and streptavidin-PE were used to identify T cells that expressed truncated human EGFR (EGFRt), and surface expression of the $CD19CAR$ was confirmed by staining with a biotinylated anti-Fc (Jackson ImmunoResearch Laboratories) and streptavidin-PE.

T cell isolation and stimulation

Leukapheresis products were obtained from the cohort of four healthy donors under protocols approved by the COHNMC Institutional Review Board. For purification of $CD8^+T_{N/SCM}$ and $CD8^+T_{CM}$ subsets, PBMCs were labeled with anti- $CD45RO$, $-CD45RA$, $-CD62L$, $-CD95$, and $-CD8$ mAbs, and $CD8^+CD62L^+CD45RA^+T_{N/SCM}$, $CD8^+CD62L^+CD45RO^+T_{CM}$ and $CD8^+CD62L^+CD45RA^+CD95^+T_{SCM}$ cells were sort-purified using a MoFlo MLS (Dako Cytomation). Freshly isolated $CD8^+T_{SCM}$, $CD8^+T_{N/SCM}$ and $CD8^+T_{CM}$ (5×10^5 each) were then propagated using the rapid expansion method (REM).¹⁷ Briefly, 10^6 T cells were stimulated with 30ng/mL anti- $CD3\epsilon$ (OKT3; Ortho Biotech), 5×10^7 γ -irradiated PBMCs (3500 cGy), and 10^7 γ -irradiated lymphoblastoid cell lines (LCLs, 8000 cGy) in 50mL culture media. Cultures were then supplemented with 50U/mL rhIL-2 (CellGenix) every 48 h for 14 d before *in vitro* analysis and adoptive transfer. In some cases, purified $CD8^+T_{N/SCM}$, $CD8^+T_{CM}$ and $CD8^+T_{SCM}$ were stimulated with 30ng/mL OKT3, γ -irradiated PBMCs at 10:1 ratio (feeders: T cells). Cultures were then supplemented with 300U/mL rhIL-2 every 48 h for 14 d.

DNA constructs

The GFP-IMPDH2dm-2A-IL-15 $_p$ cDNA3.1(+) plasmid contains a fusion of the *eGFP* cDNA, which confers fluorescence and resistance to mycophenolic acid, followed by the 2A self-cleaving peptide sequence⁴² and a human IL-15 cDNA. OKT3–2A-Hygro $_p$ EK is an expression plasmid wherein an anti-human $CD3\epsilon$ scFV:Fc:CD28 $_{TM}$ cDNA is formatted N-terminal to hygromycin phosphotransferase with an intervening 2A sequence⁴³ under the transcriptional control of the human EF-1 α promoter. The $CD19R:CD28:\zeta/EGFRt$ -epHIV7 contains: (1) the CAR sequence consisting of the V_H and V_L gene segments of the $CD19$ -specific FMC63 mAb, an IgG4 hinge-CH2CH3, the transmembrane, and cytoplasmic domains of the

costimulatory molecule CD28, and the cytoplasmic domain of the CD3 ζ chain⁴⁴; (2) the ribosomal skip T2A sequence and (3) the truncated human EGFR (EGFRt) sequence as previously described.³⁰ In some cases, the CD19R:CD28: ζ /EGFRt with mutations at two sites (L235E; N297Q) within the CH2 region on the IgG4-Fc spacers (CD19R(EQ)) was used (**Fig. S8A and S8B**).

Generation of CD8⁺ CD19-specific T_{N/SCM} and T_{CM} derived effector cells

Freshly isolated CD8⁺ T_{N/SCM} and CD8⁺ T_{CM} were activated with CD3/CD28 Dynabeads (Invitrogen), and transduced 3 d later with CD19R:CD28: ζ /EGFRt lentivirus at an MOI of 3. Ten days after the lentiviral transduction, the truncated human EGFR⁺ (EGFRt⁺) T cells were then enriched³⁰ and further expanded by REM in the presence of 50U/mL rhIL-2 and 0.5ng/mL rhIL-15 (CellGenix).

Cytokine production assays

Freshly isolated and expanded T cell products (10⁵) were cocultured in 96-well tissue culture plates with 10⁵ of the indicated stimulator cells. Supernatants were harvested 18 h after stimulation and analyzed by cytometric bead array using the Bio-Plex Human Cytokine 17-Plex Panel (Bio-Rad Laboratories) (in triplicates) according to the manufacturer's instructions. Intracellular IFN γ staining was performed and analyzed with flow cytometry.

Cytotoxicity assays

Four-hour ⁵¹Cr release assays were performed as previously described⁴⁵ using the indicated effector cells and ⁵¹Cr-labeled target cells.

Reverse transcriptase (RT) quantitative PCR (qPCR) analysis

After 14 d of stimulation, RNA was extracted with RNeasy Kits (Qiagen). RT-qPCR was performed using the RT² Profiler PCR Array (SABiosciences) designed with customized primers for sequences of transcriptionally regulated genes shown to impart differentiation, survival, and apoptotic attributes to effector CD8⁺ T cells^{11,46} and 4 control genes including house keeping genes PPIA (Peptidylprolyl isomerase A) (cyclophilin A) and RPL13A (Ribosomal protein L13a), control for reverse transcription, and positive PCR control. All the samples passed the RNA quality control, PCR array reproducibility, RT efficiency, and genomic DNA contamination according to the manufacturer's instructions. Data were analyzed by $\Delta\Delta C_t$ method using RT² Profiler PCR Array Web portal software (<http://www.sabiosciences.com>). Medians of fold change of T_{CM} versus T_{N/SCM} derived CD8⁺ effectors from 4 individual donors are presented.

Xenograft model

All mouse experiments were approved by the COH Institutional Animal Care and Use Committee. Six- to 10-week old NSG mice were injected intravenously (i.v.) on day 0 with 1 \times 10⁷ freshly thawed T cell products. Irradiated (8000 cGy) NS0-huIL15 cells (2 \times 10⁷) were given intraperitoneally (i.p.) three times a week to provide a systemic supply of human IL-15 *in vivo*, as previously described.⁵ Human T cell engraftment was determined by flow cytometric analysis of harvested tissues based on staining with antibodies specific for human CD45 and CD8⁺. For *in vivo* stimulation, 10⁷ irradiated OKT3-expressing LCLs were injected into the mice i.v. 3 d after i.v. administration of 10⁷ T cells, and human T cells in peripheral blood were analyzed 7 d after challenge.

Biophotonic tumor imaging

1 \times 10⁶ CD19⁺ firefly luciferase⁺ (ffLuc⁺) LCLs or 0.5 \times 10⁶ CD19⁺ ffLuc⁺ acute lymphoid leukemic cells SupB15 were inoculated into NSG mice by i.v. injection. Anesthetized mice were imaged using a Xenogen IVIS 100 series system (Xenogen, Alameda, CA). Photons emitted from ffLuc⁺ tumor xenografts were quantified using the software program Living Image (Xenogen), and the bioluminescence signal was measured as total photon flux normalized for exposure time and surface area and expressed in units of photons (p) per second per cm² per steradian (sr).

Statistical analysis

Analyses were performed using Prism (GraphPad Software Inc.). Mann–Whitney *t*-test was used for the statistical analysis of the *in vivo* data. Paired *t*-test (2-tailed) was used for the analysis of *in vitro* data. *p* < 0.05 was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

Contribution: X.W. designed and performed research, collected, analyzed, and interpreted data, and co-wrote the manuscript; A.M., L.E.B., C.E.B., and C.B. analyzed and interpreted data, and co-wrote the manuscript; C. W.W., R. U., E.T., B.A., and W.C. performed research and collected data; S.J. F. analyzed and interpreted data; and M.C.J. designed research, analyzed and interpreted data, and co-wrote the manuscript. The authors thank Julie R. Ostberg for assistance in generating the Figures.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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